Effect of Artemisia annua L. essential oil on toxicity, enzyme activities, and energy reserves of cotton bollworm Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae)

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Abstract: The essential oil of Artemisia annua L., a weed collected from northern Iran, was studied for its toxicity and physiological aspects on 4th instar larva of the cotton bollworm Helicoverpa armigera Hübner in controlled conditions (26±1°C, 65±10% RH and 16 L : 8 D h). The artificial diet was used as a medium for investigating the toxicity and the effect of LC10, LC30, LC50, and LC90 on the feeding efficiency of 4th instar larva. The essential oil in doses of LC10, LC30, LC50, and LC90 were estimated to be 2.01%, 3.86%, 6.07%, and 18.34%, respectively. The activity of α-amylase, protease, lipase, general esterases, and glutathione S-transferase and protein, triglyceride, glucose for treated larva were measured. The results showed that all of these parameters were decreased compared with the control. Hence, A. annua essential oil is suggested as a botanical for controlling this important pest of field crops.

Key words: Artemisia annua, cotton bollworm, essential oil, Helicoverpa armigera

Introduction
The cotton bollworm, Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) is a serious polyphagous pest of many economically significant agricultural crops in parts of Asia, Africa, Australia, and Europe (King 1994). The cotton bollworm causes severe damage to a wide range of plant species including various ornamental, medicinal, aromatic, and agricultural crops. Over the last few decades, the use of pesticides has resulted in: toxicity to non-target organisms, development of resistance by pests, resurgence and outbreak of new pests, and harmful effects on the environment affecting the sustainability of ecosystems (Jayasankar and Jesudasan 2005). Therefore, a method of controlling the pest with the use of a botanical insecticide that could affect the population of this pest with minimal effect on the environment, is welcomed by scientists. Artemisia annua L. is an aromatic annual herb which is a member of a large plant family Asteraceae (Compositae), endemic in the north of Iran, although the plant grows wild in Europe and America and is planted widely in China, Turkey, Vietnam, Afghanistan, and Australia (Bhakuni et al. 2001). The species A. annua is an important medicinal plant. Several compounds have been isolated from this species including flavonoids, coumarins, steroids, phenolics, purines, lipids, aliphatic compounds, monoterpenoids, triterpenoids, and sesquiterpenoids (Bhakuni et al. 2001; Haghighian et al. 2008; Brisibe et al. 2009). The extracts and essential oils have demonstrated antimicrobial and insecticidal activity (Deans and Svoboda 1989; Regnault-Roger et al. 2004; Pavela 2006; Mahmoodi et al. 2014). The plant’s essential oils have various insecticidal activity such as contact toxicity (Asawalam et al. 2006; Ogendo et al. 2008), repellence (Kéita et al. 2001; Rozman et al. 2007), fumigant toxicity (Lee et al. 2003; Rajendran and Muralidharan 2005; Shojaaddini et al. 2008), and antifeedant effects ( Saxena et al. 1992). Several studies have reported insecticidal effects of A. annua extract containing growth retardation, antifeedant, and larvicidal effects (Haghighian et al. 2008; Shekari et al. 2008; Hasheminia et al. 2011). Khosravi et al. (2010) observed that A. annua extract affected the nutritional indices and also showed antifeedant activities on Glyphodes pyloalis Walker. Anshul et al. (2013, 2014, 2015) showed that methanolic extract of powdered A. annua leaves adversely affect H. armigera. The extract affected toxicity, inhibition and disruption of the growth, development and histopathological and biological parameters of H. armigera. The essential oil of Artemisia judaica L. has been demonstrated to possess insecticidal activity and repellence against several insects, such as Callosobruchus maculatus (Fab.) and Sitophilus oryzae L. (Aggarwal et al. 2001; Abd-Elhady 2012). Excessive use of chemical insecticides adversely affects the environment and human health. Therefore, based on the literature review, we decided to carry out an experiment on this important pest which takes into account an almost safe method and which takes into account the toxicity and sub-lethal effects on the physiology of the pest. To the best of our
knowledge, so far no steps have been taken concerning the effect of *A. annua* products on *H. armigera*.

**Materials and Methods**

The larvae of *H. armigera* were collected from the tomato farms in the city of Astaneh-ye Ashrafiyeh (37°15’35”N 49°56’40”E) in northern Iran. The larvae were reared on an artificial diet of powdered cowpea, wheat germ powder, yeast, sorbic acid, ascorbic acid, sunflower oil, formaldehyde, and water (Shorey and Hale 1965). The insects were kept in transparent plastic containers (10 × 5 × 5 cm) at 26±1°C, and a relative humidity (RH) of 65±5%. The photoperiod was 16 L : 8 D h (Hemati et al. 2012).

**Preparation of the essential oil**

*Artemisia annua* was collected from Ramsar in northern Iran (36°54’N, 50°40’E). The herb was dried in the shade, and hand-ground to a powder. The dried herb powder (50 g) was briefly mixed with 750 ml of distilled water. After 24 h, the mixture was transferred to the Clevenger-type apparatus according to the method recommended in the British Pharmacopoeia. Distillation lasted about 2 h, and then the essential oil was obtained. This process was repeated several times in order to reach the required amount. The oil phase was isolated from the obtained solution. Sodium sulfate was used for dehydration (Yazdani et al. 2014).

**Bioassay**

Bioassay tests were carried out on one-day-old 4th instar larvae. Prior to the experimental phase, larvae were starved for 4 h. Preparatory tests were initially performed to find the effective dose ranges. Five concentrations of *A. annua* essential oil, i.e. 2.5%, 4%, 6.5%, 9.5%, and 15% were determined to be the effective doses. This experiment was performed in 3 replications with 10 larvae of 4th instars in each replication. These essential oil concentrations were adjusted to the artificial diet, and this mixture was put into the plastic jars before the release of the larvae. After 24 h, the numbers of dead larvae were recorded. An untreated diet was also provided as a control. The procedure of Bernfeld (1955) was used to measure the α-amylase activity. In the procedure, 1% soluble starch was used as the substrate. The reaction was performed by sodium phosphate buffer at 35°C with 10 µl of the enzyme, 40 µl of substrate, and 40 µl of sodium phosphate buffer (pH 9), for 30 min. To stop the reaction, 100 µl of dinitrosalicylic acid (DNS) was added and heated in boiling water for 10 min. Absorbance was recorded at 540 nm after cooling. 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. In this test, maltose was used to generate the standard curve.

**Assay of lipase activity**

The activity of lipase was estimated using the method of Tsujita et al. (1989). Ten µl of homogenate was mixed with 18 µl of p-nitrophenyl butrate (50 mM) as the substrate, and mixed with 172 µl of universal buffer (1 M) (pH 7). This mixture was incubated at 37°C. The absorbance was read at 405 nm. The activity of lipase was estimated using the method of Tsujita et al. (1989). Ten µl of homogenate was mixed with 18 µl of p-nitrophenyl butrate (50 mM) as the substrate, and mixed with 172 µl of universal buffer (1 M) (pH 7). This mixture was incubated at 37°C. The absorbance was read at 405 nm.

**Assay of protease activity**

The protease activity of larval guts was determined using azocasein 1% as the substrate (Garca-Carreño and Haard 1993). Each gut was centrifuged in 10 µl of distilled water, then 10 µl of supernatant and 15 µl of buffer (pH 8) with 50 µl of substrate were reacted for 3 h at 37°C. Proteolysis was stopped by the addition of 150 µl of 10% trichloroacetic acid (TCA). The solution was transferred to 4°C in a refrigerator for 30 min, and the reaction mixture was centrifuged at 13,000 g for 10 min. One hundred µl of supernatant was mixed with 100 µl 1 N NaOH and the absorbance was read at 440 nm.

**Estimation of glucose**

Whole body of larvae (100 µl) mixed with 500 µl 0.3 N perchloric acid and the precipitate was removed by centrifugation (10 min, 12,000 g). The supernatants were used for determination of the glucose concentrations (Siegert 1987).

**Estimation of protein**

Protein was measured based on Bradford’s method (Bradford 1976) and by utilizing a total protein assay kit (Biochem Co., Iran). In this method, proteins made a complex which was purplish-blue with an alkaline copper solution, which with its absorption value at 540 nm had a direct relation to the amount of the whole body protein.

**Estimation of triacylglyceride**

A diagnostic TAG kit (from Pars Azmoon Co., Tehran, Iran) was used to measure the amount of triacylglyceride in the 4th-instar larvae. The reagent solution contained phosphate buffer (50 mM, pH 7.2), 4-chlorophenol (4 mM), adenosine triphosphate (2 mM), Mg2+ (15 mM), glycerokinase (0.4 kU · l−1), peroxidase (2 kU · l−1), lipoprotein lipase (2 kU · l−1), 4-aminoantipyriyne (0.5 mM), and glycerol-3-phosphate-oxidase (0.5 kU · l−1). Samples
(10 µl) were incubated with 10 µl of distilled water and 70 µl of reagent for 20 min at 25°C (Fossati and Prencipe 1982). Optical densities (ODs) of samples and reagent as a standard were read at 546 nm.

**Assay of general esterase activity**

The activity of general esterases was determined according to the Van Asperen (1962) method. As substrates, α-naphthylacetate (α-NA) and β-naphthylacetate (β-NA) (10 mM) were used. Initially one insect was homogenized in 1,000 µl of 0.1 M phosphate buffer (pH 7) and Triton X-100 at the ratio of 0.01%, and centrifuged at 10,000 g for 10 min at 48°C. The supernatant was transferred to a new microtube and was diluted with phosphate buffer. Fast Blue RR Salt (1 mM) was added and the absorbance was read at 450 nm.

**Assay of glutathione S-transferase activity**

For determining glutathione S-transferase (GST) activity, the method of Habig et al. (1974) was used. As the substrate, 1-chloro-2,4-dinitrobenzene (CDNB) (20 mM) was used. Each larva was homogenized in 20 µl of distilled water and centrifuged at 12,500 g for 10 min at 4°C. Fifteen µl of supernatant was mixed with 135 µl of phosphate buffer (pH 7), 50 µl of CDNB, and 100 µl of GST. The absorbance was read at 340 nm.

**Statistical analysis**

LC10, LC30, LC50, and LC90 of toxicity bioassay were calculated with Polo-PC software (LeOra 1987). Data from the enzyme activity were compared by one-way analysis of variance (ANOVA). Differences between the various treatments were determined at 5% by Tukey’s multiple range tests using SAS software (SAS 1997).

### Results

#### Bioassay

The LC10, LC30, LC50, and LC90 values of *A. annua*, 24 h after treatments per confident limits, and the slope of line regression, are shown in table 1. They were estimated to be 2.01%, 3.86%, 6.07%, and 18.34%, respectively.

#### Effect of *Artemisia annua* essential oil on total protein, glucose, and triacylglyceride

The results of total protein, triacylglyceride, and glucose in 4th instar larvae of *H. armigera* after treatment with *A. annua* essential oil are shown in table 2. The amount of the total protein of the larva treated with LC30 (F = 16; df = 4; p = 0.008) and LC50 (F = 33; df = 4; p = 0.0076) of *A. annua* essential oil, showed significant decreases when compared to the control.

The amount of triacylglyceride of the larva treated with LC30 (F = 74.4; df = 4; p = 0.0088) and LC50 (F = 110.5; df = 4; p = 0.0014) of *A. annua* essential oil, showed a significant decrease compared with the control. Also the amount of glucose significantly decreased in comparison with the control, LC30 (F = 10.9; df = 4; p = 0.0069) and LC50 (F = 25.1; df = 4; p = 0.0055).

#### Effects of *Artemisia annua* essential oil on digestive enzymes

The effects of *A. annua* essential oil on digestive enzymes after the diet treatment with different concentrations (LC30 and LC50) of *A. annua* essential oil, are shown in table 3. The results showed that the activity of α-amylase sharply decreased in larvae treated with both the concentrations of *A. annua*, LC30 (F = 29.17; df = 4; p = 0.0057) and LC50 (F = 110.4; df = 4; p = 0.009). The activity of lipase

### Table 1. Toxicity of *Artemisia annua* to 4th instar larva of *Helicoverpa armigera*

<table>
<thead>
<tr>
<th>Essential oil</th>
<th>N</th>
<th>Slope±SE</th>
<th>α² (df)</th>
<th>LC₅₀ [mg · g⁻¹] (95% CL)</th>
<th>LC₃₀ [mg · g⁻¹] (95% CL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. annua</em></td>
<td>200</td>
<td>2.67±0.443</td>
<td>2.62(3)</td>
<td>3.86(2.82–4.75)</td>
<td>6.07(4.92–7.41)</td>
</tr>
</tbody>
</table>

N – number of insects used in the bioassay; SE – standard error

The LC₃₀ and LC₅₀ values, confidence limit (CL 95%) and regression slope after 24 h exposure to *A. annua* in larvae of *H. armigera*

### Table 2. Effect of *Artemisia annua* essential oil on protein, glucose, and triglyceride of *Helicoverpa armigera* 4th instar larvae

<table>
<thead>
<tr>
<th>Essential oil</th>
<th>Concentrations [mg · g⁻¹ diet]</th>
<th>Protein [mg · l⁻¹]</th>
<th>Glucose [mg · l⁻¹]</th>
<th>Triglyceride [µmol · l⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. annua</em></td>
<td>the control</td>
<td>4.2±0.15 a</td>
<td>2.73±0.3 a</td>
<td>2.03±0.08 a</td>
</tr>
<tr>
<td></td>
<td>LC₃₀</td>
<td>3.7±0.29 b</td>
<td>1.6±0.15 b</td>
<td>1.26±0.01 b</td>
</tr>
<tr>
<td></td>
<td>LC₅₀</td>
<td>3.27±0.14 b</td>
<td>1.7±0.27 b</td>
<td>0.94±0.05 b</td>
</tr>
</tbody>
</table>

Within columns, means followed by the same letter do not differ significantly at p < 0.05
in 4th instar larvae of *H. armigera* treated with LC<sub>30</sub> concentrations of *A. annua* essential oil, did not show significant differences compared with the control, but the LC<sub>50</sub> concentration (F = 13.83; df = 4; p = 0.0057) of *A. annua* significantly decreased the activity of this enzyme in the larvae of *H. armigera*. The activity of protease significantly decreased in the larvae treated with LC<sub>30</sub> (F = 13.64; df = 4; p = 0.0024) and LC<sub>50</sub> (F = 17.68; df = 4; p = 0.0013) concentrations of *A. annua*.

**Effects of Artemisia annua essential oil on detoxifying enzymes**

General esterases with substrate α-naphtylacetate and β-naphtylacetate substrates showed a significant decrease compared with the control at LC<sub>30</sub> (F = 17.76; df = 4; p = 0.0013) and LC<sub>50</sub> (F = 10.45; df = 4; p = 0.0031) for α-naphtylacetate and LC<sub>30</sub> (F = 8; df = 4; p = 0.047) and LC<sub>50</sub> (F = 34.75; df = 4; p = 0.0041) for β-naphtylacetate concentrations of *A. annua* essential oil (Table 4). The activity of GSTs was decreased by both concentrations of the essential oil, and the activity was significant compared with the control, LC<sub>30</sub> (F = 21.13; df = 4; p = 0.01) and LC<sub>50</sub> (F = 309.21; df = 4; p = 0.0001).

**Discussion**

In the present study, the effect of *A. annua* essential oil was investigated for its toxicity against larvae of *H. armigera*. The amounts of non-enzymatic compounds were also measured on the larvae fed different concentrations of *A. annua*. Proteins are the most important biochemical components that are necessary for insect development and growth. The results showed that the amount of all non-enzymatic components decreased in larvae which were fed all the concentrations of *A. annua* essential oil. Etebari et al. (2005) showed that insecticide decreased the protein amount of an insect’s body. Schmidt et al. (1998) observed that treatment of *Spodoptera littoralis* Fabricius (Lepidoptera: Noctuidae) and *Agrotis ipsilon* Hufnagel (Lepidoptera: Noctuidae) with azadirachtin, decreased hemolymph proteins. This phenomenon could be due to the break-down of proteins into their respective amino acids, that could help to provide energy for the insect to survive. The amount of both glucose and triacylglyceride was decreased in the treated larvae. This depletion might be due to an energy demand and an increased metabolism as a result of the oil effect (Sancho et al. 1998; Olga et al. 2006). Also glucose depletion may be due to stress conditions imposed on these insects that need more energy, so the glucose was metabolized to meet the energy expense. Similar results were observed by Khosravi et al. (2010) in *G. pyloalis* larva treated with *A. annua* extract, and by Seyoum et al. (2002) in desert locust.

For many herbivorous insects that feed on plants during their life, α-amylase is the most important digestive enzyme. Our study showed a significant decrease in α-amylase activity in the midgut of treated larvae. Mehrabadi et al. (2010) suggested that when the action of the enzyme is inhibited, nutrition of the organism is impaired – causing a shortness in energy. This suggestion is supported by Saleem and Shakoori (1987), Lee et al. (1994),

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**Table 3.** Activity digestive (α-amylase, lipase, protease) enzymes in 4th instar larva of *Helicoverpa armigera* after treatment with LC<sub>30</sub>, LC<sub>50</sub> concentrations of *Artemisia annua* essential oil

<table>
<thead>
<tr>
<th>Essential oil</th>
<th>Concentrations [mg · g&lt;sup&gt;–1&lt;/sup&gt; diet]</th>
<th>α-Amylase [nmol · min&lt;sup&gt;–1&lt;/sup&gt; · mg&lt;sup&gt;–1&lt;/sup&gt; protein]</th>
<th>Lipase [µmol · min&lt;sup&gt;–1&lt;/sup&gt; · mg&lt;sup&gt;–1&lt;/sup&gt; protein]</th>
<th>Protease [OD · min&lt;sup&gt;–1&lt;/sup&gt; · mg&lt;sup&gt;–1&lt;/sup&gt; protein]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. annua</td>
<td>the control</td>
<td>0.049±0.004 a</td>
<td>1.8±0.11 a</td>
<td>0.28±0.012 a</td>
</tr>
<tr>
<td></td>
<td>LC&lt;sub&gt;30&lt;/sub&gt;</td>
<td>0.019±0.007 b</td>
<td>1.72±0.069 a</td>
<td>0.12±0.06 b</td>
</tr>
<tr>
<td></td>
<td>LC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>0.014±0.003 b</td>
<td>0.899±0.072 b</td>
<td>0.095±0.001 b</td>
</tr>
</tbody>
</table>

**Table 4.** Activity detoxifying enzymes: general esterase and glutathione S-transferase (GST), in 4th instar larva of *Helicoverpa armigera* after treatment with LC<sub>30</sub>, LC<sub>50</sub> concentrations of *Artemisia annua* essential oil

<table>
<thead>
<tr>
<th>Essential oil</th>
<th>Concentrations [mg · g&lt;sup&gt;–1&lt;/sup&gt; diet]</th>
<th>GST [µmol · min&lt;sup&gt;–1&lt;/sup&gt; · mg&lt;sup&gt;–1&lt;/sup&gt; protein]</th>
<th>Esterase [nmol · min&lt;sup&gt;–1&lt;/sup&gt; · mg&lt;sup&gt;–1&lt;/sup&gt; protein]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>α-naphtyl acetate substrate</td>
<td>β-naphtyl acetate substrate</td>
</tr>
<tr>
<td>A. annua</td>
<td>the control</td>
<td>0.148±0.007 a</td>
<td>0.152±0.03 a</td>
</tr>
<tr>
<td></td>
<td>LC&lt;sub&gt;30&lt;/sub&gt;</td>
<td>0.094±0.009 b</td>
<td>0.064±0.005 b</td>
</tr>
<tr>
<td></td>
<td>LC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>0.0116±0.001 b</td>
<td>0.091±0.01 b</td>
</tr>
</tbody>
</table>
Shekari et al. (2008), Khosravi et al. (2010), and Valizadeh et al. (2013). Proteases are important in digesting food and converting protein into amino acids needed for the body. Lipases are enzymes that hydrolyze the outer links of fat molecules (Yazdani et al. 2014). In this study, lipase activity had no significant differences in the treated larvae in comparison with the control, but the LC50 concentration of A. annua decreased the activity of this enzyme. The decreased activity of midgut lipase could be due to the disturbance of digestion and absorption of processes (Napoleão et al. 2013). Sujatha et al. (2010) reported that an extract of Pedalium murex L. increased lipase activity in S. littoralis. Proteases play a critical role in the food digestion of insects (Terra and Ferriera 2005). When using the LC50 and LC90 concentrations of A. annua essential oil on 4th instar larvae of H. armigera, it was observed that the activity of this enzyme decreased. Reduction in digestive enzymes may be referred to the biochemical inhibition of essential oil extracted from A. annua in studies by Senthil-Nathan et al. (2004 and 2006), Zibaei and Bandani (2010a), and Khosravi and Jalali (2013) who inferred that botanical insecticides may affect the construction of certain types of proteases.

Glutathione S-transferases are a major group of detoxification enzymes found in most organisms. They help to protect cells from oxidative stress and chemical toxicants by aiding the excretion of electrophilic and lipophilic compounds from the cell (Hayes and Pulford 1995). In the present study, the activity of GST in larvae with the studied essential oil, decreased, compared to the control. Our results are different from the results obtained in other studies (Vanhaelen et al. 2001; Dugravot et al. 2004; Khosravi et al. 2010; Zibaei and Bandani 2010b). In our study, the general esterase activity decreased in the H. armigera larva with the essential oil which we used. These results show that GSTs and esterase do not play a role in the detoxification or in the metabolism of A. annua essential oils.

The present investigation indicated that A. annua essential oil affected H. armigera in a toxic way. In addition, compounds present in this essential oil effect the activity of macromolecules, digestive enzymes, and the detoxifying enzymes in this pest, posing an irreversible effect which may aid in controlling this important pest of crops.

References


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