

SHORT COMMUNICATION

Effect of *Teucrium polium* (Lamiaceae) essential oil on digestive enzyme activities and energy reserves of *Ephesia kuehniella* (Lepidoptera: Pyralidae)**M Shahriari¹, N Sahebzadeh¹, A Zibae²**¹Department of Plant Protection, Faculty of Agriculture, University of Zabol, Zabol, Iran²Department of Plant Protection, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran

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Abstract

In the current study, the effects of *Teucrium polium* L. (Lamiaceae) essential oil (*Tp* EO) and α -pinene as a major component of this essential oil, were tested against *Ephesia kuehniella* Z. (Lep.: Pyralidae) for larvicidal activity, digestive enzymes and non-enzymatic (amount of macromolecules) responses. Concentrations of 4.91 and 10.66 μ l/ml were determined as LC₅₀ values for *Tp* EO and α -pinene, respectively. The activities of α -amylase, triacylglycerol lipase, general protease, serine proteases (trypsin and chymotrypsin-like), carboxy- and aminopeptidases significantly decreased when the larvae fed on the diet supplemented with *Tp* EO and α -pinene but significant increases were observed in elastase-like proteinase. The amount of protein, glycogen and triglyceride decreased in the treated larvae by both treatments. The results showed that the digestive enzymes and non enzymatic activities of the treated larvae were decreased compared to the control. Therefore, *Tp* EO and α -pinene are suggested as botanicals for controlling flour moth larvae.

Key Words: digestive enzymes; essential oil; flour moth; macromolecules; *Teucrium polium*; α -pinene**Introduction**

In view of enzymology, digestive enzymes (such as amylases, lipases, and proteases) are commonly found in the midgut of insects. To provide metabolites and energy, these enzymes convert the food complexes into micro molecules (Klowden, 2007). α -amylase catalyzes the endo-hydrolysis of long chains such as starch and glycogen (Terra and Ferreira, 2012). This activity converts them to maltose and other oligosaccharides, which are easily absorbed by the epithelial cells of insect gut (Rahimi and Bandani, 2014). The outer links of triacylglycerols are hydrolyzed by triacylglycerol lipases (TAG-lipases). These enzymes preferentially release fatty acids (preferably unsaturated fatty acids) which are activated by calcium ions (Terra and Ferreira, 2012). Insect proteases are the diverse groups of hydrolytic enzymes, which break peptide bonds in dietary proteins (Terra and Ferreira, 2012; Subala and Shivakumar, 2015). Insects receive their required energy from ingested macromolecules such as carbohydrates, lipids and proteins. In insects, the most important lipid and

carbohydrate reserves are triglyceride and glycogen (or trehalose), respectively (Klowden, 2007; Nation, 2008). Proteins are composed of amino acids, associated to molecular transformation, cuticular sclerotization, and visual pigment synthesis, and even engaged as neural transmitters (Klowden, 2007; Ramzi *et al.*, 2014).

The flour moth, *Ephesia kuehniella* (Lep.: Pyralidae) is a polyphagous and dangerous pest on the stored products that has been observed in cereals flour. The larvae cause high levels of damage by feeding and producing frass on the stored products (Jallouli *et al.*, 2013). Currently, synthetic insecticides such as methyl bromide are most often used to control the stored products pests. However, concerns over the environmental pollution, residue levels, toxicity to non-target organisms and pesticide resistance are associated with conventional application of these insecticides, which compel us to attention to new compounds for pest control (Rajendran and Sriranjini, 2008; Muthusamy and Shivakumar, 2017).

To manage the stored product pests, the application of botanical insecticides is considered as an appropriate alternative to synthetic pesticides (Rajendran and Sriranjini, 2008; Karaborklu *et al.*, 2011). Plant secondary metabolites play insecticidal activities which could potentially act in anti-herbivore defenses against

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agricultural pests (Isman, 2006). Lamiaceae plants are quite promising to develop as novel biopesticides for the control of pest (Kim *et al.*, 2013). *Teucrium polium* is a member of this family already showing strong insecticidal activity on *Musca domestica* (Dip.: Muscidae), *Callosobruchus maculatus* (Col.: Chrysomelidae), and *Tribolium castaneum* (Col.: Tenebrionidae) (Bigham *et al.*, 2010; Hydarzade and Moravvej, 2012; Khani and Hydarian, 2014). The major constituents of its essential oil are α -pinene (12.52 %), linalool (10.63 %) and caryophyllene oxide (9.69 %) (Moghtader, 2009).

A comprehensive understanding of plant essential oils (EOs), with their active metabolites, and their interactions on physiology of the insect pests is necessary before using these compounds against the stored grain pests. Accordingly, we examined the toxicity and biochemical properties of *T. polium* essential oil (*Tp* EO) and α -pinene with a specific focus on digestive enzymes including α -amylase, triacylglycerol lipase, general protease, serine proteases (trypsin and chymotrypsin-like), carboxy- and aminopeptidases, as well as elastase-like proteinase of *E. kuehniella*. It is well documented that these enzymes alter during physiological process in insects. In addition, the effects of *Tp* EO and α -pinene on amount of protein, glycogen, and triglyceride were also considered on the treated fourth instar larvae of *E. kuehniella* under laboratory conditions.

Materials and Methods

Insect rearing

The larvae of *E. kuehniella* were collected from the infected wheat flour in Zabol, Sistan and Baluchestan province, Iran. The insects were reared in rectangular plastic boxes (17×9×9 cm³). The insects were reared in the laboratory under controlled (25 ± 1 °C, 65 ± 5 % R.H., and 15L:9D) on an artificial diet (43 gr wheat flour, 6 gr yeast, and 20 ml of glycerin) (Lima *et al.*, 2001).

Essential oil preparation

Seeds of *T. polium* were ground and 40 g of powdered seeds was incubated at 500 ml of distilled water for 4 h in 100 °C. Water of obtained sample was taken by sodium sulfate and kept at 4 °C prior to the experiment tests (Negahban *et al.*, 2007). α -pinene was purchased from Sigma-Aldrich (Spain) and were applied without further purification.

Bioassay

According to Shahriari *et al.* (2017), fourth instar larvae of *E. kuehniella* were randomly selected and were exposed to 500 mg of artificial diet containing 2, 3, 4.5, 6.7 and 10 μ l/ml of *Tp* EO, and 4, 5.6, 8, 11.3 and 16 μ l/ml of α -pinene, respectively. Control larvae were fed on the diet containing acetone as solvent for dilution of serial concentrations of *Tp* EO and α -pinene. Larval mortality was recorded after 24 h. The experiments were carried out using 180 larvae with 3 replicates in each concentration (total numbers were 360 larvae for both *Tp* EO and α -pinene concentrations).

Larval gut extraction

The treated larvae of *E. kuehniella* were randomly and individually selected and their midguts were dissected under a stereomicroscope in ice-cold distilled saline solution (NaCl, 10 mM). The midguts were rinsed in ice-cold distilled water and placed in a pre-cooled homogenizer. Then the prepared midguts were grounded prior to be centrifuged at 13,000 rpm (20 min at 4 °C). The supernatants were stored at -20 °C for determination of digestive enzyme activities (Ramzi *et al.*, 2013).

Effects of *Tp* EO and α -pinene on digestive enzymes

α -amylase assay was carried out as described by Bernfeld (1955). Ten microliter of gut extraction and 20 μ l of 1 % soluble starch as substrate were incorporated using phosphate buffer (50 μ l, 20 mM, pH 7.1), mixed thoroughly and incubated for 30 min at 35 °C. Eighty microliter dinitrosalicylic acid (to stop the reaction) was added and heated in boiling water for 10 min and the absorbance of this reaction was read at 545 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 (Ramzi *et al.*, 2014). The method described by Tsujita *et al.* (1989) was used to assay TAG-lipase activity. Twenty microliter of homogenated guts were incubated at 37°C with 100 μ l of Tris-HCl buffer (10 mM, pH 8) and 40 μ l of *p*-nitrophenyl butyrate (27 mM) as substrate. The absorbance was read at 405 nm. One unit of enzyme released 1.0 nmol of *p*-nitrophenol per min at pH 7.2 and 37 °C when *p*-nitrophenyl butyrate was used as substrate. Standard curve of *p*-nitrophenol was used to calculate the specific activity of this enzyme. General proteolytic activity was measured using azocasein (2 %) based on a method described by Elpidina *et al.* (2001). The reaction mixture consisted of 20 μ l of the enzyme solution, 100 μ l of Tris-HCl buffer (pH 8) and 40 μ l of azocasein (2 %) as substrate. The reaction mixture was incubated at 37°C for 60 min before adding 150 μ l of 10 % trichloroacetic acid (TCA) to terminate the reaction. Precipitation was achieved by cooling at 4 °C for 120 min and it was centrifuged at 13,000 rpm for 10 min. An equal volume of NaOH (2 M) was added to the supernatant and the absorbance was recorded at 440 nm. The blank consisted of all mentioned portions except the enzyme solution of guts. Trypsin, chymotrypsin and elastase-like activities were assayed using a concentration 1 mM of BApNA (Nabenzoyl-L-arginine-p-nitroanilide), 1 mM N-succinyl alanine alanine proline phenylalanine p-nitroanilide (SAAPPpNA) and 1 mM SAAApNA (N-succinyl-alanine-alanine-alanine-p-nitroanilide) as substrates, respectively. The reaction mixture included 35 μ l of Tris-HCl buffer (20 mM, pH 8 as literature recommended pH for serines in lepidopteran species), 5 μ l of BApNA, SAAPPpNA and SAAApNA substrates individually and 5 μ l of enzyme solution. This mixture was incubated at 30 °C (10 min), and its absorbance was then read at 405 nm. To prove the specific proteolytic activity, a negative control was provided for each substrate separately containing all mentioned components

besides enzyme pre-boiled at 100 °C for 30 min (Oppert *et al.*, 1997). Activities of the two exopeptidases in the midgut of *E. kuehniella* were obtained using Hippuryl-L-Arginine and Hippuryl-L-Phenylalanine for carboxy- and aminopeptidases, respectively. The reaction mixture consisted 35 µl of Tris-HCl buffer (20 mM, pH 7), 5 µl of each mentioned substrate and 5 µl of enzyme solution. Following by incubation (30 °C, 10 min), the absorbance was read at 340 nm. To prove the specific proteolytic activity, a negative control was provided as mentioned previously (Oppert *et al.*, 1997).

Effects of TP EO and α-pinene on macromolecules

The amount of protein was measured using Lowry *et al.* (1951) procedure. Twenty microliter of homogenized sample was added to 100 µl of reagent, and incubation was made for 30 min at 25 °C. The absorbance was read at 545 nm (Recommended by Ziest Chem. Co., Tehran-Iran) (Ramzi *et al.*, 2014). To determine triacylglyceride content, a diagnostic kit (Pars Azmoon Co., Tehran, Iran) was used to measure the amount of triacylglyceride in the fourth 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), peroxidase (2 kU/l), lipoprotein lipase (2 kU/l), 4-aminoantipyrine (0.5 mM), and glycerol-3-phosphate-oxidase (0.5 kU/l). Samples (10 µl) were incubated with 10 µl of distilled water and 70 µl of reagent for 20 min at 25 °C. The following equation was used to calculate the amount of triacylglyceride:

$$\text{mg/dl} = \frac{\text{OD of sample}}{\text{OD of standard}} \times 0.01126.$$

Optical densities (ODs) of samples and reagent as standard were read at 545 nm (Fossati and Prencipe, 1982). For estimation of glycogen, fat bodies of 20 larvae were cut and immersed to 1 mL of 30 % KOH w/Na₂SO₄. Tubes containing the samples were covered with aluminium foil (to avoid evaporation) and were boiled for 30 min. Tubes were shaken and cooled in ice. Then, 2 mL of 95 % EtOH was added to precipitate glycogen from digested solution. The samples were again shaken and incubated in ice for 30 min. Tubes were centrifuged 13,000 rpm for 30 min. Supernatant was removed and pellets (glycogen) were re-dissolved in

1 mL of distilled water before being shaken. Glycogen standard (0, 25, 50, 75 and 100 mg/mL) was prepared before adding phenol 5 %. The samples were incubated on ice bath for 30 min. Finally, the absorbance of standards and samples were read at 490 nm (Chun and Yin, 1998).

Data analysis

For estimation of LC₅₀ and corresponding 95 % CI values, POLO-Plus software was used. Biochemical data were estimated in a complete randomized design, and were compared by one-way analysis of variance (ANOVA) followed by Tukey's test. Differences between samples were statistically considered at a probability less than 5 % and marked in the tables and figures.

Results and Discussion

Bioassay

In this study, the lethal concentrations (LC₅₀) values were observed in 4.91 µl/ml and 10.66 µl/ml for *Tp* EO and α-pinene, respectively (Table 1). By increasing of concentration, the mortality of larvae was increased. Similarly, α-pinene was evaluated against *T. castaneum* larvae, at 24 and 48 h post-treatment, and LC₅₀ of this compound was determined as 12.85 and 8.39 µl/ml, respectively (Shahriari *et al.*, 2016). Bigham *et al.* (2010) found a significant oral toxic activity of *T. polium* on *M. domestica* (LC₅₀ = 80ppm). LC₅₀ values were showed contact toxicity of *T. polium* 1263.09 and 1469.72 µl/m² on adult males and females of *C. maculatus*, respectively (Hydarzade and Moravvej, 2012). Plant essential oils (EOs) are aromatic compounds and complex mixture of monoterpenes. It is often observed that complex essential oil compounds are more efficacious than the pure combination (Don-Pedro, 1996; Bakkali *et al.*, 2008; Kumrungsee *et al.*, 2014). In our study, the oral toxicity of *Tp* EO was higher than α-pinene on *E. kuehniella*. Similarly, Don-Pedro (1996), Ho *et al.* (1997), Hori (1998), and Kim *et al.* (2010, 2013) demonstrated that essential oil from different plant species (anise, rosemary, citrus, origanum and cumin) were more effective than their pure secondary metabolites (trans-anethole, linalool, limonene, carvacrol and p-cymene) on different pests such as *C. maculatus*, *Myzus persicae* S. (Homoptera: Aphididae), *T. castaneum* and *Sitophilus oryzae* (Col.: Curculionidae).

Table 1 Dose-response parameters of fourth instar larvae of *Ephestia kuehniella* exposed to *Teucrium polium* essential oil and α-pinene

Treatment	LC ₅₀ (µl/ml)	X ² (df)	Slope±SE
<i>Tp</i> EO	4.91 (4.16-5.87)	2.05 (3)	3.16 ± 0.504
α-pinene	10.66 (8.61-14.91)	2.37 (3)	2.28 ± 0.526

Tukey test, *p* < 0.05

Table 2 Activity of digestive enzymes in fourth instar larvae of *Ephesia kuehniella* at 24, 48 and 72 h post treatment with LC₅₀ value of *Teucrium polium* essential oil and α -pinene

Digestive enzyme	Time (h)	Control	<i>Tp</i> EO	α -pinene	F- Value	Pr
α -amylase (U/mg protein)	24	0.658±0.0049a	0.144±0.0024c	0.240±0.0053b	3794.32	0.0001
	48	0.763±0.0085a	0.355±0.0034b	0.370±0.0090b	992.23	0.0001
	72	0.762±0.0008a	0.290±0.0049c	0.417±0.0017b	6250.88	0.0001
TAG lipase (U/mg protein)	24	0.562±0.0045a	0.352±0.0045b	0.346±0.0092b	340.02	0.0001
	48	0.563±0.0046a	0.506±0.0058b	0.338±0.0052c	676.9	0.0001
	72	0.589±0.0053a	0.456±0.0035b	0.328±0.0035c	658.24	0.0001
General protease (U/mg protein)	24	0.118±0.0046a	0.059±0.0043b	0.053±0.0050b	59.38	0.0001
	48	0.120±0.0043a	0.088±0.0017b	0.074±0.0023b	50.89	0.0002
	72	0.123±0.0033a	0.106±0.0038b	0.094±0.0026b	19.07	0.0023
Trypsin-like (U/mg protein)	24	0.166±0.0032a	0.063±0.0031b	0.033±0.0038c	409.32	0.0001
	48	0.165±0.0035a	0.067±0.0037b	0.058±0.0031b	371.03	0.0001
	72	0.163±0.0040a	0.073±0.0030b	0.059±0.0030b	275.65	0.0001
Chymotrypsin-like (U/mg protein)	24	0.092±0.0029a	0.049±0.0013c	0.080±0.0038b	58.59	0.0001
	48	0.094±0.0034a	0.078±0.0003b	0.087±0.0025ab	7.33	0.0245
	72	0.092±0.0035a	0.069±0.0008b	0.086±0.0012a	29.64	0.0008
Elastase-like (U/mg protein)	24	0.103±0.0024b	0.117±0.0029a	0.119±0.0036a	7.73	0.0219
	48	0.099±0.0029b	0.125±0.0032a	0.130±0.0040a	22.44	0.0016
	72	0.096±0.0030c	0.164±0.0034a	0.132±0.0043b	60.91	0.0001
Carboxypeptidase (U/mg protein)	24	0.297±0.0031a	0.202±0.0040b	0.189±0.0031b	17.22	0.0033
	48	0.300±0.0041a	0.218±0.0018b	0.199±0.0058c	159.55	0.0001
	72	0.300±0.0041a	0.226±0.0040c	0.260±0.0030b	94.55	0.0001
Aminopeptidase (U/mg protein)	24	0.105±0.0031a	0.079±0.0029b	0.108±0.0031a	26.59	0.0010
	48	0.106±0.0041a	0.073±0.0011b	0.068±0.0028b	57.86	0.0001
	72	0.108±0.0041a	0.072±0.0080b	0.073±0.0027b	75.66	0.0001

Statistical differences have been done within each time intervals and marked by different letters in each row (Tukey test, $p < 0.05$).

Effects of *Tp* EO and α -pinene on digestive enzymes

The lower activity of α -amylases was found in the larvae fed on the diet containing LC₅₀ concentrations of *Tp* EO and α -pinene compared to the control (Table 2). Similarly, Bigham *et al.* (2010) reported that *Tp* EO decreased the activity of α -amylase in the treated *M. domestica* larvae versus control. The reduced activity of α -amylase by plant-based compounds could imply their cytotoxic effect on the midgut epithelial cells those synthesize insect α -amylase (Franco *et al.*, 2002; Senthil-Nathan *et al.*, 2006; Zibae and Bandani, 2010). This result is consistent with previous studies which were

demonstrated the reduction of α -amylase activities in pests such as *E. kuehniella* (Shahriari and Sahebzadeh, 2017), *Ectomyelois ceratoniae* (Lep.: Pyralidae) (Ramzi *et al.* 2013, 2014), *Glyphodes pyloalis* (Lep.: Crambidae) (Khosravi *et al.*, 2011; Yazdani *et al.*, 2013), and *Pieris rapae* (Lep.: Pieridae) (Hashminia *et al.*, 2011) after treatment with botanical toxins.

Midgut TAG-lipase has a primary role to obtain dietary lipid as well as strong antiviral activity. We found a significant reduction in TAG-lipase activity in the larvae of *E. kuehniella* fed on the artificial diet containing *Tp* EO and α -pinene (Table 2). Yazdani *et al.* (2013) reported that LC₅₀ concentration of some

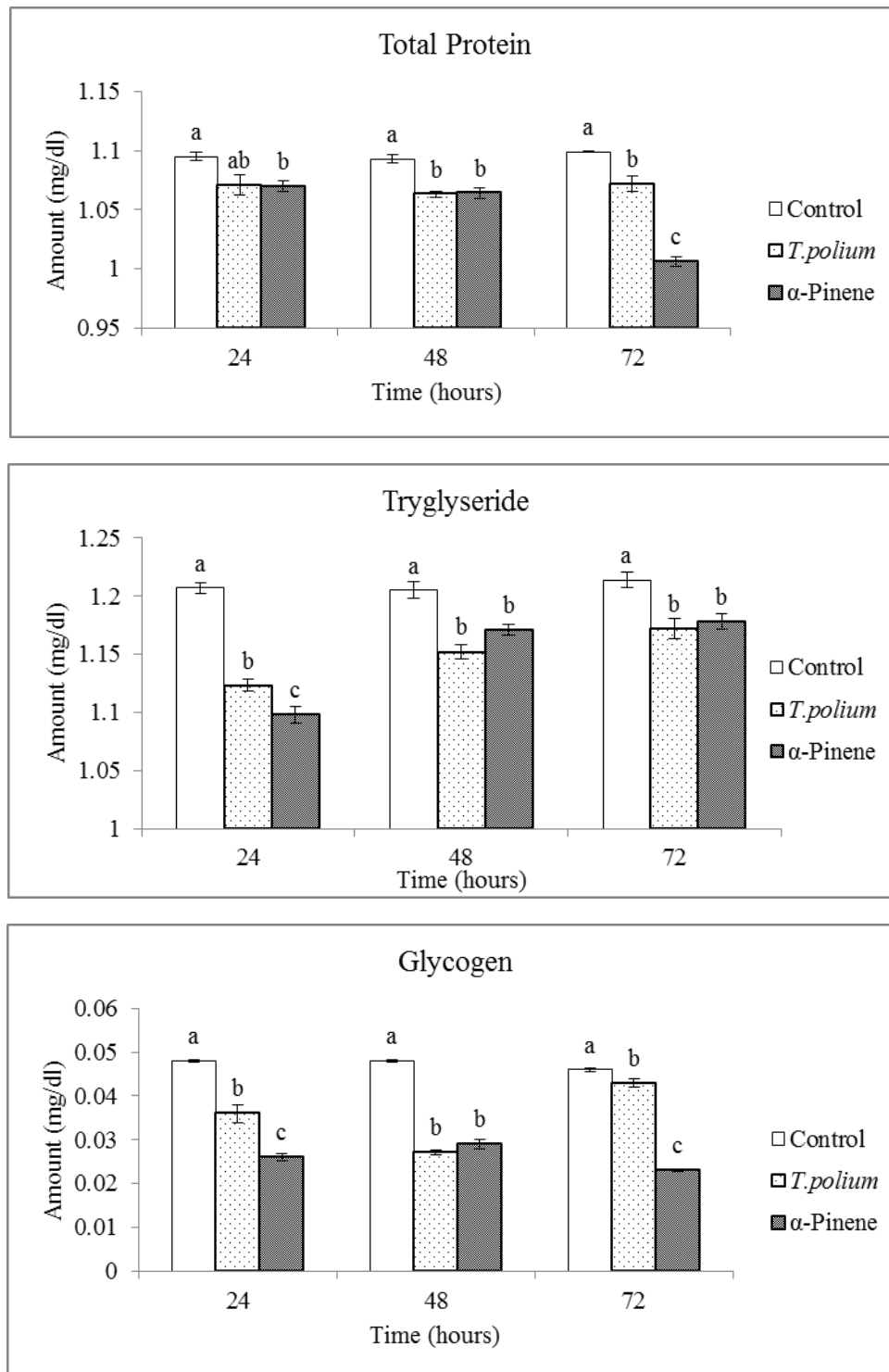


Fig. 1 Effects of essential oil of *Teucrium polium* essential oil and α -pinene on amounts of storage macromolecules (total protein, triacylglyceride, and glycogen) in the fourth instar larvae of *E. kuehniella*. Statistical differences have been done within each time intervals and marked by different letters (Tukey test, $p < 0.05$).

essential oils such as increased the activity of TAG-lipase in the treated *G. pyralis* larvae versus control. Khosravi *et al.* (2011) found similar results while Senthil-Nathan *et al.* (2006) reported the lower

activity of TAG-lipase in *Cnaphalocrocis medinalis* (Lep.: Pyralidae) midgut after treatment with *Azadirachta indica* (Meliaceae) extract. Great utilization of exogenous lipids could increase the

activity of TAG-lipase in insect midgut (Yazdani *et al.*, 2013). Decreased activity of TAG-lipase by botanical insecticide probably resulted to disturbance of digestion and absorption processes (Senthil-Nathan *et al.*, 2006; Zibae and Bandani, 2010).

Treatment of *E. kuehniella* larvae with LC₅₀ concentrations of *Tp* EO and α -pinene showed the lower general proteolytic activity (Table 2). Senthil-Nathan *et al.* (2006) found a significant reduction in protease activity of *C. medinalis* fed on a diet containing *Bt* toxins and botanical insecticides including neem seed and leaf extract of *Vitex negundo* (Verbenaceae). Digestive proteolytic activity increased in the larvae of *G. pyralis* treated by *Lavandula angustifolia* (Lamiaceae) (Yazdani *et al.*, 2013). The digestion of proteins in the first stage in insects mostly occurs under the effect of serine proteases (Terra and Ferreira, 2012). In our study, *Tp* EO and α -pinene decreased the activities of trypsin and chymotrypsin-like protease but a significant addition was observed on elastase activity of the treated larvae (Table 2). Similar to our results, Mojarab-Mahboubkar and Jalali-Sendi (2016) demonstrated that activity of serine proteases decreased by *Artemisia annua* methanolic extract except for elastase. Zibae *et al.* (2014) reported that activity of serine proteases decreased in the larvae of *Pieris brassicae* (Lep.: Pieridae) treated with 0.5, 1 and 2 % concentrations of *Polygonum persicaria* (Polygonaceae) Agglutinin. Senthil-Nathan *et al.* (2006), Zibae and Bandani (2010), and Ramzi *et al.* (2013) reported that plant-based compounds may affect the construction of some kinds of proteases and inhibition them to digestion ingestion proteins.

It was shown that activities of two exopeptidases including amino and carboxypeptidases of treated *E. kuehniella* larvae with LC₅₀ concentration of *Tp* EO and α -pinene significantly decreased in comparison with the control larvae (Table 2). Ramzi *et al.* (2013, 2014) and Zibae *et al.* (2014) found no significant differences in the activities of aminopeptidase between control and treated larvae, while carboxypeptidase activity significantly decreased 48 h post-treatment by lectins extracted from plant source. Exopeptidases hydrolyze single amino acids either from the N-terminus or from the C-terminus (aminopeptidases and carboxypeptidases, respectively) at the end of a polypeptide chain (Kanost and Clem, 2012). The potential mechanisms of EO's and plant secondary metabolites on exopeptidase activities are still lacking.

Effects of Tp EO and α -pinene on macromolecules

In this study, amounts of protein, glycogen, and triglyceride in *E. kuehniella* larvae treated by *Tp* EO and α -pinene decreased significantly versus control (Fig. 1). Several studies have been demonstrated that synthetic and botanical insecticides could alter the amount of carbohydrates, lipids and proteins in insects. The significant reductions in amount of protein in *G. pyralis* treated with lethal and sublethal doses of *L. angustifolia* EO have been reported by Yazdani *et al.* (2013). In a similar study, Zibae *et al.* (2011) showed that the amount of

protein, glycogen (trehalose) and lipid as non-enzymatic components in hemolymph and fat bodies of *Eurygaster integriceps* (Hemiptera: Scutelleridae) reduced after exposure to pyriproxifen. *Citrullus colocynthis* (Cucurbitaceae) agglutinin decreased the amount of glycogen and triglyceride levels in *E. ceratoniae* (Ramzi *et al.*, 2014). The reductions occurring by *Tp* EO and α -pinene in *E. kuehniella* might be due to accelerating in glycogenolysis at larval fat bodies, transport glycogen from fat body to hemolymph as a response to energy depletion when larvae were exposed to toxins (Zibae *et al.*, 2011). Lipid depletion after toxin treatments could be occurred because of alteration in its synthesis patterns (Klowden, 2007), hormonal dysfunction for controlling lipid metabolism (Steel, 1980) and the utilization of this metabolic reserve to generate insect energy demand (Sak *et al.*, 2006).

Results of this study indicate that *Tp* EO and α -pinene possesses larvicidal effects on *E. kuehniella*. Moreover, the results demonstrated the lower digestive enzyme activities and macromolecules amount of treated larvae compared to the controls. In general, EOs and their secondary metabolites show to be significant entomotoxic molecules that efficiently affect physiology and biology of pests without affecting non-target organisms. This study could play a significant role in integrated pest management in the stores.

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