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RESEARCH REPORT

Immunological and oxidative responses of the lesser mulberry pyralid, *Glyphodes pyloalis* by an aqueous extract of *Artemisia annua* L.

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Abstract

In this search for affordable and locally available biological substances both to farmers and environment, an aqueous extract of *Artemisia annua* L. was investigated for the first time against the lesser mulberry pyralid, *Glyphodes pyloalis* Walker a serious pest in mulberry orchards. The LC_{10}, LC_{30} and LC_{50} values were estimated 12.82 %, 20.6 % and 27.35 % (W/V) respectively. The extract adversely affected oviposition, impaired immunity through reduced granulocytes and phenoloxidase activity. The increased activity of detoxifying enzymes including esterases and glutathione *S*-transferase (GST) were also observed. The enhanced antioxidant system including peroxidase (POX), catalase (CAT), glucose 6-phosphate dehydrogenase (GPDH) and superoxide dismutase (SOD) were also observed. The results of the present study may provide a very safe way to control this pest in mulberry orchard and deserve further studies.

Key Words: antioxidant enzymes; aqueous extract; *Artemisia annua*; detoxifying enzymes; immune response; oviposition

Introduction

Nowadays, increasing populations coupled with increased food demand has been resulted in a disaster for both human health and the surrounding environment (Sparks and Lorbasch, 2017; Isman, 2020; Ali *et al.,* 2021); natural substances extracted from plants can be an alternative remedy to chemical pesticides (Isman, 2000; Govindarajan *et al.,* 2016; Verma *et al.,* 2021). *Artemisia anuua,* also known as sweet wormwood or annual wormwood, is a medicinal plant in many parts of the world and is now of great economic importance due to its biological activity against various pests (Khosravi *et al.,* 2011; Seixas *et al.,* 2018; Liu *et al.,* 2019; Oftadeh *et a*l., 2021). This plant has long been known in ancient Chinese medicine for its antimalarial activity of artemisinin against plasmodium (Meshnick *et al.,* 1996; Tu, 2016; Salehi *et al.,* 2018; Shahrajabian *et al.,* 2020). Moreover, it is used to treat fever, summer heat wounds, jaundice, lice, scabies, tuberculosis, hemorrhoids, dysentery, while in Iran is used as an antispasmodic, sedative remedy for children (Sadiq *et al.,* 2014; Septembre-Malaterre *et al.,* 2020; Feng *et al.,* 2020; Trendafilova *et al.,* 2021). The bioactivities of *A. annua* against pests have attracted the attention of many scientists, especially the research that has been done and focused in our laboratory since 2008 (Shekari *et al.,* 2008; Hasheminia *et al.,* 2011; Zibaee and Bandani, 2011).

The mulberry pyralid has become a major pest of mulberry plantations in orchards of northern Iran where the leaves are harvested for use in sericulture (Lalfelpuii *et al.,* 2014). This insect is suspected as an intermediate host of densoviruses and picornavirus to the silkworm (Watanabe *et al.,* 1988). The use of chemical insecticides is strictly prohibited because the fresh leaves are provided daily to silkworm. Farmers have their plantations adjacent to their homes which this prevents the use of synthetic chemicals (Afraze *et al.,* 2020; Oftade *et al.,* 2020) and also breed domestic animals on a smaller scale. Therefore, they preferably do not use any chemical insecticide. Therefore, plant-based products can be considered as a safe and inexpensive option for their health and that of domesticated animals.

The immune system in insects includes cellular and humoral immune responses. Cellular immunity occurs with changes in hemocyte counts, coagulation,

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Table 1 The LC values, from oral toxicity of *A. annua* aqueous extract on fourth instar larvae of *G. pyloalis*

Aqueous extract	Time*	LC_{10}	LC_{30}	LC_{50}	Slope ± SE	X^2 (df=3)	P value
Artemisia annua	48	12.82 (3.04 -18.98)	20.06 (9.40 - 27.06)	27.35 (18.13 - 39.15)	3.895 ± 0.577	5.348	1.761

*48 h after treatments; LC: lethal concentration (% W/V for oral toxicity); x^2: chi-square value; df: degrees of freedom

micro accumulation, increased phenoloxidase enzyme activity and melanin formation around external factors. While in humeral immunity, antimicrobial peptides produced by fat body cells play an effective role in eliminating toxic (Hoffmann, 2003; Beckage, 2011; Krautz *et al.,* 2014; Dubovskiy *et al.,* 2016; Baghban *et al*., 2018; Ebrahimi and Ajamhassani, 2020). Much research has been done on the use of plant pesticides, including extracts and essential oils of *A. annua* plant against insect immune system (Padmaja and Rao, 2000; Zibaee and Bandani, 2010; Ali and Ibrahim, 2018; Ramírez-Zamora *et al.,* 2020; Oftadeh *et al.,* 2020). In addition, antioxidant enzymes are considered as part of the immune response against causative agents (Beutler, 2004; Iwanaga and Lee, 2005). These enzymes are responsible for controlling ROS (Reactive oxygen species) produced by biotic and non-biotic stresses in insects (Pavlick *et al.,* 2002; Kang *et al.,* 2015; Nasi *et al.,* 2020). ROS contains oxygen ions, free radicals, and organic and inorganic molecules. The activity of these molecules increases under the influence of external factors and causes damage to the structure of cells and tissues in the insects (Lyakhovich *et al.*, 2006; Wan *et al.,* 2014). The results obtained by many researchers show that the antioxidant system act as a defense mechanism against the production of ROS produced by external factors (Dhivya *et al.,* 2018; Lin *et al.,* 2018; Manjula *et al.,* 2020; Magierowicz *et al.,* 2020).

Various studies have shown a reasonable control by *A. annua* extracts and essential oils. However, the solvent or essential oil extractions are time consuming tasks. This is our first attempt to use the aqueous extract of this precious plant against the mulberry pyralid, which is easily available inside to meet the needs of farmers. In this way, it reduces the costs and environmental side effects of solvents.

Materials and methods

Insect rearing

Different instar larvae of *Glyphodes pyloalis* were collected from infected mulberry orchards Rasht (37.1936° N, 49.6410° E.), northern Iran. The larvae were feed on fresh mulberry leaves (Ichinose Var.) at 24 ± 2 °C, 75 ± 5 % of relative humidity, and 16:8 (L:D) h photoperiod, in plastic boxes. Adults were kept in plastic receptacles (18 × 15 × 7 cm). Then a piece of cotton soaked in 10 % water and the honey solution was fed to the moths and fresh leaves were placed in containers to oviposition.

Preparation of aqueous extract

The leaves of *A. annua* were collected from the area around the Faculty of Agricultural Sciences, the University of Guilan, Rasht 37.1936° N, 49.6410° E. Then leaves were boiled with a proportionate amount of distilled water for 1 h. After passing through a strainer, the resulting solution was placed in an oven at 50 °C for 48 h. Then the residue was mixed with distilled water and used as a stock and concentrations were made from it.

Oral toxicity of aqueous extract of A. annua

In order to determine the toxicity of the extraction on fourth instar lesser mulberry pyralid larvae, leaf disc immersion method was used (Horowitz *et al.,* 2004). For this purpose, 5 concentrations (10, 20, 30, 40 and 50 % W / V) of aqueous extract were evaluated. This experiment was performed with four replications and each replication with ten larvae. The mortality was recorded after 48 h. The LC_{10}, LC_{30} and LC_{50} were estimated using Polo-Plus (2002) software.

Oviposition deterrence

This experiment was performed using a choice bioassay. For this purpose, newly emerged adults were kept in the mating cage for 24 h and used for oviposition bioassay at next day. The oviposition cage consisted of a rectangular plastic container measuring (45 cm × 20 cm × 20 cm). At the top of the cage, there was a window (10 cm × 10 cm) covered with a piece of mesh cloth to release the moths inside. Four 30 cm long glass-plastic tunnels are attached to each wall of the cage. The opening of each tunnel was opened into the cage and the end is blocked with a small fan for air circulation. In this method, leaf discs treated with aqueous extract of *A. annua* and the control treated with distilled water were placed in each tunnel. Five female moths were mated and released into the cage. After 24 h, the leaves were collected and the number of eggs laid was counted. This experiment was performed with five replications and each replication with 5 moths. The Oviposition Deterrent Index (ODI) was calculated using the following formula (Huang and Renwick, 1994).

$$ODI = Cn-Tn / Cn + Tn \times 100$$

Where, Cn and Tn represents number of eggs laid on control and treated leaves, respectively.

Table 2 Ovipositional responses of *G. pyloalis* females to different concentration of aqueous extracts of *A. annua* in choice bioassay

Concentration	Time*	Control	LC_{10}	LC_{30}	LC_{50}	F	P	Df
ODI	24	91.4 ± 3.044a	36.4 ± 6.738b	2.4 ± 1.197c	0c	277.40	0.0001	3,19

*24 h after treatments; Means (±SE) followed by the same letters in a row indicate no significant difference ($p < 0.05$) according to the Tukey test

Immunological assay

Total Hemocyte Count (THC)

Hemolymph of fourth instar larvae were prepared from the first abdominal pro leg, after 24 and 48 h. For THC a Neubauer hemocytometer (HBG, Germany) was used, Then larval hemolymph (10 µL) was mixed with 290 µL of anti-coagulant solution (0.017 M EDTA, 0.041 M Citric acid, 0.098 M NaOH, 0.186 M NaCl, pH 4.5) (Amaral *et al.,* 2010).

Differential hemocyte count (DHC)

For this purpose, the first abdominal proleg was dissected and 5 µL of hemolymph was placed on to a clean slide and then smeared using another slide. After the smear was dried, the slide was stained using diluted Giemsa's stain (1:9) for 12 minutes. They were subsequently differentiated in dilute lithium carbonate solution for red staining structures and then in HCl acidified distilled water for blue staining structures. The slides were washed in distilled water and mounted in Canada balsam (Merck, Germany). Then counting 200 hemocytes randomly from 4 corners and a central part in the slides (Wu *et al.,* 2016), these cells were identified based on morphological features under a microscope (Leica light-microscope) (Rosenberger and Jones, 1960).

Phenoloxidase activity assay

To measure phenoloxidase activity, the method of Catalan *et al.* (2012) was used with some modification. 10 µL of hemolymph was dissolved in 90 µL of phosphate buffer and L-DOPA (3, 4-dihydroxyphenylalanine) (10 mM) was used as a substrate. The samples were centrifuged at 4 °C for 5 minutes at 5000 rpm. 50 µL of the solution was mixed with 150 µL of the substrate. Enzyme activity was calculated per mg of hemolymph protein, which was also measured by the Lowry method (Lowry 1951). The specific activity of the enzyme was read at 490 nm with a micro plate reader.

Enzymatic Assays

Sample Preparation

The 4th instar larvae were homogenized in 1 ml of ice-cold 50 mM PBS with 10 % glycerol. Sample mixtures were centrifuged at 13000 rpm for 15 min in 4 °C. The collected supernatant was used for the enzymatic assay.

GST activity assay

Glutathione S-transferase activity was measured using the method Habig *et al.* (1974) with two substrates of 1-chloro-2,4-dinitrobenzene (CDNB) and 3,4-dichloronitrobenzene (DCNB). 50 µL of sample, 135 µL of phosphate buffer (pH 7), 50 µL of reagent and 100 µL of reduced glutathione were mixed together. After 5 minutes of incubation at 25 °C, the absorbance was read at 340 nm.

General esterase activity assay

General esterase activity was measured according to the method of Han *et al.* (1998). The larval midgut was homogenized with 1000 µl of 0.1 mM phosphate buffer and Triton x-100 (0.01 %), and centrifuged at 4 °C for 10 minutes. Then 10 µL of each substrate of alpha naphthyl acetate and beta naphthyl acetate (10 mM) separately with 5 µL of RR-Salt blue salt with 40 µL of phosphate buffer (20 mM) and 5 µL enzyme samples were mixed. After 5 minutes of incubation at 25 °C, the light absorption was read at 450 nm.

CAT activity Assay

The method of Wang *et al.* (2001) was used to measure the CAT activity. 500 microliters of 1 % hydrogen peroxide were added to 50 µL of sample (treated and control). The reaction mixture was incubated for 10 minutes at 28 °C. The absorbance was read at 240 nm.

SOD activity Assay

The method of McCord and Fridovich (1969), has been used to measure the SOD activity of treated and control larvae. 500 µL of SOD solution (70 µM NBT and 125 µM xanthine prepared in phosphate buffer (pH 7). Then, it was mixed with 100 µL of xanthine oxidase solution including 100 µL of xanthine oxidase (5.87 units/ml) and 10 mg of bovine serum albumin dissolved in 2 ml of PBS) were added to 50 µL of sample. The reaction mixture was. Incubated at 28 °C for 20 min in darkness. The absorbance was read at 560 nm.

POX activity Assay

50 µL of sample was added to 250 µL of buffered pyrogallol (0.05 M pyrogallol in 0.1 M phosphate buffer (pH 7.0)) (250 µL) and 250 µL of H_2O_2 (1 %). The absorbance was recorded for every 30 s up to 2 minutes at 430 nm (Addy and Goodman, 1972).

GPDH activity Assay

The procedure of Balinsky and Bernstein (1963) was adopted to calculate GPDH amount in treated and control larvae. We used 100 µL of Tris-HCl (100 mM, pH 8.2), 50 µL of NADP (0.2 mM) and 30 µL of $MgCl_2$ (0.1 M) were mixed to initiate the reaction. 100 µL of GPDH (6 mM) was added to the mixture and OD raise was measured at 340 nm.

Protein Assay

Protein content was determined by the method of Lowry *et al*. (1951) and using Ziest Chem's biochemical kit (Ziest Chem. Co., Tehran-Iran). 100 µL of reagent along with 20 µL of enzyme extracts were incubated at 25 °C for 30 minutes and the adsorption was read at 545 nm using a micro plate reader.

Statistical analyses

Determination of mortality and lethal concentrations were done by Polo-Plus (2002) software. The least significant among treatments were compared using Tukey analysis (SAS Institute, 1997). Differences among means were considered to be significant at $p \leq 0.05$. All the data in relation to immune system analyzed by T-test.

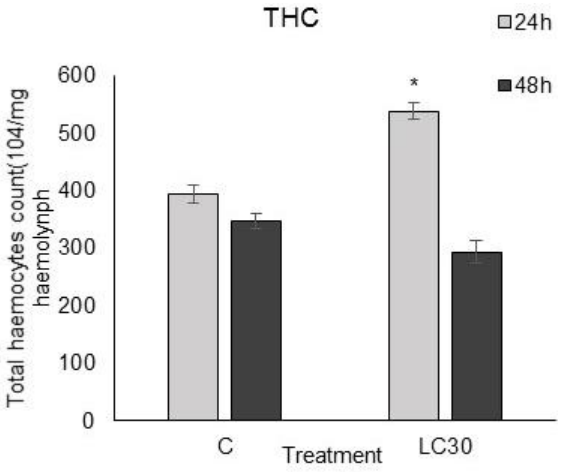

Fig. 1 Total hemocyte count (**THC**) following treatment with LC30 with *A. annua* aqueous extract compared to control (**C**) in fourth instar larvae of *G. pyloalis* after 24 and 48 h. Statistical differences have been marked asterisks ($p \leq 0.05$). According to a T-test

Results

Bioassay of A. annua *aqueous extract on* G. pyloalis *larvae*

The aqueous extract of *A. annua* caused mortality in *G. pyloalis* larvae after 48 h. The LC_{50} value was 27.35 % W/V. The rate of mortality in treated larvae was dose-dependent. The confidence limits (CL) and the slope of regression are shown in Table 1.

Effect of A. annua aqueous extract on G. pyloalis oviposition

The oviposition rate in female moths decreased significantly compared to the control after 1 d (F = 277.40, $df_{t,e}$ = 3, 19, p = 0.0001) Table 2.

Effect on THC and DHC

The number of total hemocytes in treated larvae of *G. pyloalis* increased significantly after 24 h compared to the control (*t = 7.03, df = 4, p= 0.002*) (Fig. 1), while no significant reduction was observed after 48 h (t = 2.34, df = 4, p = 0.079). The number of plasmatocyte (*t = 2.95, df = 4, p = 0.041*) and granulocyte (t = 2.82, df = 4, p = 0.047) followed the THC trend i.e. significantly increased after 24 h (Fig. 2). Whereas a significant decrease in the number of granulocytes was detected compared to the control (*t = 2.89, df = 4, p = 0.044*) (Figs. 2). The activity of phenoloxidase increased significantly after 24 h (*t = 4.90, df = 4, p = 0.008*) (Fig. 3), and the reduced activity was significant compared to the control at 48 h (*t = 3.31, df = 4, p = 0.029*) (Fig. 3).

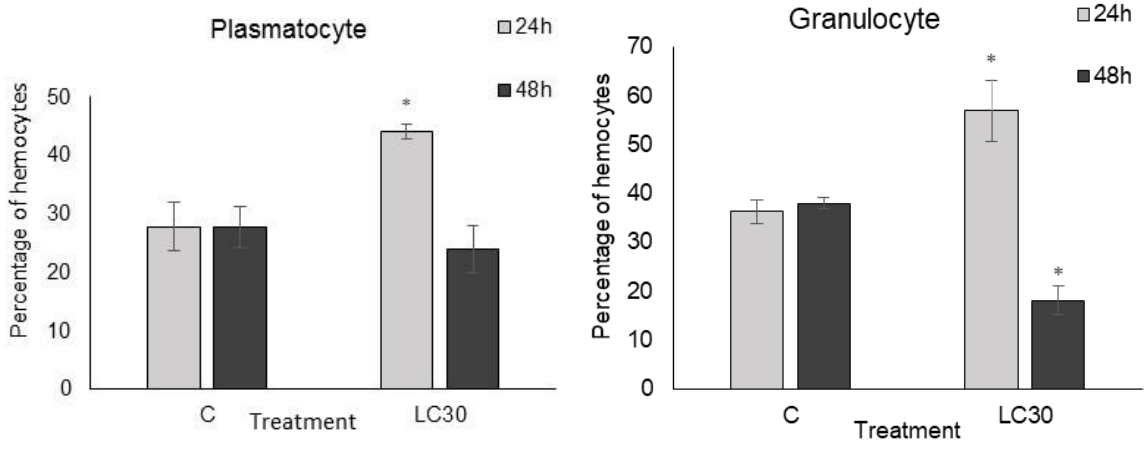

Fig. 2 The effect of LC30 of *A. annua* aqueous extract on percentages of plasmatocytes and granular cells in *G. pyloalis* after 24 and 48 h. Statistical differences have been marked with asterisks ($p \leq 0.05$) according to T-test

Effect of A. annua *aqueous extract on activity of detoxifying enzymes in* G. pyloalis *larvae*

General esterase activity in larvae treated with LC50 and LC30 aqueous extracts of *A. annua* increased after 24 and 48 h for both alpha and beta-acetyl naphthalene as substrate Table 3. While no significant difference was observed between LC10 and control. The overall results of the effect of *A. annua* aqueous extract on GST activity increased enzyme activity in treated larvae respectively (F = 20.60, $df_{t,e}$ = 3, 8, p = 0.0004), (F = 20.35, $df_{t,e}$ = 3, 8, p = 0.0004), (F = 24.47, $df_{t,e}$ = 3, 8, p = 0.0002) and (F = 34.99, $df_{t,e}$ = 3, 8, p = 0.0001) Table 3. The lowest activity was observed in the controls.

Effect of A. annua *aqueous extract on antioxidant enzymes of* G. pyloalis *larvae*

The antioxidant enzymes including PO, CAT, GPDH and SOD in the treated larvae at LC_{50} of *A. annua* aqueous extract after 24 and 48 h showed enhanced level of activity compared to control, while the LC_{30} and LC_{10} treated larvae given time showed no significant changes compared to control (Table 4).

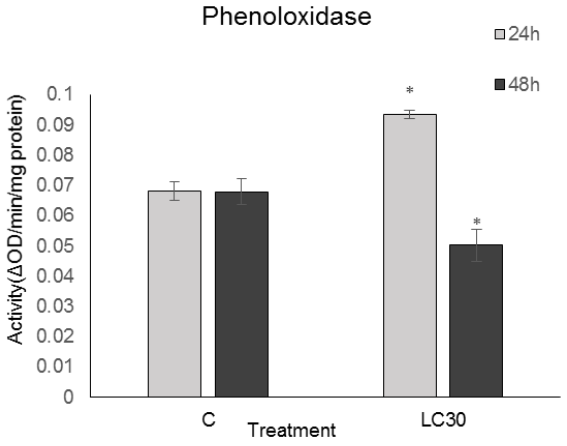

Fig. 3 The effect of LC30 of *A. anuua* aqueous extract on (PO) activity in *G. pyloalis* after 24 and 48 h. Statistical differences have been marked asterisks ($p \leq 0.05$) according to a T-test

Discussion

Many plants with insecticidal properties should be considered worthy as insect control strategies due to the excellent availability, viability and cost-effectiveness of plant resources (Idoko *et al.,* 2020; Isman, 2020). Plant extracts, due to their biodegradable nature, are an environmentally friendly approach to pest control (Vurro *et al.,* 2019; Damalas and Koutroubas, 2020). In this study, the aqueous extract of the *A. annua* leaves caused dose-dependent mortality in *G. pyloalis* larvae. Based on studies conducted by many researchers, it has been determined that the insecticidal activity of *A. annua* extract can be due to the presence of terpenes. These compounds have insect repellent properties and also affect the insect biology as antifeedants, fumigants, ovicides and contact toxicants (Khosravi *et al.,* 2010; Hasheminia *et al.,* 2011; Deb and Kumar 2019; Oftadeh *et al.,* 2020). To investigate the repellent effects of *A. annua aqueous extract*, we measured the oviposition deterrent index. Based on results, the rate of oviposition in females decreased by increasing the concentration of the extract, this may be due to the presence of repellent compounds in the extract (Milano *et al.,* 2010; Abdelgaleil *et al.,* 2019; Couto *et al.,* 2019; Vats *et al.,* 2019).

Table 3 Detoxifying enzyme activities in 4th instar larvae of *G. pyloalis* after treatment with *A. annua* aqueous extract

Detoxifying enzymes	Time*	Treatment				p	F	Df
		Control	LC_{10}	LC_{30}	LC_{50}			
Glutathione S-transferase (DCNB)	24	0.049 ± 0.0052c	0.093 ± 0.0028b	0.101 ± 0.0048b	0.127 ± 0.0047a	0.0004	20.60	3,11
	48	0.070 ± 0.0072c	0.099 ± 0.0058bc	0.102 ± 0.0057b	0.151 ± 0.0080a	0.0002	24.47	
Glutathione S-transferase (CDNB)	24	0.069 ± 0.0075c	0.070 ± 0.0047bc	0.071 ± 0.0035b	0.101 ± 0.0052a	0.0001	20.35	3,11
	48	0.048 ± 0.0058c	0.074 ± 0.001b	0.086 ± 0.0014b	0.115 ± 0.0069a	0.0004	34.99	
α-naphtyl acetate	24	0.038 ± 0.0069b	0.039 ± 0.0062b	0.068 ± 0.0024a	0.074 ± 0.0056a	0.0029	11.45	3,11
	48	0.039 ± 0.0043c	0.041 ± 0.0036c	0.070 ± 0.0046b	0.091 ± 0.0040a	0.0001	34.36	
β-naphtyl acetate	24	0.053 ± 0.0075b	0.054 ± 0.0038b	0.073 ± 0.0060ab	0.093 ± 0.0057a	0.0039	10.39	3,11
	48	0.054 ± 0.0038c	0.070 ± 0.0029bc	0.085 ± 0.0073ab	0.100 ± 0.0071a	0.0022	12.46	

Unit: (U/mg protein); * 24 and 48 h after treatments; Means (±SE) followed by the same letters in a row indicate no significant difference ($p < 0.05$) according to the Tukey test

Table 4 Antioxidant enzyme activities in forth instar larvae of *G. pyloalis* after treatment with *A. annua* aqueous extract

Antioxidant enzymes	Time*	Treatment				p	F	Df
		Control	LC_{10}	LC_{30}	LC_{50}			
POX	24	0.001 ± 0.0003b	0.001 ± 0.0003b	0.002 ± 0.0002ab	0.003 ± 0.0004a	0.0087	7.98	3,11
	48	0.001 ± 0.0001c	0.002 ± 0.0002bc	0.003 ± 0.0004ab	0.004 ± 0.0005a	0.0007	17.39	
CAT	24	0.096 ± 0.0061c	0.113 ± 0.0157bc	0.146 ± 0.0087ab	0.182 ± 0.0109a	0.0025	11.91	3,11
	48	0.096 ± 0.0083c	0.132 ± 0.0061bc	0.167 ± 0.0122b	0.218 ± 0.0116a	0.0002	27.07	
SOD	24	0.032 ± 0.0009b	0.033 ± 0.0022b	0.045 ± 0.0035a	0.047 ± 0.0014a	0.0022	12.4	3,11
	48	0.033 ± 0.0010b	0.042 ± 0.0025ab	0.046 ± 0.0023a	0.050 ± 0.0040a	0.0080	18.18	
GPDH	24	0.060 ± 0.0019b	0.061 ± 0.0036b	0.068 ± 0.0036b	0.083 ± 0.0019a	0.0022	12.44	3,11
	48	0.061 ± 0.0022c	0.061 ± 0.0020c	0.075 ± 0.0021b	0.092 ± 0.0022a	0.0001	44.14	

Unit: (ΔOD/min/mg protein);* 24and 48 h after treatments; Means (±SE) followed by the same letters in a row indicate no significant difference ($p < 0.05$) according to the Tukey test

The effect *A. annua* extract on immune system of *G. pyloalis* larvae has been investigated. Innate immune system of insects is a major defense factor against pathogens and other external factors with important effect on insect survival. This system consists of two parts, cellular and humoral, which prevent the spread of infection (Mandrioli *et al.,* 2003; Malagoli *et al.,* 2007). Cellular immunity includes phagocytosis, encapsulation and nodule formation through hemocytes and humoral immunity includes antimicrobial peptides and the prophenoloxidase system (Bulet and Stöcklin, 2005). Given the role of hemocytes in insect cellular immune responses to external factors (Lavine and Strand, 2002), changes in their number significantly affect the ability of the immune system against invading organisms (Bergin *et al.,* 2003). According to our results, the aqueous extract of *A. annua* increased THC as well as plasmatocytes and granulocytes after 24 h. There are many reports showing an increase in the total number of hemocytes affected by plant insecticides (Shaurub *et al.,* 2014; El-Sheikh, 2016; Shaurub and Sabbour, 2017; Ghoneim *et al.,* 2018; Dhivya *et al.,* 2018). In this study, based on differential hemocytes count, the percentage of granulocytes was significantly reduced compared to the control after 48 h. There are also same results reported by some authors (Azambuja *et al.,* 1991; Suyog *et al.,* 2012; Hassan *et al.,* 2013; Er and Keskin, 2016; Asiri, 2017; Er *et al.,* 2017; Manjula *et al.,* 2020). Sharma *et al.* (2008) reported that rhizome extract of *Acorus calamus* caused morphological changes in plasmatocytes and granulocytes of *Spodoptera littura* larvae and reduced the differential hemocytes count in larvae. In other study, Zibaee and Bandani (2010) reported depression in THC, DHC, in *Eurygaster integriceps* fed on *A. annua* extract. Decreased insect hemocytes count can be due to the antimitotic effects of plant extracts (Huang *et al.,* 2011). It has been reported that the insect hemocyte count are affected by the mitotic division of the circulating hemocytes (Er *et al.,* 2010). The plant extract seems to disrupt hematopoietic organs and inhibits cell division and proliferation in which leads to decreased insect hemocytes. On the other hand, the cytotoxicity effect of some plant extract has also been reported (Ghoneim, 2018). This phenomenon may be another factor in hemocyte depression in *G. pyloalis* larvae that is treated by the extract of *A. annua*. Phenoloxidases are important factors in insect cellular system which are involved in the coagulation process of hemolymph, melanization processes in nodules and capsules, as well as, wound healing (Chapman, 2013). The activity of this enzyme increased in larvae treated with *A. annua* aqueous extract after 24 h. However, 48 h later, phenoloxidase activity was significantly reduced compared to the control. Some authors have reported reduced phenoloxidase activity in insects treated by plant extracts (Liu *et al.,* 2009; Zibaee and Bandani, 2010; Zibaee and Bandani, 2012; Shayegan *et al*., 2019). The initial increase in the enzyme activity may be due to the insect immune response to the introduction of plant extract. It seems that decreased phenoloxidase activity after 48 h of larval treatment is due to the cytotoxicity effect of the plant extract which disrupts hemocytes and is responsible for enzyme production.

Esterases and glutathione S-transferase are two components that play important role in the detoxification process in insect. Due to their high substrate activity, these enzymes reduce the toxic effects of the compounds entering the insect body and eventually lead to resistance against pesticides (Alias, 2016). General esterases are type of hydrolase enzymes that breaks down many compounds, including aliphatic and aromatic esters,

choline esters and organophosphate (Ramsey *et al.,* 2010; Chapman, 2013). In this research, the activity of these enzymes increased significantly after 48 h at concentrations of LC_{30} and LC_{50} of *A. annua* aqueous extract which seems to be due to the excretion of compounds in the extract. Other studies have reported an increase in enzymes by some plant extracts (Senthil-Nathan, 2013; Yazdani *et al.,* 2013; Chen *et al.,* 2019; Murfadunnisa *et al.,* 2019; Wang *et al.,* 2020). Glutathione S-transferase is an important enzyme in detoxification process and insect antioxidant systems. This enzyme is responsible for removing the products from lipid peroxidation and hydroperoxidase from cells (Park and Tak, 2016).

Another part of the detoxification process is done by antioxidant enzymes. Enzymes such as SOD, POX, and CAT, in combination with other non-enzymatic antioxidants, are involved in the removal of toxic free radicals produced in response to exposure to toxic substances. (Nappi *et al.,* 2005; Krych *et al.,* 2014). The results show that the activity of CAT, SOD, GPDH and POX increased in *G. pyloalis* larvae treated by aqueous extract of *A. annua*. This increase was dose-dependent and may be due to the production of ROS in the insect body, which is produced under the influence of plant extracts in the body of herbivorous insects. The resultant oxidative stress activates the antioxidant system in the body of the insect (Chapman, 2013). Many studies have shown that the activity of these enzymes is increased by plant compounds (Xiong *et al.,* 2016; Pandey and Singh 2017; Lin *et al.,* 2018; Rahimi *et al.,* 2018; Dhivya *et al.,* 2018; Magierowicz *et al.,* 2020).

This study was conducted to control the disastrous mulberry pyralid in accordance with the provision of a safe, secure and cost-effective method, especially for ordinary farmers. In this study, we showed that an aqueous extract of *A. annua* leaves not only causes moderate mortality in *G. pyloalis* larvae but also shows irreversible physiological changes that also increase the hope of its continued effectiveness. The physiological changes related to immune and antioxidant systems were severely affected. In addition, the reduction of oviposition rate in female moths is consistent with the repellence effect. Overall, an aqueous extract of *A. annua* deserves further attention as a safe and inexpensive method with a simple formulation that should be included in this context.

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