Research Article

Effect of *Achillea millefolium* and *Teucrium polium* extracts on nutritional indices and α-amylase and protease activities of Egyptian cotton leafworm, *Spodoptera littoralis* (Lepidoptera: Noctuidae)

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**Abstract:** The effect of methanolic extracts of *Achillea millefolium* and *Teucrium polium* on third instar larvae of Egyptian cotton leafworm, *Spodoptera littoralis* were investigated. Methanolic plant extracts were mixed with artificial diet and then nutritional indices, glutathione-S transferase, esterase, α-amylase and protease, activity were measured 72h later. Approximate digestibility was increased significantly in larvae fed on the food containing methanolic extract of *T. polium* but there was not any significant change in insects treated with *A. millefolium*. *T. polium* significantly decreased relative growth rate (RGR), relative consumption rate (RCR), efficiency of digested food (ECD) and efficiency of conversion of ingested food (ECI) whereas *A. millefolium* only decreased RGR and RCR. Feeding on artificial diet containing plant extract decreased α-amylase and protease activities in the midgut of the insect. Furthermore, the effect of methanolic extract of plants on detoxifying enzymes showed that they have no effect on glutathione-S-transferase and esterase activities.

**Keywords:** *Achillea millefolium*, *Teucrium polium*, *Spodoptera littoralis*, digestive enzymes

Introduction

The Egyptian cotton leafworm, *Spodoptera littoralis* (Boisdual) (Lepidoptera: Noctuidae) is one of the key pest of cotton and many other crops like tomato and eggplant in many countries (Shalaby et al., 2013). Using chemical control for managing this pest has resulted in the development of resistance to almost all insecticides (Abo-Elghar et al., 2005).

Synthetic insecticides show deleterious effects on non-target organisms. Botanical products seem to be useful alternative for insecticides (Hasheminia et al., 2011). The use of botanical insecticides generally seems to be safe (Pavela, 2010). Plant extracts studied by many authors have shown insecticidal activity against different pests (Ayvaz et al., 2010). They may also show antifeedant activity which is worthy of scientists’ attention (Dolui and Debnath, 2010). Methanolic extract of *Adhatoda vasica* is shown to have antifeedant and toxicity activities against *S. littoralis* larvae (Sadik, 2003). Moreover, Methanolic extract of the stem of *Vincetoxicum hirundinaria* demonstrated antifeedant activity against *S. littoralis* larvae (Pavela, 2010). Plant extracts of *Cleome arabica* are shown to have insecticidal effect on larvae of *S. littoralis* (Ladhari et al., 2013).
Effects of plant extracts on *S. littoralis*  

Achillea, is a genus with more than 100 species and it is well known for its medicinal rhizomous herbs. Many researches have shown that the plant has insecticidal activity. Essential oil extracted from *Achillea* exhibited toxicity to *Tribolium castaneum* (Khani and Asghari, 2012) and *Pieris rapae* (Hasheminia et al., 2011). The essential oil from *Teucrium polium* was demonstrated to be toxic to *Musca domestica* (Bigham et al., 2010).

Treatment of second instar larvae of *S. littoralis* with *Sesamum indicum* leaf extract showed that *S. indicum* did not change detoxification enzyme activities in *S. littoralis* (Sintim et al., 2009).

In the present study the effect of methanolic extract of *Achillea millefolium* and *Teucrium polium* on toxicity, nutritional indices, digestive enzymatic activities in the midgut and on detoxification enzymes of *S. littoralis* was investigated.

Materials and Methods

**Insect rearing**

Insects were collected from cotton field in Dezful-Iran (32°22′57″N 48°24′07″E). They were reared on an artificial diet (Shorey and Hale, 1965) at 20 ± 1 °C, 60 ± 2% RH and photoperiod of (16:8 L: D). Twenty four houred-third instar larvae were used in all experiments.

**Methanolic extract preparation**

The foliage of *A. millefolium* and *T. polium* were collected in July 2015 from Torbat Heydariyeh (35°16′26″N 59°13′10″E) and Jiroft regions (28°40′41″N 57°44′26″E) respectively. After washing with distilled water and drying in the shade at room temperature (RT), foliage was powdered using an electric grinder. Methanolic extraction was done according to the procedure described by Warthen et al. (1984). Briefly, 30g of the powder was stirred with 300 ml of 85% methanol at RT for 1 h. It was incubated at 4 °C for 48 h followed by additional stirring for 1 h. Finally it was filtered using Whatman No. 4 filter paper. The solvent was removed using vacuum in a rotary evaporator at 40 °C. Finally, 10mL of methanol was used to dissolve the residue and which was used as a stock solution. In order to obtain different concentrations, further dilutions with methanol were made.

**Bioassays and treatment**

**Toxicity tests**

Five different concentrations of *A. millefolium* and *T. polium* extracts were used to evaluate LC<sub>50</sub>. Third instars were used in all the experiments. In each experiment 40 insects with 4 replicates were tested for each concentration. The LC<sub>50</sub> values were evaluated after 48h, using SAS 6.12 software.

**Nutritional indices assay**

Hundred microliter of *A. millefolium* and *T. polium* plant extracts were added to 0.9gr of artificial diet at the rate of 7318 and 5930ppm respectively. Instars were transferred into plastic containers (diameter: 15cm, depth: 7 cm) which contained the food. Top of the container had an opening covered by a mesh net. The experiment was repeated four times (15 larvae per replicate). After 72 h, nutritional indices were calculated according to Naseri et al. (2010).

**Protease and α-amylase assay**

In order to prepare samples for measuring protease or α-amylase activity, midgut was dissected in 50mM Tris-HCl (pH 7.4). It was incubated in the same for 30 min at RT in order to release enzyme activity into the buffer.

α-Amylase activity was measured as previously described by Mikani et al. (2015). Using Kikkoman kit (Kikkoman Corp., Japan).

Protease activity was measured by digestion of azocasein according to the method of Elpidina et al. (2001). Briefly, 60μL of the enzyme samples were incubated with 60μL of azocasein solution in Tris-HCl (pH 7.4) at 37 °C for 30min followed by adding 160μL of 20% trichloroacetic acid to stop the reaction. The plates were incubated at 4 °C for 10min. Samples were centrifuged (4000g at 4 °C, 15
min) and the supernatant was used for measuring protease activity. The absorbance was measured by microplate reader (Epoch, USA) at 335 nm.

Protein concentration of sample was measured using BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). For standard, bovine serum albumin was used.

**Detoxification enzyme activity**

**Esterase activity**

Esterase activity was determined according to the method of Van Asperen (1962) with some modification. Briefly, insect was homogenized in 300μl 0.1 M phosphate buffer (pH 7) and Trition X-100 followed by centrifugation at 15000g for 10min at 4°C. 50μL of supernatant was added to 100μL 0.1 M phosphate buffer (pH7.0) and 10μL of α-naphthyl acetate (10 mM in acetone). Then 50μL fast blue RR (0.5 mg/mL in buffer) was added and finally the released naphthol was continuously measured at 450nm for 25 min using microplate reader (Epoch, USA).

**Glutathione S-transferase activity**

Glutathione S-transferase (GST) activity was determined according to the method of Habing et al. (1974). 1-chloro-2, 4-dinitrobenzene (CDNB) was used as the substrate. Insect was homogenized in 300μL of ice-cold 10mM phosphate buffer (pH 7.0) Followed by centrifugation at 10,000g for 10min at 4°C. 10μL of the supernatant was added to 200μL of reaction mixtures [1mM 1-chloro-2, 4-dinitrobenzene (CDNB) and 5mM GSH in 0.1 M sodium phosphate buffer, pH7.0]. The change in absorbance was measured continuously for 6min at 340nm using microplate reader (Epoch, USA).

**Statistical analysis**

Data were analyzed by one-way ANOVA (Fishers LSD). For p < 0.05, differences were accepted as significant.

**Results**

**Toxicity test**

The LC50 value, regression slope and confidence limit (95%) at 72h exposure to artificial diet containing 100μL of the plant extracts are demonstrated in Table 1 and Fig. 1. The LC50 Values for of *A. millefolium* and *T. polium* were 18569 and 14275 ppm respectively (Table 1 and Fig.1).

**Nutritional indices**

The nutritional indices of larvae feeding on artificial diet containing methanolic extract of *A. millefolium* or *T. polium* at the rate of 7318 and 5930 ppm (LC50) respectively, were affected as follows. Approximate digestibility (AD) was increased significantly in larvae fed on artificial diet containing methanolic extract of *T. polium* but there was not any significant change in larvae treated with *A. millefolium*. *T. polium* significantly decreased efficiency of conversion of ingested food (ECI) and efficiency of digested food (ECD) but *A. millefolium* extract did not indicate changes in ECI and ECD. Both relative growth rate (RGR) and relative consumption rate (RCR) were decreased in larvae treated with *A. millefolium* or *T. polium* plant extract (Table 2).

Table 1 Toxicity of methanolic extracts *A. millefolium* and *T. polium* to 3rd instar larvae of *Spodoptera littoralis*, 72 h after feeding on artificial diet containing plant extracts.

<table>
<thead>
<tr>
<th>Extraction</th>
<th>N</th>
<th>γ2 (df)</th>
<th>P-value</th>
<th>Slope ± SE</th>
<th>LC20 (95% CL) (ppm)</th>
<th>LC50 (95% CL) (ppm)</th>
<th>LC80 (95% CL) (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. millefolium</em></td>
<td>200</td>
<td>1.029(3)</td>
<td>0.794</td>
<td>2.081 ± 0.346</td>
<td>34275 (11685-9723)</td>
<td>18569 (15022-18723)</td>
<td>47120 (34009-80146)</td>
</tr>
<tr>
<td><em>T. polium</em></td>
<td>200</td>
<td>1.18(3)</td>
<td>0.758</td>
<td>2.206 ± 0.341</td>
<td>5930 (3884-7680)</td>
<td>14275 (11685-17656)</td>
<td>34363 (25980-54923)</td>
</tr>
</tbody>
</table>

CL = Confidence limits.
Effects of plant extracts on S. littoralis

Figure 1 Probit analysis of mortality in 3rd instar larvae of Spodoptera littoralis, 72h after feeding on artificial diet containing 7318 and 5930 ppm (LC20) of (A) A. millefolium (B) T. polium respectively. NED refers to normalized equivalent deviation.

Table 2 Nutritional indices of 3rd instar larvae of Spodoptera littoralis, 72 h after feeding on artificial diet containing 7318 and 5930 ppm (LC20) of A. millefolium or T. polium respectively.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AD (%)</th>
<th>ECI (%)</th>
<th>ECD (%)</th>
<th>RCR (mg/mg/day)</th>
<th>RGR (mg/mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>80 ± 0.25</td>
<td>22 ± 0.2</td>
<td>27 ± 0.25</td>
<td>0.50 ± 0.002</td>
<td>0.11 ± 0.0008</td>
</tr>
<tr>
<td>T. polium</td>
<td>91 ± 0.59*</td>
<td>17 ± 0.8*</td>
<td>20 ± 0.40*</td>
<td>0.42 ± 0.010*</td>
<td>0.07 ± 0.0020*</td>
</tr>
<tr>
<td>A. millefolium</td>
<td>79 ± 0.29</td>
<td>20 ± 2.24</td>
<td>25 ± 2.83</td>
<td>0.44 ± 0.002*</td>
<td>0.09 ± 0.0100*</td>
</tr>
</tbody>
</table>

RGR: relative growth rate; RCR: relative consumption rate; ECI: efficiency of conversion of ingested food; ECD: efficiency of conversion of digested food; AD: approximate digestibility. An asterisk indicates a significant difference relative to the control treatment.

*: significant at P ≤ 0.05.

α-amylase and protease activity

The result showed that feeding on artificial diet containing 100µl of the plant extract of A. millefolium or T. polium at 7318 and 5930 ppm (LC20) respectively, decreased α-amylase and protease activities in S. littoralis. α-amylase activity from 154mU in control decreased to 106 and 76mU in insects feeding on food containing A. millefolium or T. polium respectively (Fig. 2A). It also sharply decreased protease activity in the insect. Protease activity was decreased from 125mU in control to 69 and 56mU in treatments respectively (Fig. 2B).

Esterase and glutathione s-transferase activity

There was no significant difference in GST or esterase activity between control and insects feeding on artificial diet containing either one of the plant extracts (Fig. 3 A, B).

Discussion

Plants synthesize secondary metabolites that protect them against pests and can act as antifeedant (Yazdani et al., 2014). Our results clearly show that methanolic extracts of A. millefolium and T. polium have insecticidal activity against S. littoralis at high concentration (Table. 1 and Fig. 1) and both of them have antifeedant activity at low concentrations (Table. 2). These results confirmed previous studies which indicated that A. Millefolium has antifeedant activity against Pieris rapae (Hasheminia et al., 2011). It also was shown that T. polium has antifeedant activity against Musca domestica (Bigham et al., 2010).
The effect of two plant extracts (LC$_{20}$) on nutritional indices were evaluated (Table 2). It was shown that 72 h after treatment, there was no significant change in AD which is consistent with an earlier report (Hasheminia et al., 2011). It may be concluded that AD showed no change due to the reduced RGR. Both ECD and ECI were significantly lower in larvae that fed on an artificial diet that contained T. polium extract compared with control. Shekari et al. (2008) also reported similar results showing that Artemisia annua plant extract reduced ECI and ECD in Xanthogaleruca luteola. There was no change in ECD and ECI in insects feeding on diet treated with A. Millefolium which was similar to previous study (Hasheminia et al., 2011). The reduction in both RCR and RGR due to plant extracts is consistent with the effect of A. millefolium on P. rapae (Hasheminia et al., 2011).

There are many factors that may affect digestive enzyme activity in insect midgut such as starvation. Here we confirmed that feeding S. littoralis on artificial diet containing plant extract, decreased protease and $\alpha$-amylase activities (Fig. 2 A, B). Previous results also showed that A. millefolium crude leaf extracts reduced $\alpha$-amylase activity in P. rapae (Hashemiaiaet al., 2011). Other reports also demonstrated that T. polium decreased $\alpha$-amylase activity of M. domestica (Bigham et al., 2010). There are more reports that also showed decreasing of digestive enzyme activities after treatment with various plants extract (Shekari et al., 2008; Hasheminia et al., 2011).

GST and esterase involvement in insecticide resistance have been earlier mentioned in many insects. Elevated GST activity has been associated with the resistance of many insects to insecticides (Alizadeh et al., 2011). Here we did not detect any difference between control and insect GST activity that fed on artificial diet containing either one of the plant extracts. Esterase is also another detoxifying enzyme that hydrolyzes the esteric bond in synthetic chemicals. Here we did not observe any difference between control and treatments.

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**Figure 2** $\alpha$-Amylase (A) and protease (B) activities in 3rd instar larvae of S. littoralis, 72 h after treatment with 7318 and 5930 ppm (LC$_{20}$) of A. millefolium and T. polium methanolic extracts. Each point represents the mean ± SEM. * Different letters indicate significance at $p \leq 0.05$.

**Figure 3** Glutathione s-transferase (A) and esterase (B) activities in 3rd instar larvae of S. littoralis, 72 h after treatment with 7318 and 5930 ppm (LC$_{20}$) of A. millefolium and T. polium methanolic extracts. Each point represents the mean ± SEM. * Different letters indicate significance at $p \leq 0.05$. 

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These results are in agreement with previous studies that showed the effect of *Sesamum indicum* leaf extract on second instar larvae of *S. littoralis* in that, there were no change in GST and esterase activity (Sintim et al., 2009).

In conclusion, here we showed that feeding on artificial diet containing *A. millefolium* or *T. polium* affects not only nutritional indices but also causes α-amylase and protease activities to decrease while it has no effect on GST and esterase. Taken all together feeding on artificial diet containing one of these two plant extracts caused endocrine cells to decrease α-amylase and protease activities.

**Acknowledgements**

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**References**


اثر عصاره بومادران (Achillea millefolium) و کلپوره (Teucrium polium) بر روی شاخص‌های تغذیه‌ای و فعالیت آلفاامیلاز و پروتئاز در کرم برگخوار مصری (Spodoptera Littoralis)

چکیده: اثر عصاره مناتولی بومادران و کلپوره بر روی کرم برگخوار مصری (Spodoptera Littoralis) مورد بررسی قرار گرفت. عصاره مناتولی بومادران و کلپوره با غذای مصنوعی آمیخته شده و شاخص‌های تغذیه‌ای، فعالیت آنزیم‌های گلوتاتیون اس-ترانسفراز، استراز، آلفاامیلاز و پروتئاز و 24 ساعت پس از تغذیه مورد بررسی قرار گرفت. هضم تقریبی (AD) در لاروی تغذیه شده با عصاره مناتولی کلپوره افزایش یافته در حالی که عصاره بومادران هیچ تاثیری بر روی این شاخص نداشت. کلپوره نزدیکی ۳٪ نسبی (RGR) نرخ رشد نسبی (RGR) را کاهش داد. کلپوره غذای ضایع شده (ECI) و کارایی تبدیل غذای خورده شده را کاهش داد. تقاضایی از غذاهای مصنوعی تأثیر عصاره بومادران و کلپوره میزان فعالیت استراز و پروتئاز را در مده میباینی کاهش داد. ضمناً عصاره مناتولی هیچ کدام از گیاهان تأثیری روی میزان فعالیت گلوتاتیون-اس-ترانسفراز و استراز نداشت.

واژگان کلیدی: Spodoptera littoralis, Teucrium polium, Achillea millefolium, آنزیم‌های گوارشی.