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RESEARCH REPORT

Effects of *Bacillus thuringiensis* var. *kurstaki* and medicinal plants on *Hyphantria cunea* Drury (Lepidoptera: Arctiidae)

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Abstract

The fall armyworm, *Hyphantria cunea* Drury (Lepidoptera: Arctiidae) is an insect native to North America that was recently introduced into Iran resulting in severe damage to trees and agricultural production. An experiment was conducted to examine potential effects of medicinal plants, *Artemisia annua* and *Lavandula stoechas* and the insect pathogenic bacterium *Bacillus thuringiensis* var. *kurstaki* on activities of digestive enzymes (*α*-amylase, *α*- and *β*-glucosidase, lipase and proteases) and lactate dehydrogenase (LDH) in *H. cunea* by using two hosts, mulberry and sycamore. Results showed that *B. thuringiensis* var. *kurstaki* and plant extracts when administered orally, affected the digestive enzyme profiles of *H. cunea*. Combined effect of *B. thuringiensis*, *A. annua* and *L. stoechas* extracts on mulberry decreased the activities of digestive enzymes in a dose-related manner, except for *β*-glucosidase and lipase. When larvae were treated by different concentrations of the mentioned insecticides, LDH activity increased i.e. the higher activity was obtained by *B. thurengiensis* alone and *B. thurengiensis* and *L. stoechas* extracts together. The least activity was observed in the case of *L. stoechas* extracts alone on both hosts. Physiological analysis would be particularly informative when using combination of biopesticides to enhance the efficiency of a safe management process.

Key Words: *Hyphantria cunea*; *Bacillus thuringiensis* var. *kurstaki*; *Artemisia annua* extract; *Lavandula stoechas* extract; digestive enzymes; lactate dehydrogenase

Introduction

The fall armyworm, *Hyphantria cunea* Drury (Lepidoptera: Arctiidae) is an insect native in North America that is presently distributed in many areas in the northern hemisphere (Warren and Tadic, 1970) and New Zealand (Kean and Kumarasinghe, 2007). It has been introduced to different areas of Europe and Asia (Li *et al*., 2001). Since, 2002, *H. cunea* established itself in northern areas of Iran, causing severe damage to trees. It is a multivoltine pest feeding on leaves of trees and hibernates as a pupa in soil around the damaged trees. Research has been conducted to look for natural plant protection compounds such as botanical insecticides, antifeedants and microorganisms such as fungi and bacteria.

Bacillus thuringiensis is a Gram-positive, soil-dwelling bacterium which is commonly used as a pesticide. The genus *Artemisia* is a member of a large plant family Asteraceae (Compositae) encompassing more than 300 different species of this diverse genus (Shekari *et al*., 2008). The species *A. annua*, known as sweet worm wood, grows widely in Europe and America and is grown in China, Turkey, Vietnam, Afghanistan and Australia (Bhakuni *et al*., 2001; Shekari *et al*., 2008). Several isolated compounds from this species have shown antimalarial, antibacterial, antiinflamatory, plant growth regulatory and cytotoxicity (antitumor) activities (Akhtar and Isman, 2004). *Lavandula stoechas* (French Lavender), Lamiaceae, occurs naturally in the Mediterranean region and is a perennial shrub that grows to 30-100 cm tall. It was declared a toxic weed and has potential for use as a botanical insecticide.

Reduced efficacy of synthetic insecticides has been highlighted in the last two decades. The first alternative was *B. thuringiensis* Berliner (Bt), but the increasing number of reports on the resistance to Bt led also to choose other biological insecticides such as those coming from plants (Senthil Nathana *et al.,* 2006). Several studies have investigated the

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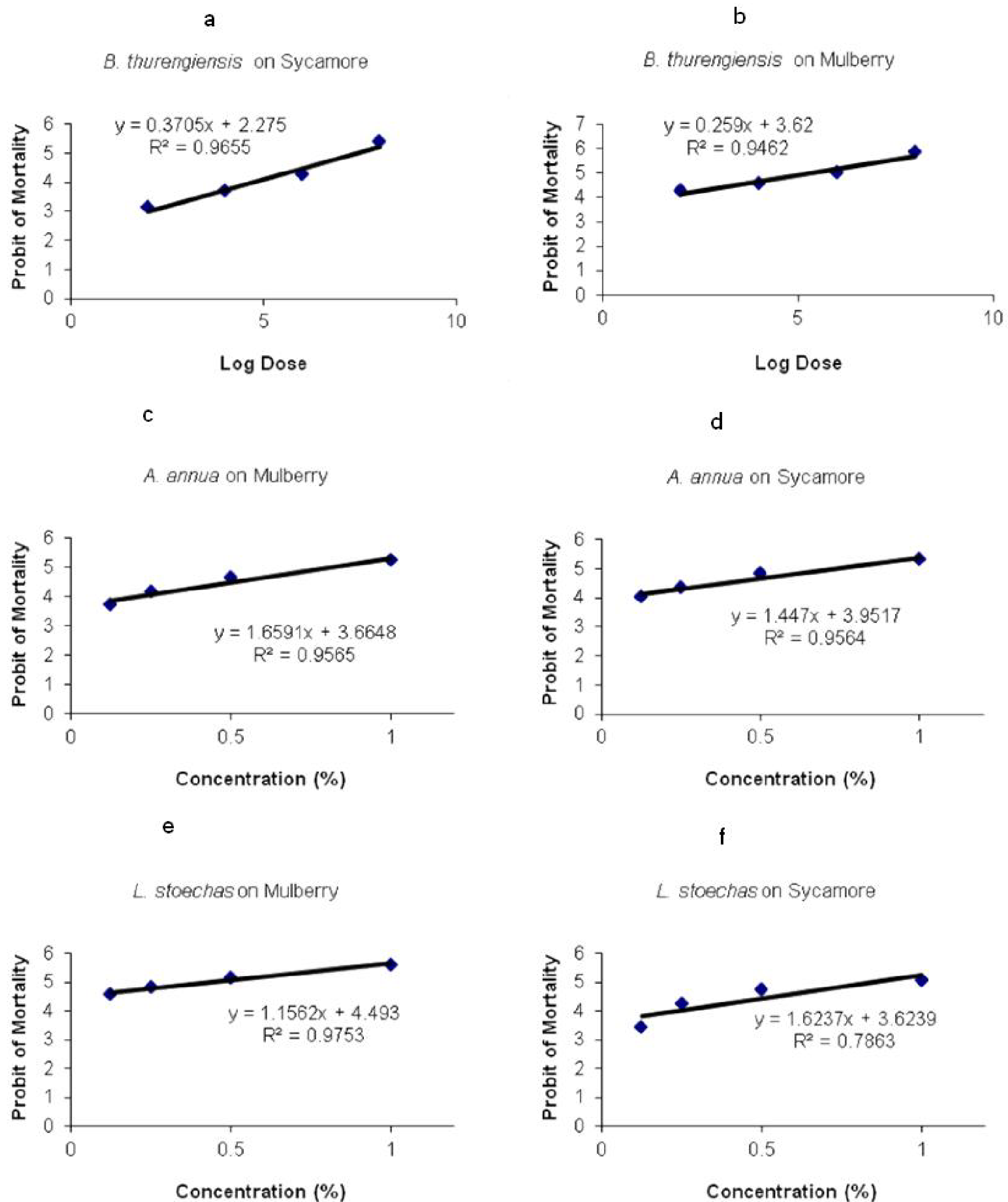

Fig. 1 Mortality probit of the *Bacillus thurengiensis*, *Artemisia annua and Lavandula stoechas* in the presence of two hosts on the larvae of *Hyphantaria cunea.*

combination of Bt with baculoviruses, however other combinations should be explored (Senthil Nathana *et al.,* 2006). It is clear that botanical insecticides and microbes such as *B. thuringiensis* affect insect physiology in different ways including decrease of digestive enzyme activities. Hence, in this paper research was conducted to examine potential effects of two botanical insecticides and bacterial toxin on activities of digestive enzymes and lactate dehydrogenase (LDH) in the *H. cunea* in the presence of two hosts, mulberry, an important tree in orchards, and sycamore, an important tree in urban areas, in order to find more suitable ways to decrease its population and damage.

Materials and Methods

Insects

First instar larvae of *Hyphantria cunea* were collected from the field and reared separately on mulberry and sycamore to reach 4th instar larvae in the laboratory at 27 ± 2 °C under a 14 h light:10 h dark photoperiod. These larvae were used to initiate the experiments.

Preparation of Bacillus thurengiensis var. kurstaki and plant extracts

A stock suspension of *Bacillus thuringiensis var kurstaki* (10^9 spore/ml) was provided by Giah company (Iran) and a serial concentration prepared using distilled water. Medicinal plant (*Artemisia annua* and *Lavandula stoechas*) leaves were collected, washed with distilled water and dried at room temperature in the shade. Methanolic extraction was carried out according to the procedure described by Shekari *et al*. (2008). Briefly, 30 g of dried leaves were stirred with 300 ml of 85 % methanol in a flask, left for 48 h at 4 °C, then filtered through Whatman No.4 filter paper. The solvent was removed by vacuum in a rotary evaporator and the dark green residue was dissolved in 10 ml acetone and used as a starting stock solution. Further dilutions with either acetone or distilled water were used to prepare different concentrations.

Bioassay

Bioassays were performed with first instar larvae of *H. cunea* using 10^2, 10^4 and 10^6 spores/ml of Bt on mulberry and 10^4, 10^6 and 10^8 spore/ml on sycamore. Concentrations of 0.09, 0.22 and 0.42 % of *A. annua* on mulberry and concentrations of 0.13, 0.28 and 0.48 % 0n sycamore were used. Concentrations of 0.02, 0.11 and 0.32 of *L. stoechas* on mulberry and concentrations of 0.13, 0.38 and 0.79 on sycamore were used. Control leaves were treated with distilled water. For each treatment 30 larvae in three replicates were used in all the experiments and whole experiments were replicated twice. During the experiments, the larvae of each experimental condition were kept separately. The effective concentration (LC_{50}) was calculated after 24 h using Probit analysis (Finney, 1971). Fresh leaves were sprayed with different concentrations of the Bt, *A. annua*, and *L. stoechas* and allowed to air dry. Control leaves were treated with methanol alone. First instar larvae were starved for 4 h and then fed on leaves treated with the different concentrations of Btk, *A. annua*, and *L. stoechas*. The uneaten leaves were removed every 24 h and the larvae were fed fresh treated leaves until larvae reached to fourth instar when the biochemical experiments were initiated.

Sample preparation for enzymatic assay

Enzyme samples from the midguts of fourth instar larvae were prepared based on Zibaee and Bandani (2009). Briefly, larvae were randomly selected and their midguts were removed by dissection under a stereo microscope in ice-cold saline buffer (6 µmol/l NaCl). The midgut was separated from the insect body, rinsed in-cold saline buffer, placed in a pre-cooled homogenizer and ground in 1 ml of universal buffer containing succinate (5 mM), glycine (2 mM) and 2-morpholinoethanensulfonic acid (pH 7.2). The homogenates from both preparations were separately transferred to the 1.5 ml centrifuge tubes and centrifuged at 15000 rpm for 20 min at 4 °C. The supernatants were pooled and stored at -20 °C for subsequent analyses (Zibaee and Bandani, 2009).

Digestive enzyme assays

α-amylase activity

α-Amylase activity was assayed by the dinitrosalicylic acid (DNS) procedure (Bernfeld, 1955), using 1% soluble starch (Merck, Darmstadt, Germany) as substrate. Twenty microliters of the enzyme were incubated for 30 min at 35 °C with 500 µl universal buffer and 40 µl soluble starch. The reaction was stopped by addition of 100 µl DNS and heating in boiling water for 10 min. DNS is a color reagent hence, the reducing groups released from starch by *α*-amylase action were measured by the reduction of DNS. The boiling water stops the *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 at least three times.

α- and β-glucosidase activity

For solubilization of membrane hydrolyses (*α*, *β*-glocusidases) in Triton X-100, membrane preparations were exposed to Triton X-100 for 20 h at 40 ˚C, in a ratio of 10 mg of Triton X-100/mg of protein, before being centrifuged at 15,000 rpm for 30 min. No sediment was visible after the centrifugation of this supernatant at 10,000 rpm for 60 min. The activity of the enzymes remains unchanged, at -20C, for periods of at least a month (Ferreira and Terra, 1983). The *α*, *β*-glucosidases activity was assayed by incubating 50 µl of enzyme solution with 75 µl of *p*-nitrophenyl-*α*-D-glucopyranoside (*p*N*α*G) (5 mM), p-nitrophenyl-*β*-D-glucopyranoside (*p*N*β*G) (5 mM) and 125 µl of 100 mM universal buffer (pH 5.0) at 37 °C for 10 min. The reaction were stopped by adding 2 ml of sodium carbonate (1 M) and read at 450 nm (Ferreira and Terra, 1983).

Lipase activity

The enzyme assays were carried out as described by Tsujita *et al*. (1989). Thirty µl of midgut tissue extracts, 0.5 ml of universal buffer solution (1M) (pH 7.2), and 100 µl of *p*-nitrophenyl butyrate (50 mM), as substrate, were incorporated, mixed thoroughly and incubated at 37 °C. After 1 min, 100 µl distilled water was added to each tube (control and experimental samples) and absorbance was read at 405 nm. One unit of enzyme release 1.0

Table 1 Toxicity of *Bacillus thurengiensis*, *Artemisia annua* and *Lavandula stoechas* extracts on the larvae *Hyphantaria cunea*

Concentration[1]	*Bacillus thurengiensis*		*Artemisia annua*		*Lavandula stoechas*	
	Mulberry	Sycamore	Mulberry	Sycamore	Mulberry	Sycamore
LD_{10} 95% confidence interval[2]	10^2 10^1-10^4	10^4 10^3-10^5	0.09 0.03-0.13	0.13 0.07-0.18	0.02 0.001-0.06	0.13 0.05-0.20
LD_{30} 95 % confidence interval	10^4 10^3-10^6	10^6 10^5-10^7	0.22 0.16-0.28	0.28 0.22-0.35	0.11 0.03-0.18	0.38 0.27-0.51
LD_{50} 95 % confidence interval	10^6 10^6-10^8	10^8 10^7-10^{10}	0.42 0.33-0.63	0.48 0.38-0.71	0.32 0.20-0.49	0.79 0.20-0.57
L_{90} 95 % confidence interval	10^9 10^8-10^{11}	10^{12} 10^{10}-10^{14}	1.93 1.06-8.14	1.76 1.05-5.48	4.12 1.69-5.46	4.77 2.28-8.17
Slope±SE	0.34-0.073	0.25-0.056	1.95±0.50	2.29±0.54	1.15±0.35	1.64±0.40
X^2 (df)	4.70	4.44	2.46	2.37	2.83	0.82
p-value	0.48	1.14	0.72	0.70	0.16	0.64

[1]Concentration of *Bacillus thurengiensis* is spore/ml and plant extracts are percentage
[2]Confidence limits have been calculated with 95 % confidence

nanomole (10-9 mole) of p-nitrophenol per minute at pH 7.2 at 37 °C using p-nitrophenyl butyrate as substrate. The negative control tube was placed in a boiling water bath for 15 min to destroy the enzyme activity and then cooled prior to be added with the substrate.

Protease activity

General protease activity of adult midguts was determined using azocasein as substrate (Elpidina *et al*., 2001). The reaction mixture was 80 µl of 2 % azocasein solution in 40 mM universal buffer of specified pH and 30 µl enzyme. The reaction mixture was incubated at 37 °C for 60 min. Proteolysis was stopped by 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 and the absorbance was recorded at 440 nm.

Lactate dehydrogenase (LDH) assay

For evaluating lactate dehydrogenase (LDH), the King's (1965) method was used. To standardize volumes, 0.2 ml NAD^+ solution was added to the test tubes and 0.2 ml of water was added to control test tubes, each containing 1 ml of the buffered substrate. 0.01 ml of the sample 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 and the incubation 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.

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 mortality and lethal concentration were obtained by using Probit analysis (Robertson *et al*., 2007) and POLO-PC software (Leora, 1987). In this case, significant differences among the concentrations were recorded when 95 % confidence intervals (CI) did not overlap. Other data were compared by one-way analysis of variance (ANOVA) followed by Tukey's studentisized test when significant differences were found at $p = 0.05$ (SAS, 1997). Differences among samples were considered statistically significant ($p < 0.05$).

Results

Dose-response relationships

The results showed that plant extracts and bacterial toxins produced a dose response in the insect species on both host species (Fig. 1, Table 1). The LD_{50} values of *B. thurengiensis* were significantly different in sycamore and mulberry (Table 1). In the case of *A. annua* extracts, no significant difference was observed between sycamore and mulberry, whereas extracts from *L. stoechas* produced effects similar to those observed for *B. thurengiensis* (Table 1).

Effect of Btk and plant extracts on digestive enzymes

Results showed that *B. thuringiensis* and plant extract affected the digestive enzymatic profiles of *H. cunea* at several concentrations by using oral ingestion treatment in the presence of two hosts (Tables 2-6). When larvae fed on leaves treated by Btk, activity of all digestive enzymes was decreased and showed a dose-related status (Table 2). *A. annua* treatment decreased digestive enzyme activities in larvae feed on both mulberry and sycamore in a dos-related manner (Table 3). Treatment of leaves by *L. stoechas* demonstrated a slightly decrease on digestive enzymes except for protease and lipase. However, the effect of *L. stoechas* extracts on enzyme activities on sycamore was more with regard to mulberry (Table 4).

Combined effect of *B. thuringiensis* and *A. annua* on mulberry showed that digestive enzyme activities decreased except for *β*-glucosidase and lipase (Table 5). Similar results were found in the case of *B. thuringiensis* and *L. stoechas* (Table 6).

Effect of Btk and plant extracts on lactate dehydrogenase activity

Table 7 shows effect of *B. thuringiensis*, *A. annua* and *L. stoechas* on LDH activity of *H. cunea*. Results demonstrated that LDH activity increased by treating different concentrations of insecticides on larvae and the higher activity was obtained by *B. thuringiensis* alone and *B. thuringiensis* and *L. stoechas* together. The least activity was observed in the case of *L. stoechas* alone. Similar results found when sycamore was used as host.

Discussion

Crude botanical components for various purposes were well known in traditional cultures for centuries (Schmutter, 1990). Their extracts and active ingredients are a good choice for different investigations including pest management tactics.

Here we observed that the treatment of *B. thuringiensis*, *A. annua* and *L. stoechas* separately as well as the combined effect of bacteria and plant extracts exerted a significant effect on *H. cunea* digestive enzyme and LDH when spread onto two plant hosts, namely mulberry and sycamore. Several studies have shown that feeding is necessary for the stimulation of digestive enzyme activities (Sibley, 1981; Broadway and Duffey, 1988). Results demonstrated that sublethal doses of these biopesticides individually decreased digestive enzyme activities such as *α*-amylase, *α*- and *β*-glucosidase, lipase and protease and increased LDH activity. Higher enzyme activities in the midgut of control insects are most probably due to consumption and utilization of large quantities of food (Senthil-Nathan *et al*., 2006). Imbalance in enzyme-substrate complex and inhibition of peristaltic movement of the gut (Hori, 1969) might have inhibited the enzyme activities in the treated insects (Zibaee and Bandani, 2009).

It is clear that exposure of diet to botanical insecticides has significant effects on several enzyme activities found in the late instar larvae of *H. cunea*. Botanical insecticides may interfere with the production of certain types of proteins (Smirle *et al*., 1996; Senthil-Nathan *et al*., 2006). In the case of decreasing activity of digestive enzymes due to *B. thuringiensis* treatment, this bacterium causes damage to the epithelial cells of the midgut through crystalline parasporal bodies, which release the active toxin after digestion by serine proteases under the alkaline conditions in the intestinal fluid (Senthil-Nathan *et al*., 2006). Therefore one would expect that such damage to the midgut would cause a decrease in digestive enzyme activities (Eguchi *et al*., 1972; Mathavan *et al*., 1989; Smirle *et al*., 1996; Senthil Nathan *et al*., 2006).

α-Amylase is an endo-digestive enzyme that catalyzes the breakdown of 1:4-α-glucosidase bonds in polysaccharides and converts starches into maltose (disaccharide) and glycogen into glucose. In the many studies, pesticides including *B. thuringiensis*, synthetic chemicals and botanical components significantly decreased the activity of *α*-amylase in the midgut of different insects (Senthil-Nathan *et al*., 2006; Shekari *et al*., 2008; Zibaee *et al*., 2008; Zibaee and Bandani, 2009). Saleem and Shakoori (1987) showed that sublethal concentrations of pyrethroids decreased the *α*-amylase activity in larval gut of the beetle *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae). Lee *et al*. (1994) showed that some IGRs decreased the activity level of α-amylase and esterase in the treated larvae. Ascher and Ishaaya (2004) showed that the activity level of this enzyme increased 30 % in *S. littoralis* Boisd (Lepidoptera: Noctuidae) treated with phentine acetate compared with control. Senthil-Nathan *et al*. (2006) found that *B. thuringiensis* decreased the activity level of this enzyme; the activity was much lower when bacterial spores and botanical components were combined. Zibaee *et al*. (2008) showed that along with elevation of spraying times, the activity level of *α*-amylase would sharply decrease in *Chilo suppressalis* Walker (Lepidoptera: Crambidae) larvae. Shekari *et al.* (2008) demonstrated that α-amylase activity level decreased 24 h after treatment and sharply increased at 48 h after treatment with *A. annua* extract of the elm leaf beetle. Similar results were found when adults of *Eurygaster integriceps* Puton (Heteroptera: Scutelleridae) fed on grain and water contained *A. annua* extract (Zibaee and Bandani, 2009). In this study we found that *B. thuringiensis*, *A. annua* and *L. lavandula*, individually, decreased activity level of *α*-amylase and the highest inhibitions were obtained when larvae had been fed on sycamore and combined exposures were made.

Table 2 Effect of *Bacillus thurengiensis* on the activity of different enzymes (μmol/min/mg protein) of *Hyphantaria cunea* larvae in the presence of two different hosts

Treatment[1]	*α*-amylase		*α*-Glucosidase		*β*-Glucosidase		Protease		Lipase	
	Mulberry	Sycamore	Mulberry	Sycamore	Mulberry	Sycamore	Mulberry	Sycamore	Mulberry	Sycamore
Control	2.44±0.20a	1.85±0.08a	2.72±0.34a	2.32±0.98a	2.91±1.35a	2.64±0.66a	3.54±0.00a	2.14±0.004a	4.00±0.008a	3.44±0.004a
LD_{10}	2.07±0.06ab	1.56±0.07ab	2.26±0.21a	1.62±0.40ab	3.74±0.36a	2.62±0.31a	3.08±0.001a	2.01±0.001ab	2.80±0.003b	2.8±0.004b
LD_{30}	1.69±0.06b	1.53±0.03b	1.60±0.32b	1.21±0.33b	2.84±0.12ab	1.77±0.60b	2.18±0.003b	1.13±0.00b	2.14±0.003c	2.02±0.002c
LD_{50}	1.15±0.14c	1.30±0.06b	1.58±0.44b	0.63±0.30c	2.45±0.30b	1.21±0.50b	0.71±0.001c	0.77±0.001c	1.49±0.003c	1.55±0.002c

[1]Concentration of is *B. thurengiensis* spore/ml. LD_{10}, LD_{30} and LD_{50} are 10^2, 10^4 and 10^6 on mulberry and 10^4, 10^6 and 10^8 on sycamore.
[2]Means ± SEM followed by the same letters indicate no significant difference ($p < 0.05$) according to the Tukey test.

Table 3 Effect of *Artemisia annua* extract on the activity of different enzymes (μmol/min/mg protein) of *Hyphantaria cunea* larvae in the presence of two different hosts

Treatment[1]	*α*-amylase		*α*-Glucosidase		*β*-Glucosidase		Protease		Lipase	
	Mulberry	Sycamore	Mulberry	Sycamore	Mulberry	Sycamore	Mulberry	Sycamore	Mulberry	Sycamore
Control	1.87±0.09a	1.75±0.28a	2.05±0.54a	1.90±0.4a	3.88±1.03a	2.67±0.28a	3.80±0.00a	2.36±0.00a	3.43±0.00a	3.34±0.00a
LD_{10}	1.44±0.05b	1.69±0.05a	1.39±0.10b	1.55±0.27b	2.48±0.07c	2.50±0.39b	3.16±0.00ab	1.62±0.00ab	2.79±0.00b	2.61±0.00ab
LD_{30}	1.13±0.00c	1.19±0.04ab	1.24±0.28b	±0.950.09c	1.39±0.14c	1.55±0.21c	1.88±0.00b	0.80±0.00b	2.00±0.00c	2.01±0.00b
LD_{50}	0.84±0.06c	0.99±0.03b	0.52±0.19c	0.14±0.08d	0.28±0.49d	1.25±0.31d	0.76±0.00c	0.50±0.00c	1.47±0.00d	0.56±0.00c

[1]Concentrations of plant extract are 0.09, 0.22 and 0.42 on mulberry and 0.13, 0.28 and 0.48 on sycamore as LD_{10}, LD_{30} and LD_{50}.
[2]Means ± SEM followed by the same letters indicate no significant difference ($p < 0.05$) according to the Tukey test.

Table 4 Effect of *Lavandula stoechas* extract on the activity of different enzymes (μmol/min/mg protein) of *Hyphantaria cunea* larvae in the presence of two different hosts

Treatment[1]	*α*-amylase		*α*-Glucosidase		*β*-Glucosidase		Protease		Lipase	
	Mulberry	Sycamore	Mulberry	Sycamore	Mulberry	Sycamore	Mulberry	Sycamore	Mulberry	Sycamore
Control	2.08±0.01a	1.97±0.03a	2.20±0.20a	1.49±0.91a	2.89±0.33a	2.61±0.21a	3.64±0.00a	3.51±0.00a	3.25±0.00a	2.77±0.02a
LD_{10}	2.05±0.03a	1.61±0.02b	1.77±0.65b	1.62±0.23	2.42±0.68a	2.46±0.48a	3.65±0.00a	3.43±0.00a	3.18±0.00a	2.49±0.00a
LD_{30}	1.89±0.03b	1.38±0.08b	2.37±0.74a	1.53±0.30a	2.71±0.12a	1.79±0.70a	3.42±0.00a	3.20±0.00a	2.91±0.00a	2.42±0.00a
LD_{50}	1.71±0.02b	1.11±0.05c	2.15±0.75a	1.54±0.34a	2.45±0.23a	1.41±0.23b	3.39±0.00a	3.25±0.00a	2.69±0.00a	2.38±0.00a

[1]Concentrations of plant extract are 0.02, 0.11 and 0.32 on mulberry and 0.13, 0.38 and 0.79 on sycamore as LD_{10}, LD_{30} and LD_{50}.
[2]Means ± SEM followed by the same letters indicate no significant difference ($p < 0.05$) according to the Tukey test.

Table 5 Combined effect of *Bacillus thurengiensis* and *Artemisia annua* extract on the activity of different enzymes (μmol/min/mg protein) of *Hyphantaria cunea* larvae on mulberry as the host

Treatment[1]	*α*-amylase	*α*-Glucosidase	*β*-Glucosidase	Protease	Lipase
Control	2.01±0.01a	1.99±0.021	3.32±0.80a	3.93±0.00a	3.16±0.00a
LD_{10}	2.00±0.018a	1.52±0.016b	2.89±0.34a	3.93±0.00a	3.14±0.00a
LD_{30}	1.72±0.03ab	1.45±0.08b	2.96±0.87a	2.82±0.00ab	3.14±0.00a
LD_{50}	0.86±0.06c	0.69±0.32b	2.61±0.87a	0.91±0.00b	3.07±0.00a

[1]Each LD value shows *B. thurengiensis* + plant extract concentration as LD_{10}: 10^2+ 0.09, LD_{30}: 10^4+0.22, LD_{50}: 10^6+0.42.
[2].Means ± SEM followed by the same letters indicate no significant difference ($p < 0.05$) according to the Tukey test.

Table 6 Combined effect of *Bacillus thurengiensis* and *Lavandula stoechas* extract on the activity of different enzymes (*μmol/min/mg* protein) of *Hyphantaria cunea* larvae in the presence of mulberry leaves as the host

Treatment[1]	*α*-amylase	*α*-Glucosidase	*β*-Glucosidase	Protease	Lipase
Control	1.93±0.03a	1.92±0.10a	2.99±0.79a	3.35±0.00a	3.30±0.00a
LD_{10}	1.75±0.11ab	1.77±0.30a	2.76±1.01a	1.05±0.00b	2.50±0.00b
LD_{30}	1.24±±0.19b	1.34±0.14ab	3.12±0.75a	0.33±0.00b	1.87±0.00b
LD_{50}	0.38±0.13c	1.20±1.08b	2.52±1.00b	0.14±0.00b	1.03±0.00c

[1]Each LD value shows *B. thurengiensis* + plant extract concentration as LD_{10}: 10^2+ 0.02, LD_{30}: 10^4+0.11, LD_{50}: 10^6+0.32.
[2]Means ± SEM followed by the same letters indicate no significant difference ($p < 0.05$) according to the Tukey test.

Table 7 Effect of *Bacillus thurengiensis*, *Artemisia annua* and *Lavandula stoechas* extract on Lactate dehydrogenase (μmol/min/mg protein) of *Hyphantaria cunea* larvae on mulberry and sycamore as the host

Treatment[1]	*Bacillus thurengiensis*		*Artemisia annua*		*Lavandula stoechas*		B.t. + *Artemisia annua*		B.t. + *Lavandula stoechas*	
	Mulberry	Sycamore	Mulberry	Sycamore	Mulberry	Sycamore	Mulberry	Sycamore	Mulberry	Sycamore
Control	0.17±0.05d	0.21±0.10d	0.19±0.08c	0.18±0.03c	0.23±0.07c	0.19±0.07b	0.20±0.04c	0.25±0.09d	0.23±0.031d	0.17±0.06c
LD_{10}	0.26±0.08c	0.35±0.08c	0.21±0.09c	0.22±0.01c	0.26±0.06b	0.21±0.03b	0.27±0.06b	0.38±0.03c	0.40±0.08c	0.34±0.02b
LD_{30}	0.50±0.10b	0.49±0.08b	0.35±0.07b	0.45±0.05b	0.27±0.07b	0.29±0.06ab	0.53±0.1bc	0.67±0.09b	0.61±0.19b	0.39±0.04b
LD_{50}	0.71±0.14a	0.95±0.02a	0.57±0.07a	0.81±0.04a	0.54±0.10a	0.48±0.07a	0.67±0.17a	0.97±0.12a	0.96±0.21a	0.86±0.09a

[1]Each LD value shows *B. thurengiensis* (B.t.) and plant extract alone and B.t. + plant extract concentration together in the presence of mulberry leaves as the host.
[2]Means ± SEM followed by the same letters indicate no significant difference ($p < 0.05$) according to the Tukey test.

The glycosidases catalyze the hydrolysis of terminal, non-reducing 1, 4-linked α-D-glucose residues with release of α-D-glucose. Study of glucosidase in herbivorous insects is important not only for understanding digestion biochemistry but also for developing insect pest management strategies. Plants produce a wide variety of allelochemicals which act as defensive compounds. These include alkaloids, cyanogenic and triterpenoid glycosides, phenols, flavenoids and nonprotein amino acids (Hsiao, 1985). Among these allomones, glycosides seem to play an important role in host plant resistance to insects. For example, tomatine, an alkaloid glycoside and rutin (quercetin 3-rutinoside) are involved in the resistance of tomato to the tomato fruitworm, *Heliothis zea* Fabricius (Lepidoptera: Noctuidae), by acting as feeding deterrents (Pratviel-Sosa *et al*., 1986). DIMBOA is another glycoside which is present in young corn tissues. The toxic action of these glycosides is due to their corresponding aglycones liberated by the action of β-glucosidase. In the current study, treatment of *H. cunea* larvae with sublethal concentrations of different biopesticides showed a reduction in the activity level of *α*- and *β*-glucosidases. The increase of plant extract concentrations on sycamore corresponded to a reduced enzymatic activity in larvae. This may be due to a drop in the consumption rates and leveling off or decline in food conversion efficiencies (Zibaee and Bandani, 2009). These decreasing activities were reported in other insects. Hemmingi and Lindroth (1999, 2000) studying effect of phenolic components in gypsy moth (Lepidoptera, Lymantriidae) and forest tent caterpillar (Lepidoptera, Lasiocampidae) demonstrated that glucosidase activities declined for both insect species when reared on diets with phenolic glycosides in addition to decreasing growth and increasing developmental time. Zibaee and Bandani (2009) found that *A. annua* extract significantly decreased activity of *α*- and *β*- glucosidases on *E. integriceps* adults so that the lowest activity was obtained at 25 % concentration of plant extract treatment of adults.

Lipases (triacylglycerol acylhydrolase; EC 3.1.1.3), which catalyses the hydrolysis of fatty acid ester bonds, are widely distributed among animals, plants and microorganisms (Zibaee *et al*., 2009). It was found that *B. thuringiensis* and plant extracts decreased the activity level of lipase but in the case of *L. stoechas* no significant differences were observed. Senthil Nathan *et al*. (2006) showed that treating *Cnaphalocrocis medinalis* (Guenee) (Lepidoptera: Pyralidae), the rice leaffolder, by Btk, NSKE, and VNLE (azadirachtin and neem components) sharply decreased the activity level of lipase in the midgut.

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). It was observed that biopesticides significantly decreased activity of protease in the midgut of *H. cunea* larvae especially on sycamore but no significant differences were obtained in the case of *L. stoechas* on both hosts. Because proteases are necessary for activation of *B. thuringiensis* protoxin to active toxin, this biopesticide could be a logical choice for control of caterpillars due to high pH value and suitable activity of proteases in the their midgut. Zibaee and Bandani (2009) found that *A. annua* extract significantly decreased the activity of protease on *E. integriceps* adults so that the lowest activity were obtained when 25 % concentration of plant extract was used on adults.

Physiological conditions of *H. cunea* affects the activity of the tested enzymes and reflects the absorption, digestion, and transport of nutrients in the midgut. *B. thurengiensis* damages the epithelial cells of the midgut through crystalline parasporal bodies, so decreasing levels of digestive enzymes. This results in reduced phosphorous liberation for energy metabolism, decreased rate of metabolism and decreased rate of metabolite transport, maybe due to the direct effects on enzyme regulation and synthesis.

LDH is an important glycolytic enzyme being present virtually in all tissues (Kaplan and Pesce, 1996). It is also involved in carbohydrate metabolism and has been used as an indicative criterion of exposure to chemical stress (Wu and Lam, 1997; Diamantino *et al*., 2001) and as an index of anaerobic metabolism (Chamberlin and King, 1998). Activity level of LDH in *Culex* after treatment with DDT, malathion and cyfluthrin decreased 58.88 %, 33.33 % and 66.66 %, respectively (Arshad *et al*., 2002). Senthil-Nathan and Kalviani (2005) showed that feeding of *Spodoptera litura* on *Ricinus communis* treated with azadirachtin and nucleopolyhedrovirus decreases the amount of this enzyme in midgut that demonstrates low nutritional efficiency of the larvae. Similar results were also observed on effectiveness of *Melia azedarach* on rice leaffolder (Senthil-Nathan, 2006).

Results in our current study showed that reduction of digestive enzyme activities due to using different bio-pesticides on larvae fed on sycamore were higher than those of mulberry. In addition, induction of LDH activity on larvae fed on sycamore was higher than that of mulberry. There may be different reasons for these differences. First of all, sycamore trees have been planted extensively around the city but mulberry orchards exist in the limited areas. Hence, sycamore trees are more available to larvae than mulberry. Secondly, almost all mulberry trees in the area have been modified genetically for silkworm rearing and have less plant secondary metabolites than sycamore. This matter is being observed more obviously on activity of digestive enzymes in control. But, treatment of sycamore leaves by biopesticides has a synergistic relationship with secondary metabolites existing in the plant tissue and caused more reduction in the digestive enzyme activities.

As a conclusion, *A. annua* and *L. stoechas* extracts had significant effects on the larvae of *H. cunea* so that they act synergistically with *B. thurengiensis* var. *kurstaki* toxin, causing reduction of digestive enzyme activity and elevation of LDH activity. *H. cunea* is a widely distributed pest which causes severe damages to trees in orchards. Hence, widely spraying by common synthetic

insecticide has high environmental risks specially on human therefore, studies on biopesticides and their combinations are necessary specially for physiological effect to decrease the population density of the pest. Physiological analysis would be particularly informative to get insight into which combinations of biopesticides enhance the efficiency of a safe management process.

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