A STUDY ON THE TOXICITY OF A MEDICINAL PLANT, *ARTEMISIA ANNUA* L. (ASTERACEA) EXTRACTS TO THE SUNN PEST, *EURYGASTER INTEGRICEPS* PUTON (HEMIPTERA: SCUTELLERIDAE)

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Abstract: A methanolic extract of *Artemisia annua* was obtained to evaluate its insecticidal activities against the sunn pest (*Eurygaster intefriceps*). Also, the responses of general esterase (EST), glutathione *S*-transferase (GST), alkaline phosphatase (ALP), acid phosphatase (ACP), acethylcholinesterase (AChE) to the plant extract were investigated. Topical application of plant extract on adults showed that the mortality was dose-dependent i.e. with increasing of plant extract concentrations more mortality achieved. Esterase and GST activities were increased in the first 24 h post-treatment. However, the enzymes activities were decreased after 24 h until 72 h. The activities of ALP, ACP and AChE in insect body decreased significantly and inhibition was higher along with increasing concentrations of plant extract. Isozyme electrophoresis profiles indicated that responses of isozymes (EST and GST) to plant extract were decreased after 48 h exposure to extract so that some enzymes bands disappeared. The results indicated that the highest concentration of *A. annua* extract was the most toxic among the four extracts. The decline of the detoxification ability in insects' tissues might be the main reason for the insecticidal activities.

Key words: *Eurygaster integriceps*, *Artemisia annua* extract, toxicity, enzyme activity, isozymes

INTRODUCTION

The control of *Eurygaster integriceps* as a major pest of wheat in middle east, eastern Europe and north of Africa, was based on intensive usage of two organophosphorus insecticides, fenthione and fenitrothione. Although, biological control by *Trissolcus* spp. was somehow effective, farmers didn't take interest in it. Today worldwide concerns about fragile ecological balance are making people re-think about synthesized pesticides usage. Chemical pesticides are generally effective against a wide range of insects, have long half-lives and often are found in streams and lakes as pollutants from ground run-off water. Hence, agrochemical research resulted in the discovery of novel insecticides that act on selective biochemical sites present in specific insect groups. This led to the increase in efforts to find and develop natural pesticides that are species specific and efficient against pathogens, those were insect growth regulators (IGRs) and botanical insecticides.

Among the plant families studied, Meliaceae, Asteraceae, Labiateae, Piperaceae and Annonaceae are most promising (Bhakuni *et al.* 2001). For example, *Artemisia annua* extract may be one of the best extracts for insect pest control. The genus *Artemisia* is a member of a large plant family Asteraceae (Compositae) incorporating more than 300 different species. The species *A. annua* known as sweet worm wood grows wild in Europe and America and is planted widely in China, Turkey, Vietnam, Afghanistan and Australia (Isman 2006). Several isolated compounds from this species were shown to have antimalarial, antibacterial, antiinflamatory, plant growth regulatory and cytotoxicity (antitumor) activities. Although many studies have reported insecticidal effects of plant extracts including growth retardation and arrest of ovarian development, their mode of action has not been elucidated (Akhtar and Isman 2004). Botanical products are useful tools in many pest management programs because they are highly effective, safe, and ecologically acceptable (Weinzierl and Henn 1991; Senthil Nathan and Kalaivani 2005).

In the present study organic solvent extract of *A. annua* was prepared and then insecticidal activities of this extract against *E. integriceps* was investigated. Also, physiological responses of insects to the toxic extract including its neurotoxicity were evaluated in the hope to find new way of integrated management of Sunn pest.

MATERIALS AND METHODS

Insects

The insects, sunn pest (*E. integriceps*), were collected from the Karaj wheat farm of Tehran Province of Iran and reared on wheat grains in the laboratory at 27±2°C under

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a 14 h light : 10 h dark (LD 14 : 10) photoperiod (Kazzazi *et al.* 2005).

Bioassay

For topical application, five concentrations of *A. annua* extract (Shekari *et al.* 2008) and one control (without plant extract) were used for determination of toxicity and LC_{50} values. In each concentration 30 insects (adults) were tested in 5 replicates per concentration. Insects were treated topically with 2 µl of each concentration and control (acetone extract) on the third thoracic sternum of adults using a microaplicator (Burkard Co., England). Mortality was recorded 24 and 48 hours post-treatment and LC_{50} was calculated using Polo-Pc software (LeOra 1987).

Enzyme assessments

Sample preparation

At 24 h post-treatment (10, 20 and 25% concentrations), 20 larvae were selected randomly from each group. The adult's hemolymph was taken through a cut in one of the front legs. From each adult, 50 µl of hemolymph were collected with a capillary tube (Sigma), and pooled hemolymph was used for biochemical measurement. To avoid prophenol oxidase activation followed by melanization of hemolymph, phenylthiourea as anticoagulant solution was added to the samples and they were centrifuged for 10 min at 10 000 g (Nath *et al.* 1997). The supernatant was transferred to new tubes and stored at –20°C as the enzyme source.

Assay of general esterase activity

Esterase activity was determined using the method described by Han *et al.* (1995). Seventy-five microliters of a-naphthyl acetate, β-naphthyl acetate (10 mm) and 75 µl fast blue RR salt (1 mm) were added into each tube. The reaction was initiated by addition of 50 µl of enzyme solution. Optical density (OD) at 450 nm was recorded at intervals of 10 s for 1 min using microplate reader.

Assay of glutathione S-transferase (2.5.1.18)

For glutathione S-transferase activity the method reported by Oppenorth (1979) was adopted. Twenty microliters of CDNB (20 mm) or DCNB (40 mm) were pipetted into a tube microplate wells, and then 100 µl of enzyme solution was added. The OD value at 340 nm was recorded at intervals of 9 s in 1 min.

Assay of estimation of acid (EC 3.1.3.2) and alkaline phosphatases (EC 3.1.3.1)

The enzyme assays were carried out as described by Bessey *et al.* (1946) The buffered substrate (phosphate buffer, 0.02 m, pH 7.2) was incubated with samples for 30 min. Alkali were added to stop the reaction and adjust the pH for the determination of concentration of the product formed. The spectral absorbance of *p*-nitrophenolate was maximal at 310 nm. The molar absorbance of p-nitrophenolate at 400 nm is about double that of p-nitrophenyl phosphate at 310 nm. On converting the p-nitrophenolate into p-nitrophenol by acidification, the absorption maximum is shifted to about 320 nm with no detectable absorption at 400 nm.

Determination of V_{max} and K_m values

Kinetic parameters of general esterases were determined by the method of Zhu and He. [15] 50 µl of appropriately diluted enzyme preparation was used in each assay. Final concentrations for substrate were 0.625, 1.25, 2.5, 5 and 10 mmol/l. The michaelis constant (K_m) and the maximal velocity (V_{max}) were estimated by Yang *et al.* (2005) procedure. The results of K_m and V_{max} are the means ±SE of six replicates (n = 6). To determine the kinetic parameters of GST, ACP and ALP, 50 µl of CDNB, DCNB (as GST substrates) and p-nitrophenol phosphate concentrations were mixed with 50 µl phosphate buffer and the reaction was initiated and monitored at 340, 320 and 400 nm at 1 min intervals, respectively.

AChE assays (3.1.1.7)

The activity of AChE was determined by the method of Li and Han (2002) using ATCh (for the first time substrate name is written completely) iodide as a substrate. Twenty adults were homogenized in 20 ml of ice-cold 0.1M phosphate buffer (pH 7.4). After filtering through cheese cloth, the homogenate was centrifuged at 10 000 rpm for 20 min. The supernatant was directly used as the AChE enzyme source. A series of LC_{50} and below LC_{50} concentrations of *A. annua* extract (10, 15 and 25%) were added to incubation mixture consisting of 1 ml of enzyme solution, 2 ml 0.1 m phosphate buffer and 100 ml were added and the samples were placed at 30°C in a shaking water bath for 10 min. Then 20 ml of 0. 75 mm acetylcholine iodide was added to the mixture. The AChE activity was spectrophotometrically measured at 412 nm (UV visible spectrophotometer). The enzyme activities were expressed as µmol/min/mg protein.

Isozyme electrophoresis assay of esterase and glutathione S-transferase

Supernatants of adults bodies treated for 24 h and 48 h was electrophoresed with a vertical electrophoresis for esterase. Polyacrylamide gel electrophoresis (PAGE) was carried out with 120 mV constant current until the tracing dye reached the bottom of the gel. The separating gel consisted of 7% acrylamide + bisacrylamide and 0.04 mol/l Tris–HCl, pH 8.8. The stacking gel consisted of 4% (acrylamide + bisacrylamide) and 0.01 mol/l Tris–HCl, pH 6.8. The buffer was Tris-glycine buffer, pH 8.3. After electrophoresis, gels were stained (Substrate 10 mm and fast blue RR salt 1mm), washed with phosphate buffer and then photographed. Acetic acid (7.5%) was used as stationary phase (Ke *et al.* 2008). SDS–PAGE to detect effects of plant extract on GST isoenzymes was performed using a 10% resolving gel and a 4% stacking gel. Coomassie Blue R250 was used to stain protein.

Protein determination

Protein concentrations were measured according to the method of Bradford (1976) using bovine serum albumin (Bio-Rad) as a standard.

Statistical analysis

For determination of mortality and lethal concentration, POLO-PC software (LeOra 1987) were used. All data were compared by one-way analysis of variance (ANOVA) followed by Tukey's test and significant differences were found at p = 0.05 (SAS 1997) Differences between samplings were considered statistically significant at a probability more than 5%. Probability levels are specified in the text.

RESULTS

Mortality bioassay

In bioassay tests, *A. annua* extract treatments were applied topically on a dorsal surface of the adults and at different concentrations and observations were made after 24 and 48 h (Table 1, Fig. 1). The LC_{10}, LC_{30}, LC_{50} and LC_{90} values were 11.91, 20.04, 32.24 and 83.4% after 24 h and 6.27, 11.42, 17.73 and 47.73% after 48 h, respectively.

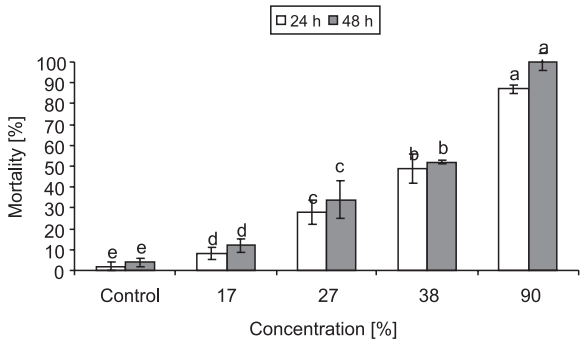

Fig. 1. Mean (±SEM) percentage mortality of *E. integriceps* adults after treatment with different concentrations of *A. annua* extract after 24 and 48 h. Means (±SEM) followed by the same letters above bars indicate no significant difference ($p < 0.05$) according to Tukey test

Mortality of insects after topical treatments with 17, 27, 38 and 90% concentrations is shown in figure 1. Results showed that the mortality of adults was dose-dependent. There were significant differences between control and treatments (Fig. 1). As can be seen in table 1 and figure 1 increasing concentrations of the plant extract exacerbate mortality.

Effect of *A. annua* extract on enzyme activities

Esterase and glutathione *S*-transferase (GST) content of hemolymph were measured in treated and control group of *E. integriceps* adults after plant extract application in order to see the differences in these enzyme activities through the plant extract mode of action (Table 2). Activity level of these enzymes (esterase and GST) in 24 h post-treatment increased significantly for both substrates of enzymes. Their activities were dose-dependent and increased with increasing of extract concentration and were significantly different from control. Figure 2 shows esterase and GST activities at different time intervals after exposure. Their activities increased until 24 h but decreased after 48 and 72 h after treatment.

Differences in alkaline phosphatase (ALP) and acid phosphatase (ALP) activities between the control and treated adults are shown in table 2. The maximal suppression of ALP and ACP activities were obtained in the 20 and 35% concentrations. Significant differences between them were observed. Figure 2 shows the activity of ALP and ACP after 12, 24, 48 and 72 h post-treatment. Their activities maximally increased after 12 and 24 h after exposure but decreased after 48 and 72 h.

A. annua extract inhibited AChE activity, over a wide range of concentrations even below the LC_{50}. The effect of doses equal to the LC_{50} were evaluated for enzyme activity of the adults. The AChE inhibition of the adults at various concentrations of plant extract is shown in table 2. The effect of plant extract on AChE enzyme activities shown as adult mortality was concentration dependent.

Table 1. Statistical comparison of different application methods of *A. annua* extract effect on *E. integriceps* adults

Treatment[1]	LC_{10}		LC_{30}		LC_{50}		LC_{90}		Slope±SE		X^2	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Topical application	11.91 b	6.27 a	20.04 ab	11.42 ab	32.24 b	17.3 b	83.4 a	47.73 b	2.98 ±0.655	2.90 ±0.451	2.68	1.72
Residue	17.63 a	8.32 a	27.87 a	14.80 a	38.27 a	22.05 a	86.73 a	58.4 a	3.80 ±0.671	3.03 ±0.431	2.85	3.84

[1] concentration per cent
[2] means followed by different letters are significantly different (Robertson *et al.* 2007).

Table 2. Effect of *A. annua* extract on detoxifying enzyme of *E. integriceps* hemolymph after 24 h

Treatment [%]	Esterase		Glutathuion *S*-transferase		Acethylcholinesterase	Alkaline phosphatase	Acid phosphatase
	α-naphtyl	*β*-naphtyl	CDNB	DCNB			
Control	3.76±0.062 a	3.34±0.035 a	2.82±0.036 a	2.84±0.036 a	7.56±0.027 a	4.92±0.024 a	3.93±0.018 a
10	4.11±0.021 b	3.50±0.012 a	2.85±0.022 b	2.92±0.022 b	7.32±0.020 b	4.91±0.020 b	3.88±0.046 b
15	4.27±0.083 b	3.86±0.048 ab	3.17±0.031 bc	3.27±0.031 bc	6.01±0.052 c	5.11±0.034 c	4.15±0.026 c
25	4.75±0.095 c	4.22±0.055 b	3.49±0.025 c	3.45±0.025 c	5.31±0.031 d	5.46±0.027 c	4.35±0.021 c

*means (±SEM) followed by the same letters indicate no significant difference ($p < 0.05$) according to the Tukey test
CDNB – 1-chloro-2,4-dinitrobenzene; DCNB – 1,2-dichloro-4-nitro-benzene

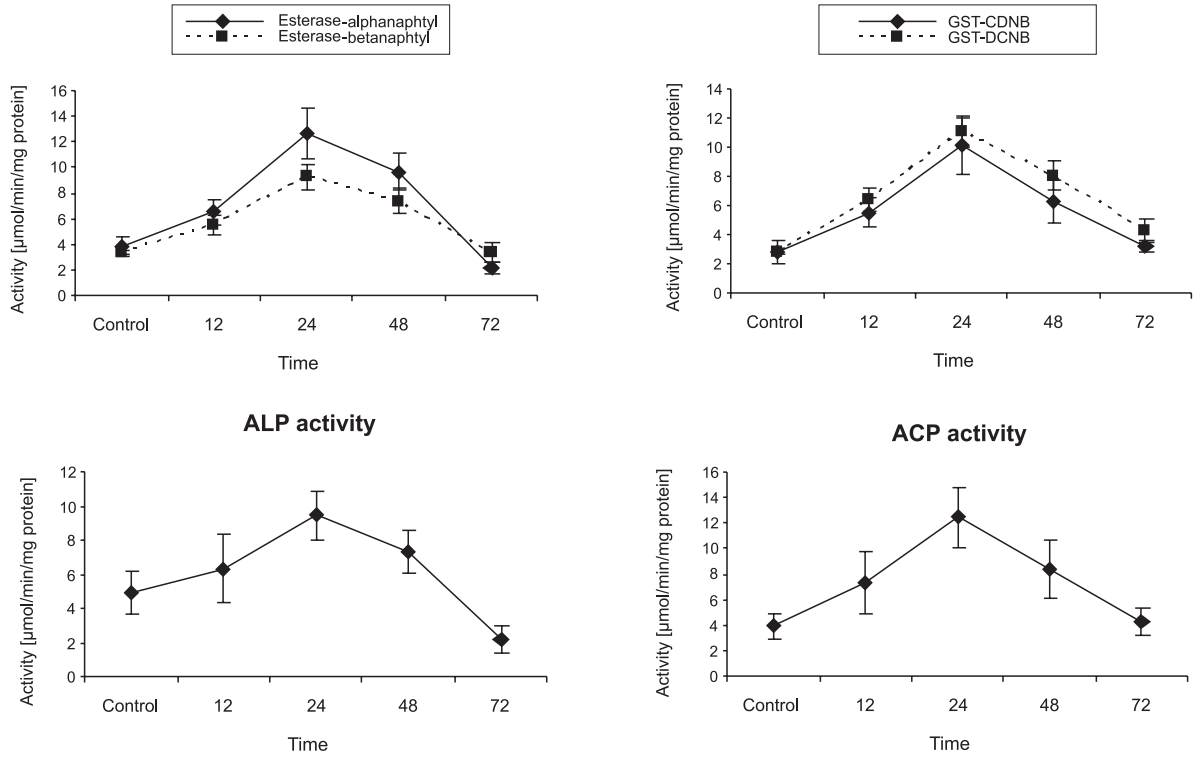

Fig. 2. Esterase, glutathione *S*-transferase, alkaline phosphatase and acid phosphatase activities of *E. integriceps* after different exposure time to *A. annua* extract. Different letters in figure indicate significant difference ($p < 0.05$)

The enzyme activity decreased as the plant metabolite dose increased (Table 2).

Table 3 shows kinetic parameters (V_{max} and K_m) of enzymes extracted from control and treated adults of *E. integriceps* at time intervals (when enzymes were extracted). Results showed that maximal velocity (V_{max}) of esterase and glutathione *S*-transferase (GST) in insects treated with different concentrations of plant extract increased but alkaline phosphatase and acid phosphatase activities were decreased. K_m values increased in all enzymes and significant differences were observed when different concentrations of *A. annua* extract were applied.

Isozyme electrophoresis assay

Esterase and GST electrophoresis profiles are shown in figure 3. In early stage (24 h) of toxicosis, enzyme activity of the treated body tissues was lower than that of the control. In late stage (48 h) of toxicosis, enzyme activity of the treated samples began to decrease and some enzyme bands completely disappeared.

Table 3. Kinetic parameters (V_{max} and K_m) of detoxifying enzymes extracted from control and treated adults of *E. integriceps* after 24 h

Treatment [%]	Esterase				Glutathione S-transferase				Alkaline phosphatase		Acid phosphatase	
	α-naphtyl		β-naphtyl		CDNB		DCNB					
	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
Control	0.59±0.12 b	3.67±0.65 a	0.53±0.09 a	4.36± 1.23 a	0.35±0.14 b	1.71±0.9 c	0.38±1.32 b	7.53±1.23 c	0.66±0.23 a	0.18±0.10 c	0.48±0.12 a	0.33±0.08 c
10	0.68±0.23 a	3.63±0.48 a	0.53±0.23 a	3.05± 2.01 b	0.37±0.08 b	2.34±0.47 b	0.44±0.36 b	12.71±2.36 b	0.58±0.47 ab	0.19±0.09 b	0.48±0.21 a	0.45±0.03 b
15	0.69±0.09 a	3.60±0.78 a	0.55±0.17 a	2.40±0.91 c	0.40±0.23 ab	2.64±0.85 a	0.56±0.89 a	18.94±7.86 a	0.58±0.32 ab	0.19±0.07 b	0.44±0.18 ab	0.44±0.17 b
25	0.74±0.36 a	3.57±1.20 a	0.57±0.47 a	2.09±0.87 d	0.46±0.18 a	2.84±1.01 a	0.65±0.59 a	16.47±10.03a	0.54±0.36 b	0.25±0.08 a	0.42±0.16 b	0.51±0.23 a

Means (±SEM) followed by the same letters indicate no significant difference ($p < 0.05$) according to the Tukey test
CDNB – 1-chloro-2,4-dinitrobenzene; DCNB – 1,2-dichloro-4-nitro-benzene

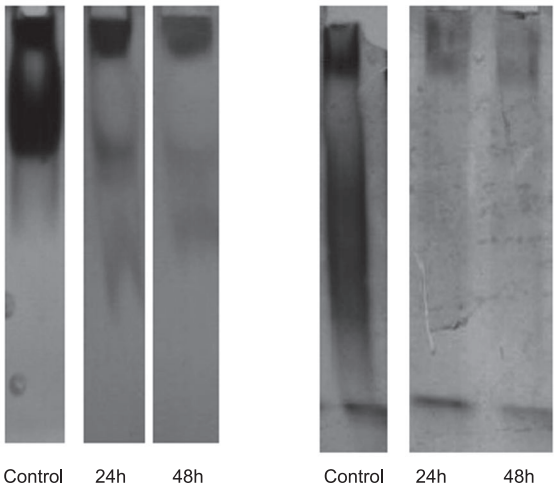

Fig. 3. Esterase and glutathione *S*-transferase (GST) isozyme profiles. Body exposure to *A. annua* extract for 24 h and 48 h.

DISCUSSION AND CONCLUSIONS

Secondary organic compounds synthesized by plants have an important role in protecting plants against insect pests. These compounds affect insects by being toxic, causing a delay in larval growth and can act as antifeedant compounds, too (Chapman 1985; Isman 2006; Senthil Nathan 2006). This study shows that the methanolic extract of *A. annua* was insecticidal when applied topically and had enzymatic inhibitory activity when used in *in vivo* experiment. The time-dependent effect of *A. annua* extract might be due to the uptake of the active compound by insect which progressively increased amount of active component in insect body with increasing exposure period. Simililar reports were shown by other studies in which *A. annua* extract had a dose- and time-dependent influence on insects (Tripathi *et al.* 2000; Jalali *et al.* 2005; Shekari *et al.* 2008).

Esterase (EST) is an important detoxifying enzyme *in vivo* which hydrolyzes the esteric bond in synthetic chemicals. Also, esterase is one of the enzymes showing the strongest reaction to environmental stimulation (Hemingway and Karunatne 1998). The responses of EST to *A. annua* extract were significantly due to using different concentrations of extract and after long exposure. In the early stage, plant extract stimulated the expression of EST body to increase the detoxification ability. In the late stage, because of a toxic effect and time EST activity was suppressed.

Glutathione *S*-transferases (GST) are mainly cytosolic enzymes that catalyze the conjugation of electrophile molecules with reduced glutathione (GSH), potentially toxic substances become more water soluble and generally less toxic (Grant and Matsumura 1989). GSTs play an important role in insecticide resistance and are involved in the metabolism of organophosphorus and organochlorine compounds. Other xenobiotics such as plant defence allelochemicals against phytophagous insects induce GST activity (Yu 1982; Vanhaelen *et al.* 2001). In this study, activity level of GST in 24 h post-treatment increased significantly for both substrates of the enzyme. Its (two or one) activity was dose-dependent and increased with increasing of extract concentration. Vanhaelen *et al.* (2001) showed that Brassicacea secondary metabolites induced GST activity in *Myzus persicae* and several Lepidopteran species such as *Heliothis virescens* Fabricius, *Trichoplusia ni* Hubner and *Anticarsia gemmatalis* Hubner. The influence of plant allelochemicals on GST activity is not limited to the herbivores and was observed in several predators, too (Francis *et al.* 2000).

Alkaline phosphatase (ALP, *E.C.3.1.3.1*) and acid phosphatase (ACP, *E.C.3.1.3.2*) are hydrolytic enzymes, which hydrolyze phosphomonoesters under alkaline or acid conditions, respectively. ALP is primarily found in the intestinal epithelium of animals and its major function is to provide phosphate ions from mononucleotide and ribonucleo-proteins for a variety of metabolic processes. ALP is involved in the transphosphorylation reaction and the midgut has the highest ALP and ACP activity as compared to other tissues (Sakharov *et al.* 1989). The overall activity of ALP and ACP decreased due to increasing of plant extract concentrations so that there were significant differences among control and three treatments. These findings coincided with other reports of plant extract treatments of insects. For example, Senthil Nathan (2006) showed that treatment of rice plants with *Melia azedarach* Juss (Meliaceae) extracts decreased the activity level of ALP in *Cnaphalcrocis medinalis* (Guenee). These authors reported that feeding *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) on *Ricinus communis* L. treated with azadirachtin decreases the amount of this enzyme in the midgut (Senthil Nathan and Kalaivani 2005).. Changes in ALP and ACP activities after treatment with *A. annua* extract indicate that changing the physiological balance of the midgut might affect these enzymes.

AChE is a key enzyme that terminates nerve impulses by catalyzing the hydrolysis of neurotransmitter, acetylcholine, in the nervous system in various organisms (Oehmichen and Besserer 1982; Grundy and Still 1985; Wang *et al.* 2004). In this study, *A. annua* extract inhibited the AChE activity in higher doses which coincided with other reports about effect of botanical insecticides on AChE inhibition. The alteration of AChE was observed in the cockroach, *Periplaneta americana* L., at 4 ppm of AZA, (Shafeek *et al.* 2004) and the snail, *Limnaea acuminata* Lamarck, at 40% and 80% concentrations of neem oil (Singh and Singh 2000). It was also observed that 25 g of distilled water extracts of the botanicals *Punica granatum* L., *Thymus vulgaris* L., and *Artemisia absinthium* L., significantly inhibited the AChE activity of nematodes at 100% concentrations (Korayem *et al.* 1993). Senthil Nathan *et al.* (2008) demonstrated that LC_{50} concentrations of AZA significantly inhibited the activity of AChE compared with control.

In case of majority of enzymes, the presence of plant extract decreased the value of V_{max} and increased K_m since the K_m has an inverse relationship with the substrate concentration required to saturate the active sites of the enzyme, this indicates a decreased enzyme affinity for substrate (Morris 1978). In other words, K_m is the measurement of stability of the enzyme-substrate complex and a high K_m would indicate a weak binding and a low

K_m strong binding (Stryer 1995). The effect of *A. annua* extract on the V_{max} shows that it interferes with the rate of breakdown of the enzyme-substrate complex. Thus, plant extract inhibits the enzymes by increasing K_m and decreasing affinity of the enzyme to substrate. Plant extract also diminished the V_{max} value which further indicates that they interfere with the rate of breakdown of the enzyme-substrate complex (Wilson 1986).

In conclusion it may be stated that, *A. annua* extract had strong insecticidal activities against *E. integriceps* and the insecticidal activities were time and dose-dependent. Plant extract affects normal physiological metabolism of adults by changing some key enzymes activities such as esterase, glutathione *S*-transferase, alkaline phosphatase, acid phosphatase and acethylcholinesterase.

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POLISH SUMMARY

BADANIE TOKSYCZNOŚCI WYCIĄGÓW Z ROŚLINY LECZNICZEJ *ARTEMISIA ANNUA* L. W STOSUNKU DO SZKODNIKA *EURYGASTER INTEGRICEPS* PUTON (HEMIPTERA: SCUTELLERIDAE)

Metanolowy wyciąg z *A. annua* uzyskano w celu oceny jego aktywności owadobójczej wobec szkodnika *Eurygaster integriceps*. Badano również reakcje ogólnej esterazy (EST), S-transferazy glutationu (GST), alkalicznej fosfatazy (ALP), kwaśnej fosfatazy (ACP) i acetylholinoesterazy (AChE) w wyciągu roślinnym. Zastosowanie wyciągu roślinnego na dorosłe osobniki wykazało, że śmiertelność zależała od dawki, to znaczy, zwiększenie stężenia wyciągu zwiększało śmiertelność. Aktywność esterazy i GST była zwiększona po 24 do 72 godzinach. Aktywność ALP, ACP i AChE w ciele owada istotnie spadała i inhibicja była wyższa w miarę zwiększania stężeń wyciągu roślinnego. Profile elektroforezy izozymów wskazywały, że reakcje izoerozymów (EST i GST) na wyciąg roślinny spadały po 48 godzinach ekspozycji na wyciąg tak, że niektóre pasma enzymów zanikły. Wyniki wskazywały, że najwyższe stężenie wyciągu *A. annua* było najbardziej toksyczne spośród czterech stężeń. Spadek zdolności detoksyfikacyjnej w tkankach owadów może być główną przyczyną obserwowanej aktywności owadobójczej.