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EFFECT OF NEEM PESTICIDE (ACHOOK) ON MIDGUT ENZYMATIC ACTIVITIES AND SELECTED BIOCHEMICAL COMPOUNDS IN THE HEMOLYMPH OF LESSER MULBERRY PYRALID, *GLYPHODES PYLOALIS* WALKER (LEPIDOPTERA: PYRALIDAE)

*Roya Khosravi, Jalal Jalali Sendi**

Department of Plant Protection, College of Agriculture, University of Guilan, 41635–1314 Rasht, Iran

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Abstract: Synthetic organic compounds and those of plant origin used in insect pest control are known to affect digestive enzymes and biochemical compounds. The lesser mulberry pyralid *Glyphodes pyloalis* Walker is a monophagous and dangerous pest of mulberry that has been recently observed in Guilan province, northern Iran. In this study the effect of the neem formulation, Achook (0.03% azadirachtin) was studied on nutritional physiology and gut enzyme activity of the lesser mulberry pyralid *G. pyloalis*. The LC_{25}, LC_{50}, and LC_{90} values on 4th instar larvae were estimated as 113.6, 256.84, and 1,210.02 ppm, respectively. The neem insecticide exhibited a significant antifeedant activity when used at the highest concentration. When *G. pyloalis* larvae were provided with mulberry leaves treated with the neem extract, all nutritional indices, except approximate digestibility, decreased. Neem was found to affect digestive enzyme activities in the midgut of treated larvae. When larvae were fed on treated leaves, biochemical compounds in the hemolymph, such as protein, lipid, and glucose decreased but the amount of uric acid increased compared with the control.

Key words: Achoock, antifeedant, biochemical compounds, enzyme activity, mulberry pyralid, nutritional indices

INTRODUCTION

Pesticides of biological origin have been intensively investigated for the past 30 years. An effort has been made to find an alternative to conventional insecticides. The alternative should be able to reduce health and environmental impacts (Copping and Menn 2001). There has been a worldwide interest in the development of alternative strategies, including the re-examination of using plant derivatives against agriculturally important insect pests. Plant-derived materials are more readily biodegradable. Some have low toxicity to humans as well as natural enemies, and are more selective in action (Keita *et al*. 2001; Tapondjou *et al.* 2002; Rahman and Siddiqui 2004).

Among the plant families, Meliaceae and Rutaceae have attracted most of the entomologists' and phytochemists' attention because they produce chemicals known as triterpenes (Connolly 1983). Many of these compounds have been demonstrated to affect insect growth and behavior, acting as antifeedants, toxins, and insect growth regulators (Champagne *et al*. 1992; Senthil-Nathan *et al*. 2005a, 2005b, 2006a, 2006b).

In the Meliaceae family, the neem tree is native to the Indian sub-continent and also grows in many countries in the world, including subtropical countries such as Africa, Central and South America, and Australia (Schmutterer 1990). The insecticidal effect of the compound azadirachtin was first reported by Ruscoe (1972). The trees produce yellow oval shaped fruits once or twice per year. Although several potentially insecticidal active ingredients occur in neem seed extract, the principal active ingredient in most formulations is tetranortriterpene azadirachtin (AZA). Azadirachtin is active in nearly 550 insect species (Schmutterer 1990; Mordue and Blackwell 1993),

The lesser mulberry pyralid, *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae) is a specialist insect on mulberry. This insect is widely distributed throughout Asia and the northern province of Iran. This pest has caused severe damage to farm fields of mulberry in northern Iran and has posed a serious concern to silkworm growers. The amount of food eaten by the first and second instar larvae is negligible, but feeding increases in later instars. Fourth and fifth instar larvae secrete fine threads to fold the leaf and feed on the mesophyll inside the folds. Fifth instar larvae feed on the whole leaf until only the ribs remain. Each generation takes 25 days and it can have five generations in a year so that damage from the fourth and fifth generations is more important (Khosravi and Jalali Sendi 2010).

The aim of this study was to identify the effect of azadirachtin on the food consumption of the fifth instar larvae of *G. pyloalis* and its effects on digestive enzymes and biochemical compounds in the hemolymph. In this study we tried to throw some light on the possible mechanisms involved in azadirachin's effect on this noxious pest of mulberry.

*Corresponding address:
jjalali@guilan.ac.ir

MATERIALS AND METHODS

Laboratory mass culture of *G. pyloalis*

The larvae of *G. pyloalis* were collected from mulberry orchards near the city of Rasht in northern Iran. They were reared on fresh mulberry leaves (Kenmochi variety) in the laboratory at 24±1°C, 75±5% relative humidity (RH) and a 16:8 (L:D) hour photoperiod. The larvae were kept in transparent plastic boxes (18x15x7 cm). The boxes were covered with muslin cloth for aeration. As adults emerged, they were separated and placed in transparent plastic boxes (18x7 cm) with a 10% honey solution on cotton wool for feeding, and mulberry leaves for oviposition. Using moist cotton wool, leaves with eggs were transferred to the transparent plastic boxes (18x15x7 cm).

Bioassays

For bioassay experiments, Azadirachtin-rich neem formulation, Achook (Registered No. CIR-23, 925/96, 0.03% azadirachtin, India), was used and diluted with distilled water. Four concentrations of azadirachtin (800, 400, 200, 100 ppm) in distilled water with emulsifier (0.5% of Triton X-100) were used for the evaluation of LC_{50} values, with one distilled water and emulsifier as the control. A leaf dip experiment was carried out using freshly cut leaves of mulberry that were dipped in a solution of azadirachtin (treatment) or distilled water (the control) for 10 s, and dried for 20 minutes at room temperature. Newly molted fourth instar larvae were starved for 4 h. Then these larvae were allowed to feed on azadirachtin treated leaves for 24 h. The tests were conducted in four replicates. There were 10 larvae for each replicate. Mortality was recorded after 24 h. The LC_{25}, LC_{50}, and LC_{90} values, and 95% confidence limit were calculated from probit regressions using the Polo-PC program (LeOra 1987).

Antifeedant activity

The antifeedant activity of azadirachtin was carried out by a leaf-disc choice test. For this purpose four concentrations (800, 400, 200, 100 ppm) of azadirachtin were prepared. The leaf discs (R = 5 cm) were dipped for 10 s in the desired concentrations. The control leaf disc was dipped in water. One treated leaf disc and one control which was dried at room temperature, were randomly placed in a transparent plastic box 21x15x5 cm over moist filter paper to avoid dryness. This experiment was replicated 4 times by 10 fifth instar larvae starved for 4 h before the start of the experiments. The dishes were similarly placed in a controlled room as above. Consumption was recorded by using a digital leaf area meter (ADC, bioscientific Ltd., England) after 24 h. The feeding deterrence index (FDI) was calculated according to Isman *et al.* (1990):

$$FDI = (C - T)/(C + T) \times 100$$

where:
C – the consumption of the control leaf,
T – the treated leaf.

Probit analysis was used to determine FD_{50} (concentration required to produce a 50% feeding deterrence compared to untreated discs).

Quantitative food utilization

The newly molted fifth instar larvae were used for this experiment. After measuring the initial weight of the larvae, they were individually introduced into separate containers. The larvae were starved for 4 h (10 larvae/concentration) and then allowed to feed on weighed quantities of treated and untreated leaves for 24 h. Four replicates were carried out. The uneaten leaves were removed after 24 h, and replaced with fresh untreated leaves. The experiment was continued for four days. Dry weights of larvae and leaves at the outset of the experiment were calculated using a wet weight/dry weight conversion, established using a separate cohort of larvae and leaves.

Nutritional indices were calculated in a traditional manner such as: Relative consumption rate (RCR) = E/TA, Relative growth rate (RGR) = P/TA, Approximate digestibility, (AD) = 100(E – F)/E, Efficiency of conversion of ingested food (ECI) = 100 P/E, Efficiency of conversion of digested food (ECD) = 100 P/(E – F), where: A – mean dry weight of animal during T, E – dry weight of food eaten/number of alive larvae, F – dry weight of faeces produced, P – dry weight gain of insect, and T – duration of experimental period (Waldbauer 1968).

Preparation of enzyme extract

Fourth instars of treated *G. pyloalis* were used to quantify the enzyme and biochemical compound. Individuals were dissected under a stereomicroscope (OLYMPUS SZX12, Japan) in ice-cold saline buffer (6 µmol/l NaCl). The entire digestive tract was dissected out, in ice-cold insect Ringer's solution. The gut was homogenized for 3 min at 4°C in ice-cold citrate-phosphate buffer (pH 6.8) using a tissue grinder. The homogenate was centrifuged at 13,000 rpm for 15 min and the supernatant was used as the enzyme source.

Assay of α-amylase activity

α-amylase activity was assayed by the dinitrosalicylic acid (DNS) procedure (Bernfeld 1955), using 1% soluble starch (Merck, Darmstadt, Germany) as the substrate. Twenty micro liters of the enzyme were incubated for 30 min at 35°C with 100 µl universal buffer and 40 µl soluble starch. The reaction was stopped by adding 100 µl DNS and heating in boiling water for 10 min. Since DNS is a color reagent, the reducing groups released from starch by α-amylase action were measured by the reduction of DNS. The boiling water stops α- amylase activity, and catalyzes the reaction between DNS and the reducing groups of starch. Absorbance was then read at 540 nm. One unit of α-amylase activity was defined as the amount of enzyme required to produce 1 mg maltose in 30 min at 35°C. A blank sample without substrate, with α- amylase extract, and a negative control containing no α-amylase extract with substrate, were run simultaneously. All assays were performed in duplicate and each assay was repeated three times.

Assay of α- and β-glucosidase activity

The activities of α- & β-glucosidases were measured with pNαG and pNβG as substrate, respectively. The homogenate (10 µl) were incubated for 30 min at 37°C with 45 µl of substrate (25 mM) and 115 µl of 40 mM glycine-phosphate-acetic-citric buffer. The reaction was stopped by the addition of 600 µl of NaOH (0.25 M). Optical density was measured at 405 nm using a microplate reader (Stat Fax 3200, Awareness Technology, USA) after 10 min. A standard curve of absorbance against the amount of p-nitrophenol released, was constructed to enable the calculation of the amount of p-nitrophenol released during the α- & β-glucosidases assays.

Assay of lipase activity

The activity of lipase was determined by the method of Tsujita *et al*. (1989). Ten µl of midgut tissue extracts was mixed with 18 µl p-nitrophenyl butyrate (50 mM) as a substrate, and 172 µl of universal buffer solution (1 M) (pH = 7). This was then incubated at 37°C. The absorbance was read at 405 nm. One unit of enzyme releases 1.0 nmol of p-nitrophenyl per minute at pH 7.2, at 37°C using p-nitrophenyl butyrate as the substrate.

Assay of protease activity

General protease activity of adult midguts was determined using azocasein as the substrate (Garcia-Carreno and Haard 1993). The reaction mixture was 80 µl of 2% azocasein solution in 40 mM universal buffer of specified pH and 30 µl enzymes. The reaction mixture was incubated at 37°C for 60 min. Proteolysis was stopped by the addition of 300 µl of 10% trichloroacetic acid (TCA). Appropriate blanks in which TCA was added first to the substrate, were prepared for each assay. Precipitation was achieved by cooling at 4°C for 120 min, and the reaction mixture was centrifuged at 16,000 rpm for 10 min. An equal volume of 1 N NaOH was added to the supernatant. The absorbance was recorded at 440 nm.

Hemolymph extraction

The hemolymph was collected by capillary tubes and poured in Eppendorf tubes containing several crystals of phenylthiourea to prevent melanization. The hemocytes were sedimented by centrifuging at 4°C, 12,000 rpm for 5 min. Supernatant was kept at – 20°C and used in the subsequent experiments.

Lactate dehydrogenase (LDH) assay

The King (1965) method was used for evaluating lactate dehydrogenase (LDH). To standardize volumes, a 0.2 ml NAD^+ solution was added to the test tubes, and 0.2 ml of water was added to the control test tubes, each containing 1 ml of the buffered substrate. The sample (0.01 ml) was also added to the test tubes. Test tube samples were incubated for exactly 15 min at 37°C and then arrested by adding 1 ml of color reagent (2, 4-dinitrophenyl hydrazine) to each tube. The incubation then continued for an additional 15 min. After the contents were cooled to room temperature, 10 ml of 0.4 N NaOH was added to each tube to make the solutions strongly alkaline. At exactly 60 s after the addition of alkali to each tube, the intensity of color was measured at 440 nm.

Estimation of glucose, cholesterol and uric acid

Glucose was analyzed as described by Siegert (1987) and total cholesterol was analyzed as was described by Richmonds (1973) hydrolyzing cholesterol esters with cholesterol oxidase, cholesterol esterase and peroxidase. Uric acid contents were determined using uricase as described by Valovage and Brooks (1979). This enzyme produces a purplish color which has a direct correlation (at 500 nm) with uric acid concentration.

Protein determination

Protein concentrations were measured according to the method of Bradford (1976), using bovine serum albumin (Bio-Rad, München, Germany) as a standard.

Statistical analysis

The data were statistically analyzed separately for each experiment and were subjected to analysis of variance (ANOVA) using SAS software. The difference between the treatments was determined by Tukey's multiple range tests (SAS 1997). Differences among means were considered significant at $p \leq 0.05$.

RESULTS

The digestive effects of azadirachtin on fourth instar larvae of *G. pyloalis* are presented in table 1. The dose response effect of azadirachtin on the mortality of larvae is shown in figure 1. Concentrations at 800, 400, and 200 ppm of azadirachtin caused 85, 62, and 37% mortality, respectively when compared with the control.

The leaf-disc choice assay showed feeding deterrence in the azadirachtin treatment. The assay resulted in 50% feeding inhibition in fourth instar *G. pyloalis* larvae for azadirachtin (FI_{50} = 79.3 ppm).

Nutritional experiments showed that both the relative consumption and growth rate of fifth instar *G. pyloalis* larvae were reduced by azadirachtin insecticide. Efficiency of conversion of ingested food ($F = 28.44$, $df = 4$, $p \leq 0.001$) and ECD ($F = 30.87$, $df = 4$, $p \leq 0.001$) values declined significantly compared with the control, while AD increased

Table 1. The LC_{25}, LC_{50}, LC_{75}, and LC_{90} values, confidence limit (95%) and regression slope of Achook on fifth-instar larvae of *G. pyloalis* after 24 h (on ppm basis)

Toxic material	No.	LC_{25} (95% C.L.)	LC_{50} (95% C.L.)	LC_{90} (95% C.L.)	Slope ±S.E.	X^2 (df)
Neem	200	113.6 (65.44–156.03)	256.84 (194.46–333.74)	1210.02 (779.38–2796.23)	1.9±0.33	0.76 (2)

C.L. (confidence limit) which has been calculated with 95% confidence; ±S.E. – standard error

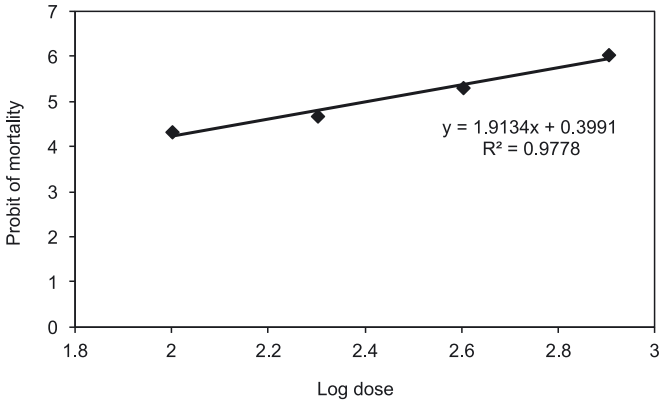

Fig. 1. Mortality probit of achook on fifth instar larvae of *G. pyloalis*

significantly following treatment with azadirachtin insecticide (F = 19.4, df = 4, p ≤ 0.001) (Table 3).

Results showed that azadirachtin affected the digestive enzymatic profiles of *G. pyloalis* at several concentrations when using the oral ingestion treatment. The activity level of α-amylase decreased in treated larvae and at 24 h (F = 71.87, df = 4, p ≤ 0.001) (Fig. 2). The activity level in insects treated with 800 ppm of neem was 0.02 nmol/min/mg protein. It was found that azadirachtin decreased the activity level of α- and β-glucosidases (Fig. 2). Activity of protease decreased after treatment with all concentrations of azadirachtin, so that the lowest activities were observed in concentration of 400 and 800 ppm (F = 42.12, df = 4, p ≤ 0.001). Feeding of *G. pyloalis* larvae by different concentration of azadirachtin insecticide caused a significant decrease in lipase activity especially after using concentrations of 400 and 800 ppm of neem (F = 39.13, df = 4, p ≤ 0. 01).

Different concentrations of azadirachtin decreased the activity of lactate dehydrogenase (LDH) in hemolymph of *G. pylolais* larvae. The lowest activity of LDH was observed in the 400 ppm concentration (F = 25.92, df = 4, p ≤ 0. 01). In the present study, in addition to enzymatic activity in the midgut, the amounts of non enzymatic compounds were measured in the hemolymph of larvae fed on different concentrations of neem. Results showed that the amounts of cholesterol, glucose, and total protein in the hemolymph of *G.pyloalis* larvae were significantly affected by different concentrations of neem insecticide. Concentrations of 400 and 800 ppm significantly decreased the amount of trehalose in comparison with the control (F = 4.58, df = 4, p ≤ 0.01). In the case of uric acid, all concentrations of neem increased the amount of uric acid (F = 20.74, df = 4, p ≤ 0.01). Our results showed that the amount of all non-enzymatic components such as protein, trehalose, and lipid, decreased in the hemolymph of larvae fed on all concentrations, except for uric acid. The amount of total protein in the hemolymph of treated larvae decreased as concentrations increased.

Table 2. Effect of Achook on feeding by *G. pyloalis* in the leaf disc choice assay

Dosage of neem [ppm]	AI%
800	91
400	75
200	69
100	56

Table 3. Nutritional indices of fifth instar larvae of *G. pyloalis* after treatment with Achook insecticide

	AD	ECI	ECD	RCR	RGR
Control	82.08±1.39 c	5.05±0.73 a	6.16±0.85a	5.43±0.73 a	1.37±0.18 a
100	82.97± 1.57 c	4.46±0.72 a	5.38±0.78 a	4.13±0.72 b	0.92±0.18b
200	86.67±1.53 bc	3.12±0.92 b	3.62±1.03 b	4.03±0.85 b	0.62±0.19c
400	89.62±1.51 ab	2.9±0.69 b	3.24±0.74 b	3.32±0.72 b	0.47±0.12 c
800	93.74±1.74 a	1.11±0.85 c	1.21±0.9 c	3.54±1.03 b	0.18±0.15d

Within columns, means followed by the same letter do not differ significantly (Tukey's test, p ≤ 0.05).
RGR – relative growth rate; AD – approximate digestibility; ECI – efficiency of conversion of ingested food;
ECD – efficiency of conversion of digested food

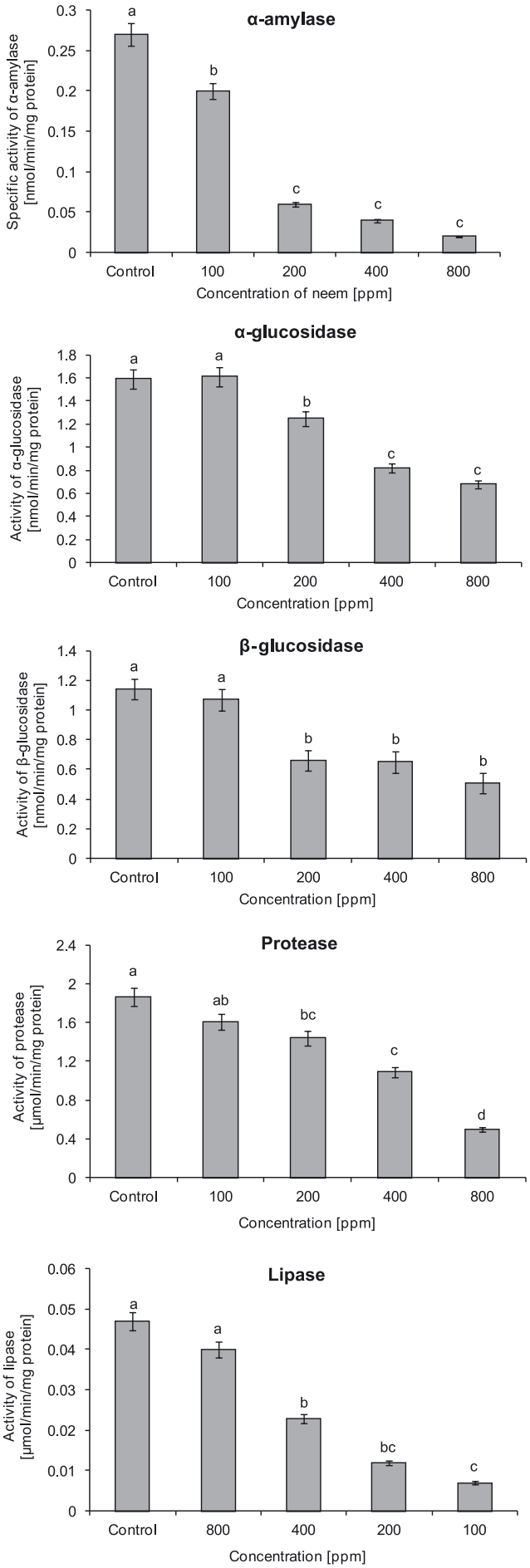

Fig. 2. Digestive enzyme activities in larvae of *G. pyloalis* after treatment with different concentrations of Achook. Statistical differences are shown by a different letter in Tukey's test ($p < 0.05$)

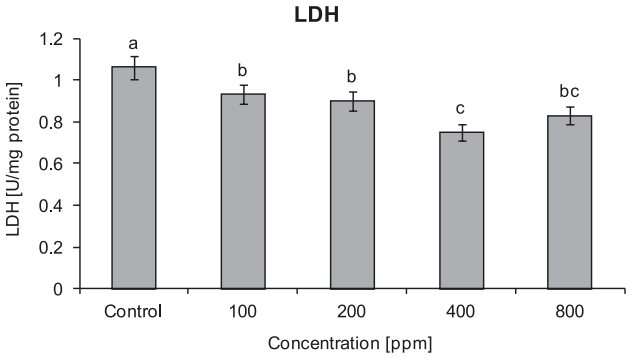

Fig. 3. Effect of different concentrations of neem on Lactate dehydrogenase activity in the hemolymph larvae of *G. pyloalis* after treatment with different concentrations of Achook. Statistical differences are shown by a different letter in Tukey's test ($p < 0.05$)

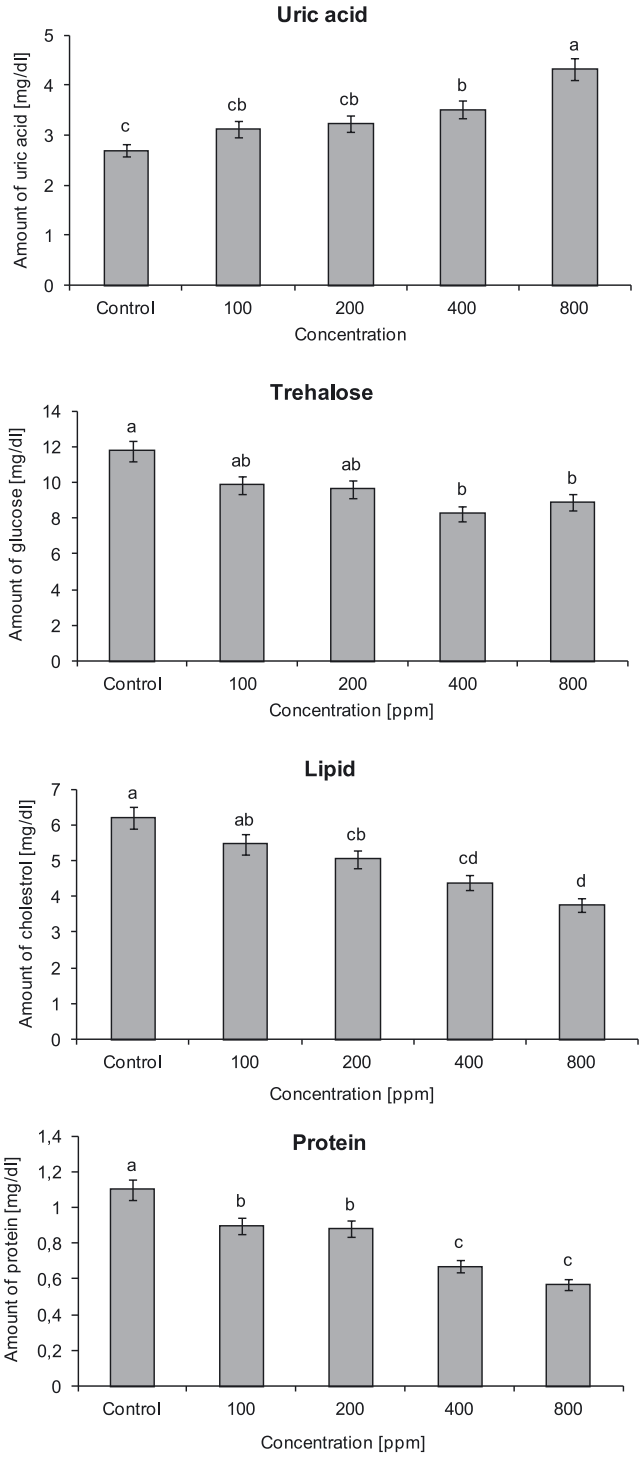

Fig. 4. Changes of biochemical compounds in hemolymph of *G. pyloalis* larvae due to different concentrations of Achook. Statistical differences are shown by a different letter in Tukey's test ($p < 0.05$)

DISCUSSION

Azadirachtin is the predominant biologically active chemical in most plant-based bioassays and is known as the 'most potent insect antifeedant discovered to date' (Miller *et al.* 2006). Our study showed that by increasing concentration, deterrence index increases and a good dose-response is achieved. *Spodoptera littoralis* Boisduval, *S. frugiperda* Smith, *Heliothis virescens* Fabricius, and *Helicoverpa armigera* Hübner all respond behaviorally to azadirachtin and do not feed on discs impregnated with the compound at concentrations of 0.1–10 ppm dependent on species (Blaney and Simmonds 1988). Feeding behavior depends upon both neural input from the insect's chemical senses (taste receptors on tarsi, mouthparts, and oral cavity) and central nervous integration of this 'sensory code'. Azadirachtin stimulates specific 'deterrent' cells in chemoreceptors and also blocks the firing of 'sugar' receptor cells, which normally stimulate feeding (Simmonds and Blaney 1984; Blaney *et al.* 1990; Simmonds *et al.* 1990). This results in an inhibition of feeding, culminating in the starvation and death of these species by feeding deterrence alone (Koul and Wahab 2004).

Based on nutritional analysis, neem insecticide significantly reduced relative consumption and growth rate. These results are similar to our previous work in which we used *Artemisia annua* L. methanolic extract (Khosravi *et al*. 2010). The reductions caused by neem may be due to the dangerous effects of plant allelochemicals on the peritrophic membrane, and damage to cellular surfaces of the midgut. Low RGR with increasing concentration, shows a low quality of food probably acting as an inhibitor. Lack of specific components, particularly nitrogen or water, leads to a lower growth rate and lower metabolic efficiency (Schoonhoven *et al*. 2005). Similarly, Senthil-Nathan (2006b) reported that crude extract of *Melia azedarach* L. reduced RGR, ECI, ECD, and CI (consumption index) in *Cnaphalocrosis medinalis* Guenée larvae. The percentage of reduction in ECI and ECD results from a foodstuff conversion deficiency, which promotes growth, perhaps through a diversion of energy from biomass production into detoxification (Senthil-Nathan and Kalaivani 2005). The effects of azadirachtin on consumption and inhibition of growth of *G. pyloalis* could be attributed to the lowered activities of both the proteases and α-amylase in the midgut, and the inhibition on feeding behavior.

In the present investigation, the activity of digestive enzymes was used as a parameter for studying the effect of azadirachtin. α-amylase (a-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1) is an enzyme that degrades starch (first to oligosaccharides and then in turn to maltose and glucose), by hydrolyzing α-1,4-glucan bonds (Terra and Ferriera 2005). In our study, treatment of fifth instar *G. pyloalis* larvae with azadirachtin showed a reduction in the activity level of α-amylase. This reduction increased with higher concentrations of the plant extract. Shekari *et al.* (2008) also showed the α-amylase activity level in elm leaf beetle treated with *A. annua* extract, decreased after 24 h and sharply increased after 48 h. *Artemisia annua* extract caused the reduction of α-amylase activity in *Pieris rapae* L. as was shown by Hasheminia *et al.* (2011). The reduction of α-amylase activity by plant extracts could be due to the plant defense compounds that act on insect gut enzymes, α-amylases, and proteinases (Ryan 1990; Franco *et al*. 2002). Also, the reduction of this enzyme activity could be due to a cytotoxic effect of different extracts on epithelial cells of the midgut, which synthesize α-amylase (Jbilou *et al*. 2008).

After amylase, glycosidases digest carbohydrate oligomers to monosaccharides (Terra and Ferriera 2005). Glycosidase is an exo-type carbohydrolase that catalyzes the liberation of α-glucoside from the non-reducing end of the substrate (Kimura 2004).

It was found that neem decreased the activity level of α- and β-glucosidases. Such a decrease in activity was reported in other insects. The results of Zibaee *et al*. (2010) showed that treatment of *H. cunea* Drury larvae with sublethal concentrations of different biopesticides showed a reduction in the activity level of α- and β-glucosidases. It was demonstrated by Zibaee and Bandani (2009) that *A. annua* extract significantly decreased the activity of α- and β-glucosidases on *E. integriceps* Puton. Increasing plant extract concentrations enhanced the enzyme inhibition that emphasizes disruption of consumption rates and food conversion efficiencies.

Proteases hydrolyze proteins to amino acids, classified as endopeptidases (EC 3.4.21-24) and exopeptidases (EC 3.2.4.11-19), based on their catalytic mechanism (Pascual-Ruiz *et al.* 2009). Our results showed that azadirachtin caused a reduction of the protease activity in *G. pyloalis*. This reduction increased with higher concentrations of neem, which coincides with other reports on insects. It has also been reported that digestive protease of *C. medinalis* was suppressed by extracts of *Vitex negundo* L. and *Azadirachta indica* A. Juss (Senthil-Nathan *et al.* 2006c). Compared with the controls, protease activity was reduced using 0.013 and 0.026 (gram leaf equivalent/ml) gle/ml concentrations of *A. annua* methanol extract 48 h post-treatment in *G. pyloalis* larvae as was demonstrated by Khosravi *et al.* (2011). Johnson *et al*. (1990) showed that botanical insecticides may interfere with the production of certain types of proteases and disable them to digest ingested proteins.

Lipases play a major role in storage and lipid mobilization. These enzymes are also the basic components in many of physiological process like, reproduction, growth, and defense against pathogens. Senthil-Nathan *et al*. (2006c) showed that treating *C. medinalis*, the rice leaf folder, with *Btk* (*Bacillus thuringiensis* Kurstaki), NSKE (neem seed kernal), and VNLE (*Vitex negundo* leaf extract) (azadirachtin and neem components) sharply decreased the activity level of lipase in the midgut. Azadirachtin inhibits peristalsis, reduces enzyme production as food moves through gut, inhibits midgut cell replacement, and reduces feeding (Anuradha and Annadurai 2008).

Lactate dehydrogenase (LDH) (EC 1.1.1.28) is an important glycolytic enzyme that is present in virtually all tissues (Kaplan and Pesce 1996). It is involved in carbohydrate metabolism and has been used as an indicative criterion of exposure to chemical stress (Diamantino *et al*. 2001). Lactate dehydrogenase is involved in the production of energy, being particularly important when a con-

siderable amount of additional energy is rapidly required. By treating neem on fifth instar larvae of *G. pyloalis*, the activity of this enzyme was found to decrease. Senthil-Nathan and Kalviani (2005) showed that the activity level of LDH in *Spodoptera litura* Fabricius decreases in midgut after feeding on *Ricinus communis* L. treated with azadirachtin and nucleopolyhedrovirus, which demonstrates the low nutritional efficiency of the larvae.

Huang *et al*. (2004) showed that azadirachtin significantly influenced the protein level in *Spodoptera littoralis* Fabricius. Other research where azadirachtin interfered with protein synthesis, is in the desert locust (Annandurai and Rembold 1993), and hemolymph of *S. litura* (F.) (Li *et al*. 1995). This phenomenon is probably due to the breakdown of proteins into free amino acids which are shunted into the TCA cycle as keto acids. Proteins are key organic constituents, and play a vital role in the compensatory mechanisms of insects during stress. There is an adverse correlation between the amount of protein and uric acid in hemolymph; it means those larvae with lowered protein have higher uric acid. That is because of the use of protein in the recovery of the damaged tissues, and producing of uric acid as the side product of protein catabolism (Zibaee *et al*. 2011). Lipids have different advantages in the physiological processes of insects. There is a higher energy production in comparison with carbohydrates and proteins, a source of metabolic water in dry conditions as well as the ability of lipids to be the basement molecule to produce carbohydrates (Chapman 1998). Different concentrations of neem decreased the amount of lipid in the hemolymph of fifth instar larvae of *G. pyloalis*. Khosravi *et al*. (2010) showed that the total lipid of fifth instar larvae decreased after feeding on treated mulberry leaves with *A. annua* extract. Zibaee *et al*. (2011) demonstrated that with the highest pyriproxifen concentration, the amount of lipid significantly decreased in the hemolymph of *E. integriceps*. Several activities of insects depend on carbohydrate metabolism. The amount of glucose shows the availability of this sugar for carbohydrate metabolism in insect cells (Satake 2000). Our results show that the amount of glucose decreased in larvae treated with different concentrations of azadirachtin, but a significant difference was only observed with the 400 and 800 ppm concentrations.

CONCLUSIONS

In conclusion, the feeding deterrence and nutritional physiology effects of azadirachtin reported in the present study, demonstrate its potential for controlling the mulberry pyralid population. A higher enzyme activity in the midgut of the control insects is most probably due to consumption and utilization of large quantities of food. Enzyme production is clearly related to the feeding behavior (amount of food that passes through the alimentary canal). There is a significant variation among the properties of insect digestive enzymes. For this reason, it is necessary to have more information on the gut enzymatic activity of insects to devise a rational strategy for insect pest control utilizing plant extracts. The activity of these enzymes is related to the physiological condition of the lesser mulberry pyralid and reflects the absorption, digestion, and positive transport of nutrients in the midgut.

REFERENCES

Annadurai R.S., Rembold H. 1993. Azadirachtin A modulates the tissue-specific 2D polypeptide patterns of the desert locust, *Schistocerca gregaria*. Naturwissenschaften 80 (3): 127–130.

Anuradha A., Annadurai R.S. 2008. Biochemical and molecular evidence of azadirachtin binding to insect actins. Curr. Sci. 95 (11): 1588–1593.

Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. 72 (1–2): 248–254.

Bernfeld P. 1955. α-amylases. Meth. Enzymol. 1: 149–151.

Blaney W.M., Simmonds M.S.J.1988. Food selection in adults and larvae of three species of Lepidoptera: a behavioural and electrophysiological study. Entomol. Exp. Appl. 49 (1–2): 111–121.

Blaney W.M., Simmonds M.S.J., Ley S.V., Anderson J.C., Toogood P.L. 1990. Antifeedant effects of azadirachtin and structurally related compounds on lepidopterous larvae. Entomol. Exp. Appl. 55 (2): 149–160.

Champagne D.E., Koul O., Isman M.B., Scudder G.G.E., Towers G.H.N. 1992. Biological activity of limonoids from the Rutales. Phytochem. 31 (2): 377–394.

Chapman R.F. 1998. The Insect: Structure and Function. 4th ed. Cambridge University Press, 788 pp.

Connolly J.D. 1983. Chemistryof the Meliaceae and Cneoraceae. p. 175–213. In: "Chemistry and Chemical Taxonomy of the Rutales" (P.G. Waterman, M.F. Grunden, eds.). Academic Press, London, 255 pp.

Copping L.G., Menn J.J. 2001. Biopesticides: a review of their action, applications and efficacy. Pest Manage. Sci. 56 (8): 651–676.

Diamantino T.C., Amadeu E., Soaresa M.V.M., Guilherminoc L. 2001. Lactate dehydrogenase activity as an effect criterion in toxicity tests with *Daphnia magna* Straus. Chemosphere 45 (4–5): 553–560.

Franco O.L., Riggen D.J., Melo F.R., Bloch C., Silva C., Grossi de sa M.F. 2002. Activity of wheat α-amylase inhibitors towards Bruchid α-amylases and structural explanation of observed specificities. Eur. J. Biochem. 267 (8): 2166–2173.

García-Carreño F.L., Haard N. 1993. Characterization of protease classes in langostilla *Pleuroncodes planipes* and crayfish *Pacifastacus astacus* extracts. J. Food Biochem. 17 (2): 97–113.

Hasheminia S.M., Jalali Sendi J., Talebi Jahromi K., Moharramipour S. 2011. The effect of *Artemisia annua* L. and *Achillea millefolium* L. crude leaf extracts on the toxicity, development, feeding efficiency and biochemical activities of small cabbage *Pieris rapae* L. (Lepidoptera: Pieridae). Pestic. Biochem. Physiol. 99 (3): 244–249.

Huang Z.W., Shi P., Dai J.Q., Du J.W. 2004. Protein metabolism in *Spodoptera litura* (F.) is influenced by the botanical insecticide azadirachtin. Pestic. Biochem. Physiol. 80 (2): 85–93.

Isman M.B., Koul O., Łuczyński A., Kamiński A. 1990. Insecticidal and antifeedant bioactivity of neem oils and their relationship to azadirachtin content. J. Agric. Food Chem. 38 (6): 1406–1411.

Jbilou R., Amri H., Bouayad A., Ghailani A., Ennabili A., Sayah F. 2008. Insecticidal effects of extracts of seven plant species on larval development, α-amylase activity and off spring production of *Tribolium castaneum* (herbst) (insecta: *Coleoptera*: *Tenebrionidae*). Biores. Technol. 99 (5): 959–964.

Johnson D.E., Brookhart G.L., Kramer K.J., Barnett B.D., McGaughey W.H. 1990. Resistance to *Bacillus thuringiensis* by the Indian meal moth *Plodia interpunctella*: Comparison of midgut proteinase from susceptible and resistant larvae. J. Inver. Pathol. 55 (2): 235–244.

Kaplan L.A., Pesce A.J. 1996. Clinical Chemistry – Theory Analysis and Correlation. Mosby-Year Book, MO, 1064 pp.

Keita S.M., Vincent C., Schmit J.P., Arnason J.T., Belanger A. 2001. Efficiency of essential oil of *Ocimum basilicum* L. and *O. gratissimum* L. applied as an insecticidal fumigant and powder to control *Callosobruchus maculatus* (Fab.) (Coleoptera: Bruchidae). J. Stored Prod. Res. 37 (4): 339–349.

Khosravi R., Jalali Senni J. 2010. Biology and demography of *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae) on mulberry. J. Asia-Pac. Entomol. 13 (4): 273–276.

Khosravi R., Jalali Sendi J., Ghadamyari M. 2010. Effect of *Artemisia annua* L. on deterrence and nutritional efficiency of lesser mulberry pyralid *Glyphodes pyloalis* (Lepidoptera: Pyralidae). J. Plant Prot. Res. 50 (4): 423–428.

Khosravi R., Sendi J.J., Ghadamyari M., Yezdani E. 2011. Effect of sweet wormwood *Artemisia annua* crude leaf extracts on some biological and physiological characteristics of the lesser mulberry pyralid, *Glyphodes pyloalis*. J. Insect Sci. 11 (156): 1–13.

Kimura K., Lee J.H., Lee I.S., Lee H.S., Park K.H., Chiba S., Kim D. 2004. Two potent competitive inhibitors discriminating α-glucosidase family I, from family II. Carbohydr. Res. 339 (6): 1035–1040.

King J. 1965. The dehydrogenases or oxidoreductases. Lactate dehydrogenase. p. 83–93. In: "Practical Clinical Enzymology" (D. van Nostrand, ed.). Van Nostrand company Ltd., London, 214 pp.

Koul O., Wahab S. 2004. Neem: Today and in the New Millenium. pp. 244–291. In: "Present Concepts of the Mode of Action of Azadirachtin from Neem" (R.J. Mordue, ed.). Kluwer Academic Publishers, 291 pp.

LeOra Software, Polo-Pc .1987. A User Guide to Probit or Logit Analysis, LeOra software, Berkeley, California, 22 pp.

Li X.D., Chen W.K., Hu M.Y. 1995. Studies on the effects and mechanisms of azadirachtin and rhodojaponin- on *Spodoptera litura* (F.). J. South China Agric. Uni. 16 (2): 80–85.

Miller T.A., Lampe D.J., Lauzon C.R. 2006. Transgenic and paratransgenic insects in crop protection. p. 87–103. In "Insecticide Design Using Advanced Technologies" (I. Ishaaya, R. Nauen, R. Horowitz, eds.). Germany: Springer-Verlag, Heidelberg, 267 pp.

Mordue A.J., Blackwell, A. 1993. Azadirachtin: an update. J. Insect Physiol. 39 (11): 903–924.

Pascual-Ruiz S., Carrillo L., Alvarez-Alfagemeyy F., Ruiz M., Castan P., Ortego F. 2009. The effects of different prey regimes on the proteolytic digestion of nymphs of the spined soldier bug, *Podisus maculiventris* (Hemiptera: Pentatomidae). Bull. Entomol. Res. 99 (5): 487–491.

Rahman M.F., Siddiqui M.K.J. 2004. Biochemical effects of vepacide (from *Azadirachta indica*) on Wistar rats during subchronic exposure. Ecotoxicol. Environ. Saf. 59 (3): 332–339.

Richmond W. 1973. Preparation and properties of cholesterol oxidase from *Nocardia* sp. and its application to enzymatic assay of total cholesterol in serum, Clinic. Chemis. 19 (12): 1350–1356.

Ruscoe C.N.E. 1972. Growth disruption effects of an insect antifeedant. Nature New Biol. 236 (5343): 159–160.

Ryan C.A. 1990. Protease inhibitors in plants: genes for improving defenses against insects and pathogens. Annu. Rev. Phytopath. 28: 425–449.

SAS Institute. 1997. SAS/STAT User's Guide for Personal Computers, SAS Institute, Cary, NC, 18 pp.

Satake S., Kawabe Y., Mizoguchi A. 2000. Carbohydrate metabolism during starvation in the silkworm *Bombyx mori* L. Arch. Insect Biochem. Physiol. 44 (2): 90–98.

Schmutterer H. 1990. Properties and potential of natural pesticides from the neem tree, *Azadirachta indica*. Annu. Rev. Entomol. 35: 271–297.

Schoonhoven L.M., van Loon J.J.A., Dicke M. 2005. Insect-Plant Biology. 2nd ed. Oxford University Press, 409 pp.

Senthil-Nathan S., Kalaivani K. 2005. Efficiency of nucleopolyhedrovirus (NPV) and azadirachtin on *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae). Biol. Con. 34 (1): 93–98.

Senthil-Nathan S., Kalaivani K., Chung K., Murugan K. 2006a. Effect of neem limonoids on lactate dehydrogenase (LDH) of the rice leaffolder, *Cnaphalocrocis medinalis* (Guenee) (Insecta: Lepidoptera: Pyralidae). Chemosphere 62 (8): 1388–1393.

Senthil-Nathan S., Kalaivani K., Chung K., Murugan K. 2006b. The toxicity and behavioural effects of neem limonoids on *Cnaphalocrocis medinalis* (Guenee) the rice leaffolder. Chemosphere 62 (8): 1381–1387.

Senthil-Nathan S., Chunga P.G., Muruganb K. 2006c. Combined effect of biopesticides on the digestive enzymatic profiles of *Cnaphalocrocis medinalis* (Guenee) (the rice leaffolder) (Insecta: Lepidoptera: Pyralidae). Ecotoxic. Environ. Saf. 64 (3): 382–389.

Senthil-Nathan S., Kalaivani K., Murugan K., Chung P.G. 2005a. The toxicity and physiological effect of neem limonoids on *Cnaphalocrocis medinalis* (Guenee) the rice leaffolder. Pestic. Biochem. Physiol. 81 (2): 113–122.

Senthil-Nathan S., Kalaivani K., Murugan K., Chung P.G. 2005b. Efficacy of neem limonoids on *Cnaphalocrocis medinalis* (Guenee) (Lepidoptera: Pyralidae) the rice leaf folder. Crop Prot. 24 (12): 760–763.

Shekari M., Jalali Sendi J., Etebari K., Zibaee A., Shadparvar A. 2008. Effects of *Artemisia annua* L. (Asteracea) on nutritional physiology and enzyme activities of elm leaf beetle, *Xanthogaleruca luteola* Mull. (Coleoptera: Chrysomellidae). Pestic. Biochem. Physiol. 91 (1): 66–74.

Siegert K.J. 1987. Carbohydrate metabolism in Manduca sexta during late larval development. J. Insect Physiol. 33 (6): 421–427.

Simmonds M.S.J., Blaney W.M. 1984. Some effects of azadirachtin on lepidopterous larvae. p. 163-180. In: "Natural Products from the neem Tree and Other Tropical Plants" Proceedings 2nd International Neem conference, 25–28 May, Germany, 345 pp.

Simmonds M.S.J., Blaney W.M., Ley S.V., Anderson J.C., Toogood P.L. 1990. Azadirachtin: structural requirements for reducing growth and increasing mortality in lepidopterous larvae. Entomol. Exp. Appl. 55 (2): 169–181.

Tapondjou L.A., Adler C., Bouda H., Fontem D.A. 2002. Efficacy of powder and essential oil from *Chenopodium ambrosioides* leaves as post-harvest grain protectants against six-stored product beetles. J. Stored Prod. Res. 38 (4): 395–402.

Terra W.R., Ferriera C. 2005. Biochemistry of digestion. p. 171–224. In: "Comprehensive Molecular Insect Science" (I.G. Lawrence, I. Kostas, S.G. Sarjeet, eds.). Vol. 3, Elsevier,

Tsujita T., Ninomiya H., Okuda H. 1989. P-nitrophenyl 865. butyrate hydrolyzing activity of hormone–sensitive lipase 866 from bovine adipose tissue. J. Lipid Res. 30 (7): 997–1004.

Valovage W.D., Brooks M.A. 1979. Uric acid quantities in the fat body of normal and aposymbiotic german cockroaches *Blatella germanica*. Annu. Entomol. Soc. Am. 72 (5): 687–689.

Waldbauer G.P. 1968. The consumption and utilization of food by insects. Adv. Insect Physiol. 5: 229–288.

Zibaee I., Bandani A.R., Sendi J.J., Talaei-Hassanlouei R., Kouchaki B. 2010. Effects of *Bacillus thurengiensis* var. *kurstaki*, and medicinal plants (*Artemisia annua* L.) and (*Lavandula stoechas* L.) extracts on digestive enzymes and Lactate dehydrogenase of *Hyphantria cunea* Drury (Lepidoptera: Arctiidae). Invertebr. Surv. J. 7 (2): 251–261.

Zibaee A., Bandani A.R. 2009. Effects of *Artemisia annua* L. (Asteracea) on the digestive enzymatic profiles and the cellular immune reactions of the Sun pest, *Eurygaster integriceps* (Heteroptera: Scutellaridae), against *Beauveria bassiana*. Bull. Entomol. Res. 100 (2):185–196.

Zibaee A., Zibaee I., Sendi J.J. 2011. A juvenile hormone analog, pyriproxifen, affects some biochemical components in the hemolymph and fat bodies of *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae). Pestic. Biochem. Physiol. 100 (3): 289–298.