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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)

Mojtaba Nakhaie Bahrami, Azam Mikani* and Saied Moharramipour

Department of Entomology, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran.

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* (Boisduval) (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 (Sadek, 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).

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* **Corresponding author**, e-mail: a.mikani@modares.ac.ir
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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 hour-aged 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_{50}. Third instars were used in all the experiments. In each experiment 40 insects with 4 replicates were tested for each concentration. The LC_{50} 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

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min) and the supernatant was used for measuring protease activity. The absorbance was measured by microplate reader (Epoch, USA) at 335nm.

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 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 LC_{50} 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 LC_{50}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 5930ppm (LC_{20}) 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.

Extraction	N	χ2 (df)	P-value	Slope ± SE	LC20 (95% CL) (ppm)	LC50 (95% CL) (ppm)	LC80 (95% CL) (ppm)
A. millefolium	200	1.029(3)	0.794	2.081 ± 0.346	7318 (4458-9723)	18569 (15022-18723)	47120 (34909-80146)
T. polium	200	1.18(3)	0.758	2.206 ± 0.341	5930 (3884-7680)	14275 (11685-17656)	34363 (25980-54923)

CL = Confidence limits.

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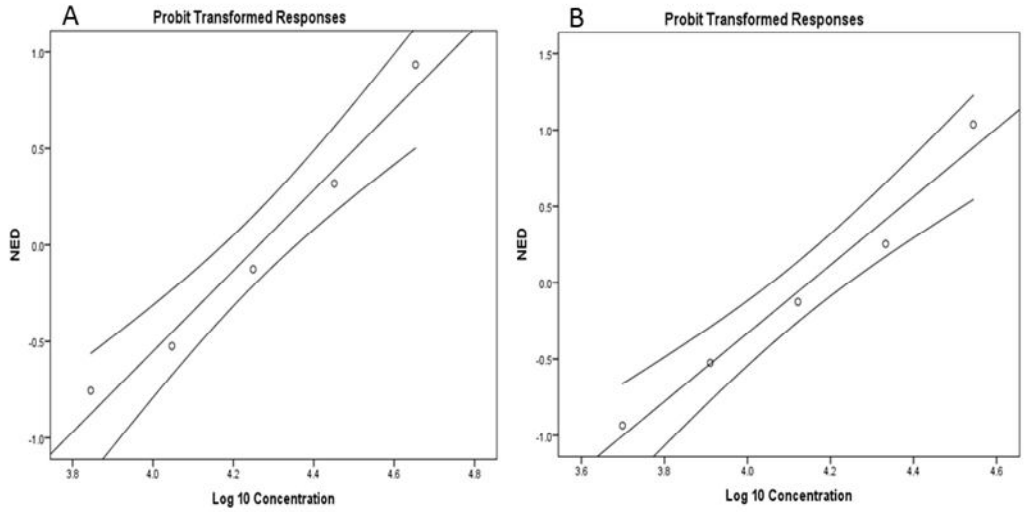

Figure 1 Probit analysis of mortality in 3rd instar larvae of *Spodoptera littoralis*, 72h after feeding on artificial diet containing 7318 and 5930 ppm (LC_{20}) of (A)*A. millefolium*or (B) *T. polium* respectively. NED refers to normalized equivalent deviation.

Table 2 Nutritional indices of 3rd instar larvae of *Spodoptera littoralis*, 72 h after feeding on artificial diet containing 7318 and 5930 ppm (LC_{20}) of *A. millefolium* or *T. polium* respectively.

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

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 $P \leq 0.05$.

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 (LC_{20}) 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).

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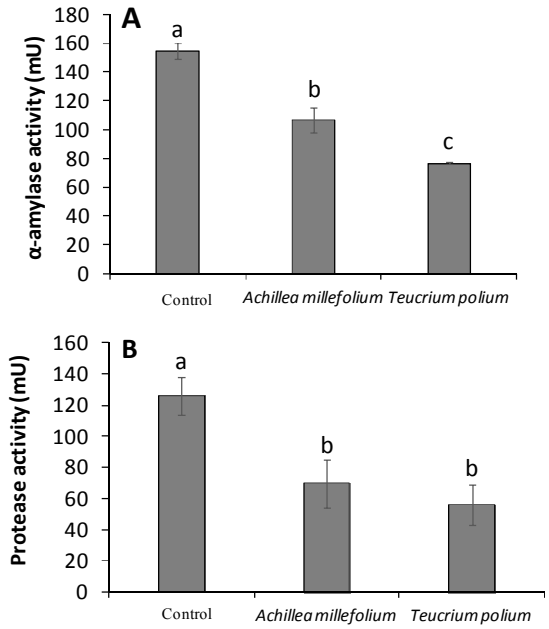

Figure 2 α-Amylase (A) and protease (B) activities in 3rd instar larvae of *S. littoralis*, 72 h after treatment with 7318 and 5930 ppm (LC_{20}) of *A. millefolium and T. polium*methanolic extracts. Each point represents the mean ± SEM. * Different letters indicate significance at $p \leq 0.05$.

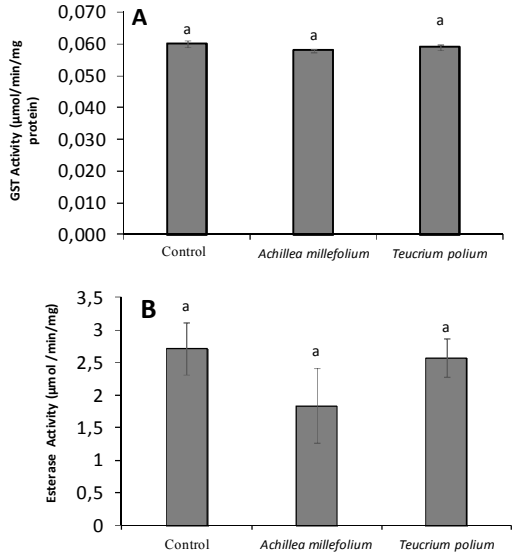

Figure 3 Glutathione s-transferase (A) and esterase (B) activities in 3rd instar larvae of *S. littoralis*, 72 h after treatment with 7318 and 5930 ppm (LC_{20}) of *A. millefolium and T. polium* methanolic extracts. Each point represents the mean ± SEM. * Different letters indicate significance at $p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

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 α-amylase activities (Fig. 2 A, B). Previous results also showed that *A. millefolium* crude leaf extracts reduced α-amylase activity in *P. rapae* (Hasheminia*et al*., 2011). Other reports also demonstrated that *T. polium* decreased α-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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These results are in good 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.

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

مجتبی نخعی بهرامی، اعظم میکانی*و سعید محرمی‌پور

گروه حشره‌شناسی کشاورزی، دانشکده کشاورزی، دانشگاه تربیت مدرس، تهران، ایران.
* پست الکترونیکی نویسنده مسئول مکاتبه: a.mikani@modares.ac.ir
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چکیده: اثر عصاره متانولی بومادران (*Achillea millefolium*) و کلپوره (*Teucrium polium*) بر روی لارو سن سه کرم برگخوار مصری *Spodoptera Littoralis* مورد بررسی قرار گرفت. عصاره متانولی بومادران و کلپوره با غذای مصنوعی آمیخته شده و شاخص‌های تغذیه‌ای، فعالیت آنزیم‌های گلوتاتیون اس-ترانسفراز، استراز، آلفاآمیلاز و پروتئاز، ۷۲ ساعت پس از تغذیه مورد بررسی قرار گرفت. هضم تقریبی (AD) در لارو تغذیه شده با عصاره متانولی کلپوره افزایش یافته در حالی‌که عصاره بومادران هیچ تأثیری بر روی این شاخص نداشت. کلپوره نرخ رشد نسبی (RGR)، نرخ مصرف نسبی (RCR)، کارایی غذای هضم شده(ECD) و کارایی تبدیل غذای خورده شده (ECI) را کاهش داد، در حالی‌که بومادران تنها RGR وRCR را کاهش داد. تغذیه از غذای مصنوعی حاوی عصاره بومادران و کلپوره میزان فعالیت آلفاآمیلاز و پروتئاز را در معده میانی کاهش داد. ضمناً عصاره متانولی هیچ‌کدام از گیاهان تأثیری روی میزان فعالیت گلوتاتیون-اس ترانسفراز و استراز نداشت.

واژگان کلیدی: *Achillea millefolium*، *Teucrium polium*، *Spodoptera littoralis*، آنزیم‌های گوارشی

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