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α-Amylase Activity of Stored Products Insects and Its Inhibition by Medicinal Plant Extracts

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ABSTRACT

The experiment was conducted to determine α-amylase activity and the effect of seven plant species extracts including *Punica granatum* L. (Punicaceae), *Rheum officinale* B. (Polygonaceae), *Rhus coriaria* L. (Anacardiaceae), *Artemisia sieberi* B. (*Compositae*), *Peganum harmala* L. (Nitrariaceae) , *Datura stramonium* L. (Solanaceae) and *Thymus vulgaris* L. (Lamiaceae) on α-amylase activity of four stored insect pests including *Callosobruchus maculatus* F. (Coleoptera: Bruchidae), *Rhyzopertha dominica* F. (Coleoptera: Bostrichidae), *Sitophilus granarius* L. (Coleoptera: Curculionidae), and *Trogoderma granarium* E. (Coleoptera : Dermestidae). Also, gut pH and optimum temperature for α-amylase activity of these insects were determined. It was found that α -amylases midgut pH of all four insect species was acidic and optimum temperature was between 30 and 40 °C. Beyond these temperatures, the α-amylases activities sharply decreased. Plant extracts caused inhibitory activity on insect α-amylases varying from nearly 4% to 95% inhibition. *D. stramonium* and *R. officinali* extracts had the highest amylase inhibitory activity among the tested extracts, while methanolic extracts of *P. harmala*, and *T. vulgaris* (except for *S. granaries* α-amylase) showed the lowest inhibitory activity. Gel assays revealed that more than one isoform of α -amylase detected in midgut crude extracts of the four insect pests examined could be inhibited by the plant extracts.

Keywords: Gut pH, Plant extracts, Stored-product insects, α –amylase.

INTRODUCTION

Synthetic insecticides and fumigants are the main chemicals used for pest control in stored products. However, because of many problems associated with the use of synthetic pesticides in integrated pest management approaches, use of chemicals to protect grains and grain products against stored-product pests is limited (Yildirim *et al*., 2001). The public concern over the residual toxicity of insecticides applied to stored grain, the occurrence of insecticide resistant insect strains, and the necessary precautions to work with traditional chemical insecticides call for more environmentally-benign alternatives to control stored-product insect pests (Hagstrum and Subramanyam, 1996; Yildirim *et al*., 2001). Recently, considerable studies have been done on plant derived materials for potentially useful products as bio-insecticides since development of resistance by pests and vectors against plant extracts has not been reported (Regnault-Roger and Philogene, 2002; Isman, 2006).

Botanical insecticides have long been attractive alternatives to synthetic chemical insecticides for pest management because

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botanicals reputedly pose little threat to the environment or to human health. Several plant extracts have been evaluated against various stored-products insect pests (Prakash and Rao 1997, Weaver and Subramanyam 2000, Athanassiou *et al*. 2005; Kavallieratos *et al*. 2007). In the context of agricultural pest management, botanical insecticides are best suited for use in the post-harvest protection of stored food products in developing countries and in organic food production in industrialized countries (Isman, 2006).

These extracts are insecticidal because they form complexes with digestive enzymes, which are stable and dissociate slowly. Inactivation of digestive enzymes by inhibitors results in blocking of gut amylases and other digestive enzymes such as proteinases, leading to poor nutrient utilization, development retardation, and death because of starvation (Isman, 2006; Hosseini-Naveh *et al*., 2007). The major insect pests of stored products are weevils in the genus *Sitophilus granaries* L. (Coleoptera: Curculionidae), *Rhyzopertha dominica* Fabricius (Coleoptera: Bostrichidae), *Trogoderma granarium* Everts (Coleoptera : Dermestidae) and *Callosobruchus maculatus* Fabricius (Coleoptera: Bruchinae). These insects, which constitute serious pests of grains, live on a polysaccharide-rich diet and are dependent on their α-amylases for survival (Mendiola-Olaya *et al*., 2000).

Since there is significant variation among the properties of insect digestive enzymes, it is necessary to have more information on the gut enzymatic activities of insects to devise a rational strategy for insect pest control utilizing plant extracts. Thus, the aim of the current study was to determine α-amylase activity and gut pH of four species of stored-products insects including *C. maculatus* , *R. dominica*, *S. granarius*, and *T. granarium*. Also, the effects of medicinal plant extracts including *Punica granatum* L. (Punicaceae), *Rheum officinale* Baill (Polygonaceae), *Rhus coriaria* L. (Anacardiaceae) , *Artemisia sieberi* Besser (Compositae), *Peganum harmala* L. (Nitrariaceae) , *Datura stramonium* L. (Solanaceae) and Thymus *vulgaris* L. (Lamiaceae) on amylolytic activity of stored product insects were evaluated.

MATERIALS AND METHODS

Insect

Population of *C. maculatus*, *R. dominica*, *S. granarius* and *T. granarium* were obtained from the insect physiology laboratory cultures (University of Tehran) and maintained on wheat grains in a plastic container in the laboratory conditions at 25 ± 2˚C, photoperiod of 14: 10 (L:D) and 55% RH.

Gut pH Determination

A series of standard indicator dyes was used to determine the pH of the gut lumen according to Bignel and Anderson, (1980) and Elpidina *et al*. (2001), with some modifications to suit our conditions. Indicators used were 0.1 % bromophenol blue (pH 3.0 – 4.6), 0.1 % methyl red (pH 4.4 – 6.2), 0.1 % bromcresol purple (pH 5.2 – 6.8), 0.1 % bromophenol blue (pH 6.2 – 7.6), 0.1 % natural red (pH 6.8 – 8.0), 0.1 % cresol red (pH 7.2 – 8.8), 0.1 % thymol blue (8.0 – 9.6) and 0.1 % Alizarin yellow (pH 10 – 12). The following two methods were used for the pH determination.

Initially, last–larval instars starved for 48 h and, then, they were given wheat grains containing pH dyes to feed on for 12 hours. Afterwards, they were dissected and the developed colour in their gut was recorded by comparison to respective standards, prepared in different pHs. The second method was based on the procedures described by Bignell and Anderson (1980). Last-larval instars were dissected and their midguts separated using fine forceps. Five larval midguts were pooled in 10 ml of distilled water and mounted on a microscope

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slide and broken open with a needle. Aliquots of one milliliter of each pH indicator solution were added to the midgut sections and the developed colour was recorded.

Enzyme Preparation

Insect α-amylase extraction was based on procedures described by Bandani *et al*. (2009). Adults of *C. maculatus*, *R. dominica*, *S. granarius* and *T. granarium* were randomly selected, cold-immobilized on ice container (4 °C) for 5 minutes, dissected under a stereoscopic microscope (OLYMPUS SZ11), and the midguts removed in distilled water. The midguts were placed in a pre-cooled homogenizer (Teflon pestle, 0.1 mm clearance) and ground in one ml of universal buffer containing succinate, glycine, 2-morpholinoethanesulfonic acid at pH 6.5. The homogenates were centrifuged at 15000 (g) at 4 °C for 15 min. The resulting supernatants were transferred to a new tube and frozen at -20 °C for further use as an enzyme source.

Plant Material and Extraction Procedure

Seeds of *Peganum harmala* L. (Zygophyllaceae), *Datura stramonium* L. (Solanace), *Punica granatum* L. (Punicace) and stems and leaves of of *Thymus vulgaris* L. (Lamiaceae), *Rosmarinus officinali* L. (lamiaceae), *Rhus coriaria* L. (Anacardiaceae) and *Artemisia siberia* L. (Compositae) were used for extraction.

Plants were rinsed with distilled water, dried in an oven (Heraeus) at 40 °C for 48 h, grounded to a powder with an electrical blender (KML-ZZJ-06) and stored at cold room temperature (4°C) in sealed plastic bags prior to extraction. Thirty grams of dried leaves were stirred with 300 ml of methanol (99.8%) in a flask overnight, then, filtered through Whatman No.4 filter paper. The solvent was removed by vacuum in a rotary evaporator (Hydolf laborota 4001) at 40 °C and the residue was dissolved in the least amount of methanol (99.8%) and used as a starting stock solution. Further dilutions in the distilled water were used to prepare suitable concentrations used in the enzyme assays (10%) (Warthen *et al*., 1984).

Determination of Amylolytic Activity

The α-amylase activity was assayed by the dinitrosalicylic acid (DNS) procedure (Bernfeld 1955), using 1% soluble starch (Merck) as a substrate described by Bandani *et al*. (2009). Ten microliters of the enzyme was incubated for 30 min at 30˚C with 500 µl universal buffer and 40µl soluble starch. The reaction was stopped by addition of 100 µl DNS and heated in boiling water for 10 min. Then, absorbance was read at 540 nm after cooling in ice for 5 min. One unit of α-amylase activity was defined as the amount of enzyme required to produce 1 mg maltose in 30 min at 30˚C. A standard curve of absorbance against the amount of maltose released was constructed to enable calculation of the amount of maltose released during α-amylase assays. Serial dilutions of maltose (Merck) in the universal buffer at pH 6.5 were made to give the following range of concentrations of 2, 1, 0.5, 0.25, 0.125 mg ml^{-1}. Suitable blanks (a blank without plant extract but with α-amylase extract and a blank containing no α-amylase extract but with substrate) were run simultaneously with the reaction mixture. All assays were performed in duplicate and each assay was repeated at least five times.

Determination of Optimal pH and Temperature

Optimum pH and temperature for different insect α-amylases were determined. Optimal pH for amylase activity was determined using universal buffer with pH set at 2, 3, 4, 5, 5.5, 6, 6.5, 7, 7.5, 8, 9 and 10. Enzymes were dissolved in universal buffer with distinct pH and were pre-incubated for 15min at 30 °C and then substrate was added

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to the reaction and the amylolytic activity was measured as described before.

The effect of temperature on α-amylase activity was determined by incubating the reaction mixture at 10, 20, 30, 35, 40, 45, 50, 55, 60 and 70°C for 30 min, then, the amylolytic activity was measured as described above.

α-Amylase Inhibitory Activity of Plant Extract

In-vitro *α*-amylase inhibition assay was conducted as follows: 500 µl of plant extract (10%) was pre-incubated with enzyme solution (500 µl) at 30 °C for 30 min. Then, substrate (1% starch solution) was added to the mixture and further incubation was done at 30 °C for 30 min followed by addition of DNS and measurement of absorbance at 450 nm as described above.

Appropriate blanks were included in the experiments, too. The percent inhibition of α-amylase was calculated as follows:

$$\% I_{\alpha\text{-amylase}} = 100\times[(\Delta A_{540\ Control} - \Delta A_{540\ Exp.})/\ \Delta A_{540\ Control}]$$

In Gel Amylase Assay

Enzyme extract (30 µl) was pre-incubated with plant extracts (60 µl, 10%) for 30 min at 30 °C. Then, the remaining amylase activity was determined by SDS-polyacrylamide gel electrophoresis (Native Page). The same procedures were done for the controls, but without addition of plant extracts. SDS-PAGE was carried out using the procedure described by Laemmli (1970) and Campos *et al*. (1989), which has been modified for the Sunn pest (*E. integriceps*) (Mehrabadi *et al*., 2009). SDS-PAGE was performed in 10% (w/v) gel with 0.05% SDS for separating gel and 5% for stacking gel with.0.05 % SDS. The electrode buffer was prepared based on the method of Laemmli (1970) but SDS was not used. The sample buffer contained 25% stacking buffer (0.5 M Tris-Hcl ,pH 6.8), 20% Glycerol, 2% SDS, 0.005%(w/v) bromophenol blue, but without mercaptoethanol and heating. Electrophoresis was conducted at 4°C with a constant voltage of 120V until the blue dye reached the bottom of the slab gel. To prepare gels for α-amylase assay, the gel was rinsed with water and washed by shaking gently with 1% (v/v) Triton X-100 in phosphate buffer contained 2mM CaCl2 and 10mM NaCl for 1.5 hour. Then, the gel was incubated with starch (1%) in phosphate buffer for 1.5 hour. Finally, the gel was rinsed with water and treated with a solution of 1.3% I2, 3% KI to stop the reaction and to stain the un-reacted starch background. Zones of α-amylase activities appeared at light band against dark background.

Protein Determination

Protein concentration was measured according to the method of Bradford (1976), using bovine serum albumin (Bio-Rad, Munchen, Germany) as a standard.

RESULTS

Determination of the Midgut pH

It was found that the four species of stored-insect's midgut pH was acidic. The gut pH of *C. maculatus*, *R. dominica*, *S. granarius* and *T. granarium,* as determined with the feeding method, were 4.8 ± 0.2 (mean± *SE*), 6.1± 0.15, 5.7± 0.09 and 6.1± 0.3, respectively. When larvae were dissected and their contents were mixed with dye indicators, it was found that pH were 5.5± 0.3, 6.7± 0.25, 6.4± 1.7 and 6.5 ± 0.1 for *C. maculatus*, *R. dominica*, *S. granarius* and *T. granauium,* respectively (Figure 1).

Optimum Conditions for α -Amylase Activity

Differences at α -amylases pH optima from the guts of three species of stored pest insects were observed (Table 1). In all cases,

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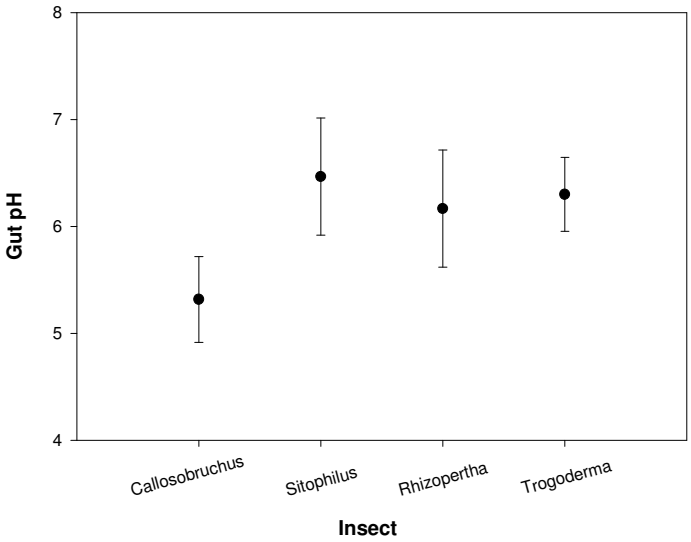

Figure 1. Gut pH of *C. maculates*, *R. dominica, S. granaries and T. Granarium*. Gut pH was determined by both in vivo (feeding) and in vitro (pulled gut) methods using pH indicators.

Table 1. The properties of a-amylases from different stored product insect species.

Insect species	α-Amylase				
	Specific activity ($\mu Mmin^{-1}ml^{-1}$)	Unite activity ($\mu Mmin^{-1}insect^{-1}$)	Optimal pH	Optimal Temperature (°C)	Number of isozymes
C. maculatus	0.0195 [c]	0.00173 [d]	5.5 [b]	34 [b]	2
R. dominica	0.0242 [b]	0.00243 [b]	6.5 [a]	38 [a]	2
S. granarius	0.038 [a]	0.00274 [a]	6.5 [a]	30 [c]	3
T. granarium	0.0114 [d]	0.00196 [c]	7.0 [a]	37 [a]	NA

NA: Not assayed. SE was 5 -15% of the means. Different letters show significant differences Tukey ($p < 0.01$), n = 5.

α -amylase activities were drastically reduced at extreme pHs. The effect of temperature on enzymatic activity of these coleopteran pests α -amylases were also examined showing that all tested α–amylases were thermo-labile, with optimum temperatures between 30 and 40 °C (Table 1). Beyond these temperatures, the α-amylases activities sharply decreased (data not shown).

Effects of Plant Extracts On α -Amylases

Inhibitory effect of extracts from different plant species on the insect pest α-amylases are shown in Table 2. Against *C. maculatus* α-amylase the highest inhibition effects was observed when extracts of *P. granatum*, *R. officinali*, *R. coriaria*, and *D. stramonium* were used resulting in 90%, 94.6%, 95.15% , and 94.56 % inhibition, respectively. In contrast, extraction of *A. Siberia*, *P. harmala*, and *T. vulgaris* had 19.22%, 4.58%, and 7.22 % inhibition, respectively (Table 2).

Extracts of *R. officinali*, *R. coriaria*, and *D. stramonium* had more than 72 % inhibition on α-amylase of *R. dominica*, whilst the extracts of *P. granatum*, *A. Siberia*, *P. harmala*, *P. harmala* and *T. vulgaris* had less than 11 % inhibition of α-amylase of *R. dominica*.

α-Amylase of *S. granarius* was inhibited more than 7 % by extracts of *P. granatum*, *R. officinali*, *R. coriaria*, *D. stramonium*, and *T.*

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Table 2. The effect of various methanolic plants extracts on different stored insect pests α-amylases. *Pg, Ro, Rc,As, Ph, Ds* and *Tv* correspond to the methanolic extracts of *P. granatum*, *R. officinali*, *R. coriaria*, *A. Siberia*, *P. harmala*, *D. stramonium* and *T. vulgaris*, respectively. Control incubations represent 100% by replacing extracts with distilled water.

Insect species	% of inhibition of plant extracts on α-amylase(αA)						
	αA+Pg	*αA+Ro*	*αA+Rc*	*αA+As*	*αA+Ph*	*αA+Ds*	*αA+Tv*
Control	100 [a]	100 [a]	100 [a]	100 [a]	100 [a]	100 [a]	100 [a]
C. maculatus	90.02 [b]	94.61 [b]	95.15 [b]	19.22 [b]	4.58 [d]	94.56 [b]	7.22 [d]
R. dominica	10.34 [e]	81.43 [c]	72.33 [d]	12.71 [d]	9.09 [b]	86.62 [c]	3.16 [e]
S. granarius	70.03 [c]	95.65 [b]	86.37 [c]	5.59 [e]	7.28 [c]	93.45 [b]	83.23 [b]
T. granarium	65.20 [d]	79.08 [c]	73.12 [d]	17.03 [c]	5.07 [d]	84.32 [c]	15.97 [c]

SE was 5–15% of the mean. Different letters show significant differences Tukey ($p < 0.01$), n = 5.

vulgaris, while extracts of *A. Siberia* and *P. harmala* had less than 8 % inhibition on α-amylase of *S. granarius*.

T. granarium α-amylase was inhibited more than 65% by *P. granatum*, *R. officinali*, *R. coriaria*, and *D. stramonium* extracts, while extracts of *A. Siberia* and *P. harmala* had less than 18% inhibition on *T. granarium* α-amylase (Table 2).

Zymogram Analysis of Digestive Insect α -Amylases

Zymogram pattern revealed that the number of α-amylases varied from 2 to 3 in different insect species. Midgut preparations of *C. maculatus* and *R. dominica* represented two iso-amylases whereas preparation of *S. granarius* showed three isozymes. The inhibitory activity of plant extracts were observed by reduction of intensity of α-amylases activity (Figure 2B, C) and in some cases i.e. *R. officinali*, *R. coriaria* and *D. stramonium,* complete deletion of bands (Figure 2 A, C).

DISCUSSION

The physico-chemical midgut conditions, especially pH of gut contents, is a major factor that affects digestive enzymes (Terra and Ferreira, 1994). Our data showed that the midgut pH in larvae of *R. dominica*, *S. granarius* and *T. granarium* were slightly acidic and midgut pH in larvae of *C. maculatus* was more acidic in comparison with the other three species. Acidic midgut pH have been reported for coleopteran pests of stored products such as *Prostethanus truncates* Horn (Bostrichidae), *Tribolium confusum* Jacquelin du Val (Tenebrionidae), *T. castaneum* Herbst, *Dermestes maculatus* DeGeer (Dermestidae), *Trogoderma versicolor* Creutz (Dermestidae), *Oryzaephilus mercator* Fauvel (Silvanidae) and *Lasioderma serricorne* Fabricius (Anobiidae) (Hosseini-Naveh *et al*., 2007; Krishna and Saxena, 1962; Murdock *et al*., 1987; Caldeira *et al*., 2003). However, some members of the family Dermestidae such as *Attagenus megatoma* (Brahm) show alkaline pH in their midguts (Johnson and Rabosky, 2000). These discrepancies in midgut pHs may be related to the different feeding habits and feeding sources.

α-Amylases from various coleopteran pests of stored products were characterized (Hosseini-Naveh *et al*., 2007; Caldeira *et al*., 2003 and references therein), showing higher activity in a pH range of 5.5 to 7.0 for different species. Interestingly, these results are corresponding to the results achieved from gut content pH, indicating that the α-amylases of each species are adapted with its gut pH. Coleopteran insect α -amylases are well adapted to acidic physiological environment of larval midgut, with optimum pHs in acidic nature such as α-amylases from *T. granarium* (PH 6.0-7.0), *C. chinensis* L. (pH 5.2–5.4) and *T. castaneum* (pH 4.6 to

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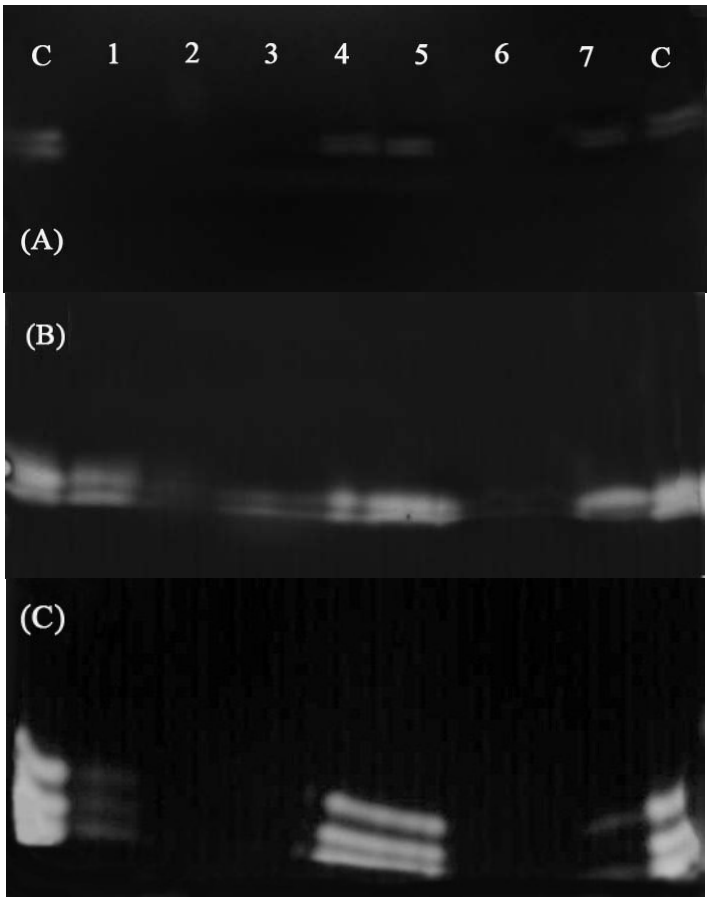

Figure 2. Determination of the effects of plant extracts on the α-amylases of *C. maculatus* (A), *R. dominica* (B) and *S. granarius* (C) in the gel assay. For more information regarding Gel electrophoresis see Materials and Methods section.

5.2) (Hosseini-Naveh *et al*., 2007; Baker, 1985, 1982). Optimal temperatures for α-amylases activity from the mentioned species were analyzed, showing higher activities between 34-38 °C. Similar results were observed for α-amylases from other coleopteran insects such as *Zabrotes subfasciatus* Boheman (Chrysomelidae), *T. castaneum*, and *T. molitor* L., which showed higher activities at 37 °C (Campos *et al*., 1989; Cinco-Moroyoqui *et al*., 2006).

Many of the natural plant compounds and organic compounds used in the control of insect pests are known to affect digestive enzymes. Secondary organic compounds synthesized by plants have an important role in protecting plants against insect pests (Athanassiou *et al*., 2005; Pare and Tumlinson, 1999). These compounds affect insects by causing a delay in larval growth and can act as antifeedant (Shekari *et al*., 2008). The current results showed that all the plant extracts had inhibitory activity on insect α-amylases varying from nearly 4% to 95% inhibition. Shekari *et al*. (2008) suggest that *Xanthogaleruca luteola* Mull α-amylases activity level decreases 24 h after treatment with *A. annua* L. extract and sharply increases at 48 h. Jbilou *et al*. (2008) found that larvae of *T. castaneum* fed on diet treated with methanol extracts from seven plants species had lower α-amylase activity than larvae fed on untreated diet. It has also been reported that digestive α-amylase, Lipase and protease of *Cnaphalocrocis medinalis* Guenée were suppressed by extracts of *Vitex*

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negundo L. *Azadirachta indica* A. Juss (Senthil-Nathan, 2006). The reduction of α-amylase activity by plant extracts could be due to the plant defense compounds that act on insect gut enzymes, α -amylases and proteinases (Ryan, 1990; Franco *et al*., 2002). Also, the reduction of this enzyme activity could be due to a cytotoxic effect of different extracts on epithelial cells of midgut that synthesize **α** -amylase (Jbilou *et al*., 2008).

The presence of a large number of α-amylases isozymes is an efficient insect strategy to escape from inhibitor toxicity (Silva *et al*., 1999). Production of isozymes was detected for other insects i.e. *S. zeamais* Motsh (Baker, 1983), *R. dominica*, *C. maculatus*, *Z. subfasciatus* and *Acanthoscelides obtectus* Say (Silva *et al*., 1999; Franco *et al*., 2005). Additional experimental studies by screening plants and plant extracts for insecticidal properties could lead to discovery of new agents for an IPM-based control of stored-products pests.

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فعالیت آلفا آمیلاز حشرات آفت محصولات انباری و مهار آن بوسیله عصاره گیاهان دارویی

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چکیده

هدف این آزمایش بررسی اثر عصاره هفت گونه گیاهی شامل *Punica granatum* L. (Punicaceae), *Rheum officinale* B. (Polygonaceae), *Rhus coriaria* L. (Anacardiaceae), *Artemisia sieberi* B. (*Compositae*), *Peganum harmala* L. (Nitrariaceae) , *Datura stramonium* L. (Solanaceae) and *Thymus vulgaris* L.(Lamiaceae) روی فعالیت آلفا آمیلاز چهار گونه حشره شامل *Callosobruchus maculates* F. (Coleoptera: Bruchidae), *Rhyzopertha dominica* F. (Coleoptera: Bostrichidae), *Sitophilus granarius* L. (Coleoptera: Curculionidae), and *Trogoderma granarium* E. (Coleoptera : Dermestidae) بود. همچنین فعالیت آلفا آمیلاز، pH دستگاه گوارش و دمای مناسب فعالیت آلفا آمیلاز این گونه های حشرات تعیین شدند. آزمایشات نشان داد که pH دستگاه گوارش کلیه چهارگونه مورد آزمایش اسیدی بود و دمای مناسب فعالیت آلفا آمیلاز بین ۳۰ تا ۴۰ درجه سانتی گراد بود. ماورای این دماها فعالیت آلفا آمیلاز بشدت کاهش پیدا میکرد. عصاره گیاهان دارای اثرات مهارکنندگی روی آلفا آمیلاز این حشرات بود که میزان مهارکنندگی از۴ تا ۹۵ درصد متفاوت بود. عصاره گیاهان *D. stramonium* و *D. stramonium* دارای بیشترین مقدار مهارکنندگی روی آلفا آمیلاز و عصاره های *P. harmala* و *T. vulgaris* دارای کمترین اثر مهارکنندگی روی آلفا آمیلاز بودند. آزمایش ژل الکتروفورز نشان داد که درکلیه چهارگونه حشره بیشتر از یک ایزوفرم آلفا آمیلاز درعصاره استخراج شده ازمعده میانی حشرات دیده شد.

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