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Research Article

Xanthogalerucella luteola (Col.: Chrysomelidae) α-amylase affected by seed proteinaceous extract from datura, wild oat and amaranth seeds

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Abstract: The elm leaf beetle, *Xanthogalerucella luteola* (Muller) (Col.: Chrysomelidae) is a serious pest of elm trees and it has been distributed all over the world. The current study was undertaken to investigate the inhibitory effects of protein extracts of three weed seeds including datura *Datura stramonium* L., amaranth *Amaranthus retroflexus* L. and wild oat *Avena fatua* L. against *X. luteola* α-amylase using spectrophotometric assay as well as in gel assays. The effects of five concentrations of each seed proteinaceous extracts were tested on α-amylase activity of the larval gut. The results showed a dose dependent manner in inhibition of the insect enzyme. At the highest concentration of protein extracts (12 µg protein) of all three seed extracts including amaranth, wild oat and datura, the inhibition was 71, 79 and 31%, respectively. Whilst, at low concentration (0.75 µg protein), the inhibition observed was 15, 36 and 5%, respectively. Thus, the greatest inhibition percentage was obtained when proteinaceous extract of wild oat seed was used. These results were confirmed when in gel assays were performed. All three seed proteinaceous extracts had an optimum pH inhibition of 6.0. Thus, it is concluded that wild oat seed proteins are potentially good for detailed investigation in order to get a clear picture of its active compound/s and its structure-function relationship.

Keywords: elm leaf beetle, seed proteinaceous extract, α-amylase, pH, In gel assay

Introduction

The elm leaf beetle, *Xanthogalerucella luteola* (Muller) (Col.: Chrysomelidae) is the most serious pest of urban area on elm trees that has been distributed all over the world (Tatli *et al*., 2013). The adult insect and its three larval instars feed on the parenchyma of leaves causing severe damage to tree. Its damage on elm tree affects all the foliage, leaves become skeletised and drop. In case of severe damage in some consecutive years, the trees develop deformed canopies as well as physiological disorders thus showing reduced photosynthesis and may eventually die (Huerta *et al*., 2010). The infested trees become particularly susceptible to scolytid beetles carrying spores of the fungus *Ceratocystis novoulmi* Brasier, which produces disease on the elm tree (Romanyk and Cadahia, 2002; Muñoz *et al*., 2003). Infestation with this pest will reduce the aesthetic value of elm trees in urban areas (Dreistadt *et al*., 2001).

The control measures against this pest vary based on the location of the infestation. Thus, pesticide use has its limitations in the urban areas. So, a more benign control method should be used in the infested areas in order not to

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pollute the environment, which could be based on physiological methods (Isman, 2000; Regnault-Roger *et al*., 2002). Digestive enzymes, especially α-amylase and protease, are potentially a good target for the insect control by taking advantage of natural enzyme inhibitors present in the plant especially in the seeds (Giri and Kachole, 1996; Hao *et al*., 2009). Thus, the study of insect digestive enzymes and their inhibitors makes sense since these enzymes play an important role in the insect growth and development. Understanding digestive enzyme function is necessary when developing insect control methods based on enzyme function and inhibition (Morton *et al*., 2000; Bandani *et al*., 2009). α-amylases are key enzymes in insect digestion and in the elm leaf beetle as well. Therefore, α -amylase inhibitors could be used for pest control since they form complexes with digestive enzymes, resulting in blocking and inactivation of the enzymes leading to poor nutrient utilization, development retardation, and even in extreme cases death because of starvation (Isman, 2006; Rahimi and Bandani, 2014; Harrison and Bonning, 2010).

In recent years considerable investigations have been done on plant-- and microorganism-derived metabolites for potentially useful products and genes in order to be used in pest management programs. As a result, many plant species have received toxic protein encoding genes as a strategy to induce resistance or protection against insect pests and pathogens. Examples of these genes which enhance resistance against pests, are lectins (Gatehouse et al., 1999), α-amylase inhibitors (Mehrabadi et al., 2012), protease inhibitors (Saadati and Bandani., 2011), toxins from *Bacillus thuringiensis* (Bt toxins) (Sharma and Ortiz., 2000), and even fusion proteins consisting of plant lectin, *Galanthus nivalis* agglutinin (GNA) linked to toxic peptide (Fitches *et al.*, 2004; Down *et al.*, 2006; Fitches *et al.*, 2008).

Since there is no study regarding the effect of the enzyme inhibitor on elm leaf beetle, the current study was undertaken to investigate the inhibitory effects of protein extracts of three weed seeds including datura *Datura stramonium* L., amaranth *Amaranthus retroflexus* L. and wild oat *Avena fatua* L. against *X. luteola* α-amylase using spectrophotometric assay as well as in gel assays. The results obtained in these studies eventually will be used in devising new control methods for this insect pest.

Materials and Methods

Insect rearing

Elm beetles were collected from the garden in the faculty of agriculture, the University of Tehran at Karaj. The collected insects were reared under laboratory conditions at 25± 2 °C with 16:8 h photoperiod and 65 ± 5% humidity as described by Tatli *et al*., (2013).

Extraction of seed proteinaceous inhibitors

Inhibitors were extracted from datura, amaranth and wild oat according to Baker (1987). Briefly, Seeds were powdered thoroughly, and then 30 g of powdered seeds from each plant separately was mixed with a solution of 0.1 M NaCl and stirred for 3 h, followed by centrifugation at 8000 ×g for 30 min. The pellet was discarded, and the supernatant was placed at 70 °C for 15 min to inactivate enzymes in the seed extract. Seed protein was extracted using a saturation of 70% ammonium sulphate followed by centrifugation at 8000 ×g for 30 min at 4 °C. The pellet containing the highest fraction of amylase inhibitors was dissolved in ice-cold sodium phosphate buffer (0.02 M and pH 7.0) and was dialyzed against the same buffer for overnight. This dialyzed solution was used as proteinaceous extract in the enzyme inhibition assays.

Enzyme preparation

Dissection of the insect gut and sample preparation was done based on Kazzazi *et al*., (2005). Briefly, last instar larvae gut was removed by dissection under a light microscope. Then, the removed guts were placed in pre-cooled homogenizer. Homogenization was done in ice cold distilled water and samples were centrifuged at 15,000 × g for 15 min at 4 °C, the supernatant was removed and stored at -20 °C for subsequent analyses.

α-amylase activity assay

α-Amylase activity assay was performed using the dinitrosalicylic acid (DNS) procedure Bernfeld (1955), using 1% soluble starch (Merck, Darmstadt, Germany) as the substrate. 10 µl of the enzyme were incubated for 30 min at 35 °C in 500 µl MES buffer (pH 6) and 40 µl soluble starch. The reaction was stopped by adding 100 µl DNS and heating in hot water for 10 min. Absorbance was then measured at 540 nm.

Effect of proteinaceous extracts on α-amylase activity

Effect of the seed extracts on α-amylase activity was determined as described by Mehrabadi *et al.* (2011) with slight modification. 10 µl of the enzyme extracts was pre-incubated with 10 µl proteinaceous extract of different concentrations (12, 6, 3, 1.5, 0.75 µg protein) for 30 min at 35 °C followed by determination of the enzyme activity as described before using dinitrosalicylic acid (DNS) method. Appropriate blanks were included in the experiments as well.

Effect of pH and temperature on inhibitory activity of the seed extracts

To determine the effect of pH on inhibitory activity of the seed extracts, highest concentration of (12 mg/ml protein) each seed proteinaceous extract was incubated along with the enzyme for 30 min at pH set at 3-9 using universal buffer (Hosseinkhani and Nemat-Gorgani 2003), and then enzyme activity was recorded. The effect of temperature on α-amylase activity was determined by incubating the reaction mixture at 20, 25, 30, 35, 40, 45 and 50 °C for 30 min, followed by measurement of activity in 540 nm. Controls were run at each temperature and pH value with midgut α-amylase alone as a control, and the percentages of inhibition were calculated from the controls vs. inhibited midgut α-amylase values measured at each temperature and pH.

In gel inhibitory assay of α-amylase

The effect of two seed extracts including Amaranth and Wild oat were tested in the gel assays. Since Datura seed extract did not show a very clear inhibition in the spectrophotometric assay it was excluded from the gel assays. The concentrations of protein extracts for both Amaranth and Wild oat used in gel assays were 12, 6, 3, 1.5, 0.75 µg protein. Electrophoretic detection of amylolytic activity in the gel was done based on the procedures described by Laemmli (1970) Briefly, PAGE was performed in 10% (w/v) gel for separating gel and 5% for stacking gel with 0.05% SDS. Electrophoresis was conducted at a voltage of 90 V until the blue dye reached the bottom of the gel. The gel was rinsed with distilled water and washed with 1% (v/v) Triton X-100 for 20 min. Then, the gel was incubated in MES buffer (pH 5.5) containing 1% starch solution, 2 mM CaCl2 and 10 mM NaCl for 1.5 h. Finally, the gel was treated with a solution of 1.3% I2 and 3% KI to stop the reaction and to stain the un-reacted starch background. Zones of α-amylase activity appeared at the light band against the dark background.

Protein determination

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

Statistical analysis

Data were analyzed based on a completely randomized design using SAS software. Mean comparison was done using Duncan's test.

Results

The effect of seed proteinaceous extracts on α-amylase activity

The effect of five concentrations of each seed proteinaceous extract was tested on α-amylase activity of the larval gut. The results showed a dose dependent manner in inhibition of the insect enzyme. Amaranth seed proteinaceous extract at concentrations of 12, 6, 3, 1.5, 0.75 µg protein inhibited the insect amylase activity 73, 61, 42, 29 and 15%, respectively (Fig. 1). Wild oat seed

proteinaceous extract at the same concentrations inhibited the insect amylase activity 79, 70, 53, 44 and 36%, respectively (Fig. 3). Also, Datura seed proteinaceous extract at the same concentrations inhibited the insect amylase activity 31, 19, 13, 8 and 5%, respectively (Fig. 4). At the highest concentration of protein extracts (12 µg protein) of all three seed extracts the inhibition was 73, 79 and 31%, respectively. Whilst, at low concentration (0.75 µg protein), the inhibition observed was 15, 36 and 5%, respectively (Fig. 2, 3 and 4). Thus, proteinaceous extract of wild oat seed resulted in greatest inhibition percentage.

Effect of pH and temprature on inhibitory activity of the seed extracts

Effect of pH on the inhibition of the α-amylase by protein extracts was tested. All three seed proteinaceous extracts showed the same optimum pH for the inhibition assays i.e. optimum pH of the all three seed extracts was 6.0 (Fig. 5). Also, the inhibitory rate decreased as the optimum pH was changed. In addition, the optimum temperature, at which there was maximum inhibitory rate, was determined as 35 °C (Fig. 6).

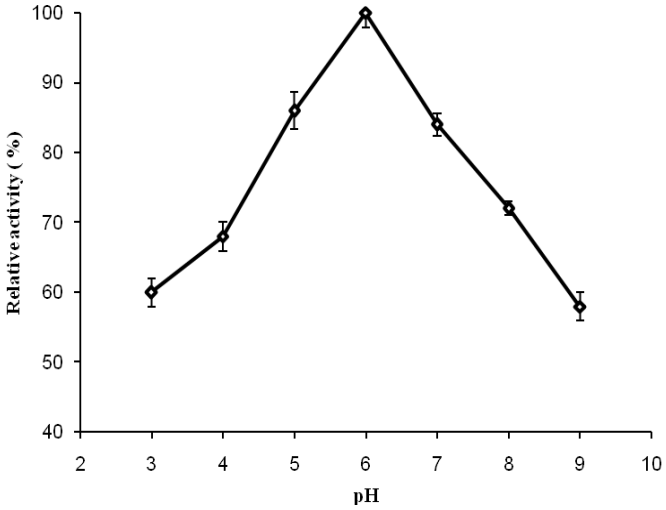

Figure 1 Effect of pH on the α-amylase activity. Activity was determined using universal buffer. Each point represents the average of three measurements.

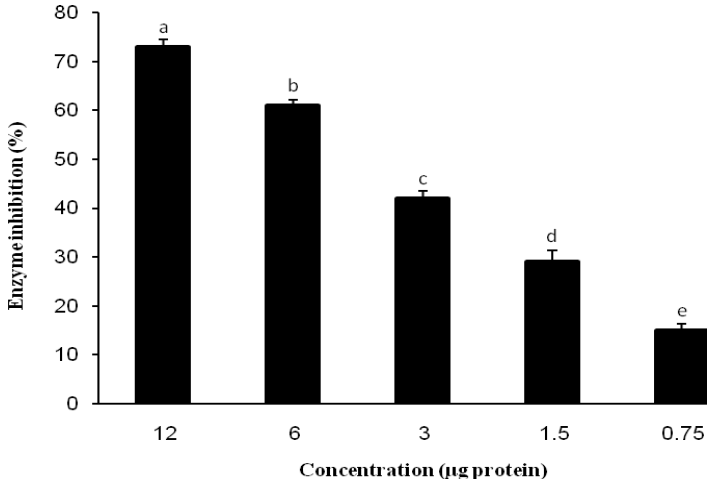

Figure 1 Inhibition of *Xanthogalerucella luteola* α-amylase activity by different concentrations of Amaranth inhibitor.

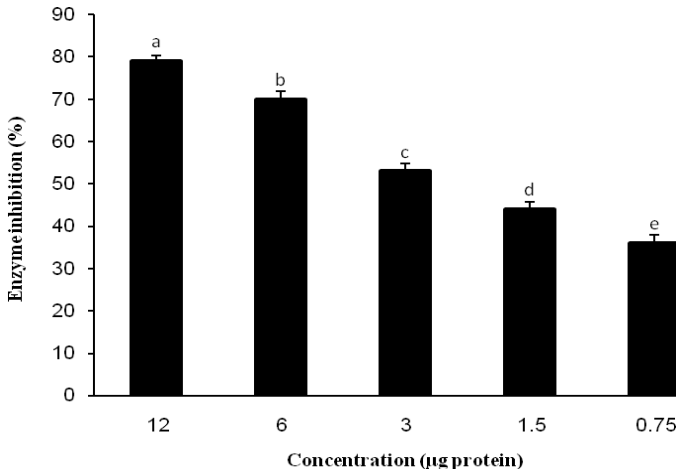

Figure 3 Inhibition of *Xanthogalerucella luteola* α-amylase activity by different concentrations of Wild oat inhibitor.

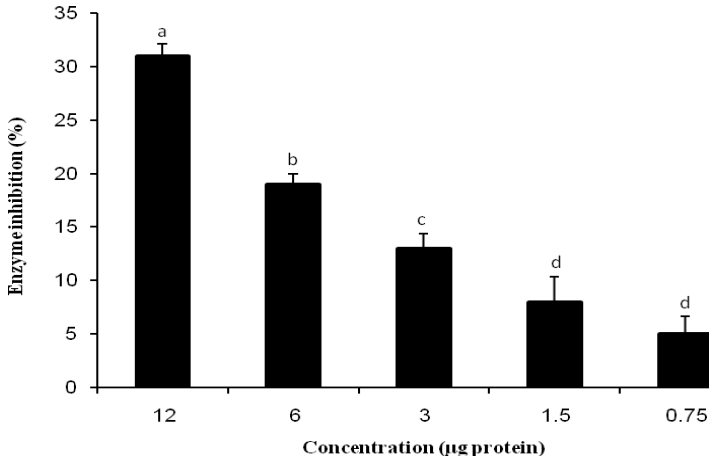

Figure 4 Inhibition of *Xanthogalerucella luteola* α-amylase activity by different concentrations of Datura inhibitor.

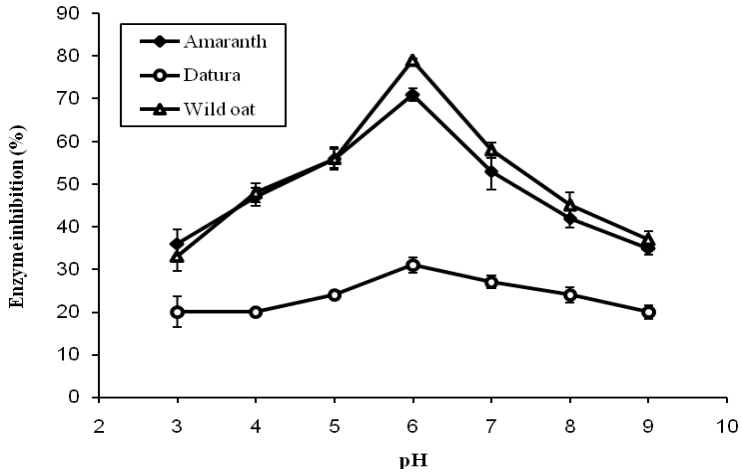

Figure 5 Effect of pH values on the inhibitory activity of amaranth, wild oat and datura α-amylase inhibitor towards *Xanthogalerucella luteola* α-amylase.

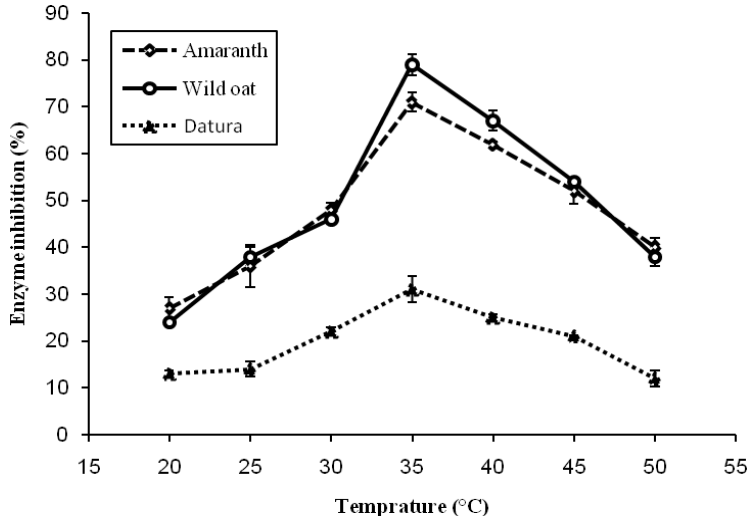

Figure 6 Effect of temperature on the inhibitory activity of amaranth, wild oat and datura α-amylase inhibitor towards *Xanthogalerucella luteola* α-amylase.

In gel inhibition assay

Gel assays showed that α-amylase of the insect gut was affected by the presence of the seed extracts as it has been shown in the spectrophotometric assays. The enzyme was subjected to a series of non-denaturing PAGE after the incubation of enzyme extract with different concentrations (including 12, 6, 3, 1.5, 0.75 µg protein) of each seed extract. In both seed extracts, when a high dose was used, almost no α-amylase band was observed and as the doses were lowered, α-amylase bands appeared (Figs. 7 and 8). Interestingly, wild oat seed extract showed better inhibition of the amylase in the gel assay as it did in the spectrophotometric assay. Thus two assays supported each other well.

Discussion

In the current study, it was found that seed proteinaceous extracts of three plant species namely amaranth, wild oat, and datura affected α-amylase activity of the elm leaf beetle gut. However, their effectiveness was not the same. For example the effect of datura on the insect amylase at the highest used concentration (12 µg protein) was 31%, whilst at the same concentration the effect of amaranth and wild oat seed proteins was 73 and 79%, respectively. These differences indicate that different plant species produce different metabolites with different specificities in order to protect themselves. Mehrabadi *et al*., (2011) tested the effect of different plant extract 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) against *Callosobruchus maculatus* (F.), *Rhyzopertha dominica* (F.), *Sitophilus granaries* (L.) and *Trogoderma granarium* Everts. They found that extract of each plant species showed a great specificity toward insect species. For example they showed that *Thymus vulgaris* inhibited the α-amylase of *S. granarius* and *R. domonica* 83 and 3%, respectively. Also, *Punica granatum* plant extract inhibited α-amylase activity of *C. maculatus* and *R. dominica* 90.0 and 10.0% respectively. Interestingly, only extract of two plant species including *Rosmarinus officinalis* L. and *D. stramonium* caused great inhibition (more than 80%) of the α-amylase activity in all insect species (*C. maculatus, R. dominica, S. granarius and T. granarium*) tested. Sharifi *et al*., (2011) tested the effect of purified inhibitor from

common bean with an ion-exchange DEAE cellulose column on *X. luteola* α-amylase and found that the inhibitor from common bean showed good inhibitory activity on gut α-amylase. Valencia *et al*., (2000) studied effect of common bean and amaranth amylase inhibitors on α-amylase of the coffee berry borer *Hypothenemus hampei* (Ferrari). Their results showed the coffee berry borer α-amylase activity was inhibited substantially and at relatively low levels of the amylase inhibitor from the common bean and much less so by the amylase inhibitor from *Amaranthus*. In another study, metabolites extracted from different wheat cultivars showed varying specificity toward α-amylase of *Tenebrio molitor* L. (Dastranj *et al*., 2013). They found at concentration of 14 µg protein from seed extract of the different wheat cultivars including MV17, Aflak, sivand, saymon, zare inhibited the α-amylase activity by 58.3, 56.2, 58.5, 57.2, and 48.5, respectively.

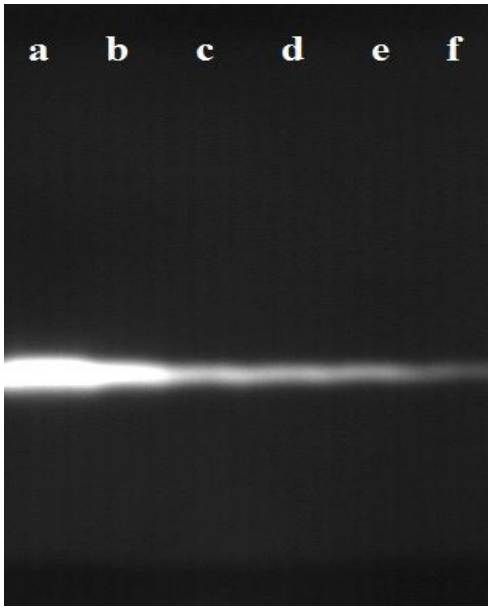

Figure 7 In gel assay of the effect of amaranth extract on the *Xanthogalerucella luteola* α-amylase activity. 10 µl extract was pre-incubated with 10 µl enzyme for 30 min, and then loaded in the gel. First column on the left shows control (a). With increasing the inhibitor concentrations the amount of the enzyme activity decreases. 12 µg protein extract (f), 6 µg protein extract (e), 3 µg protein extract (d), 1.5 µg protein extract (c), 0. 75 µg protein extract (b).

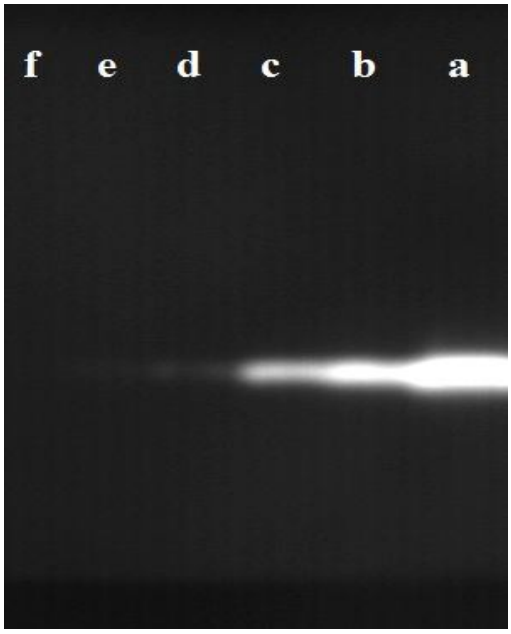

Figure 8 In gel assay of the effect of Wild oat extract on the *Xanthogalerucella luteola* α-amylase activity. 10 µl extract was pre-incubated with 10 µl enzyme for 30 min, and then loaded in the gel. First column from the right shows control (a). With increasing the inhibitor concentrations the amount of the enzyme activity decreases. 12 µg protein extract (f), 6 µg protein extract (e), 3 µg protein extract (d), 1.5 µg protein extract (c), 0. 75 µg protein extract (b).

Investigation of the effect of pH and temperature on inhibitory activity of α-amylase by Amaranth, Datura and Wild oat proteinaceous extracts showed that the greatest inhibition of α- α-amylase was observed at pH 6 and 35 °C which are the optimum pH and temperature for the activity of this enzyme in in vitro condition. There are several studies indicating pH and temperature dependence of seed extracts inhibitory effect on α-amylase activity. For example, there is an optimum pH of 5.5 for inhibition of porcine pancreatic alpha-amylase (PPA) by kidney bean, *Phaseolus vulgaris* α-amylase inhibitor varies between 4.5 to 5.5 depending on the strain used (Marshall and Lauda 1975; Powers and Whitaker 1977; Lajolo and Finardi-Filho 1985), and an optimum pH of 5.0 for inhibition of coffee berry borer (*H. hampei*) amylase

inhibitor from the common bean, *P. vulgaris* and Amaranthus (Valencia *et al*., 2000).

Inhibitory effects of the seed extract also were studied in the gel and showed that the effect of the seed extract on the enzyme was dose dependent (Sivakumar *et al*., 2006; Mehrabadi *et al*., 2012; Majidiani *et al.,* 2014).

Properties in the gel were studied and the concentration-dependent inhibition was observed in the gel that corresponded with other reports (Sivakumar *et al*., 2006; Mehrabadi *et al*., 2012; Majidiani *et al*., 2014). In this study the two assays (in gel and spectrophotometric) supported each other well.

It could be concluded that seed extracts from different species have different effects on the insect alpha amylase because of their unique metabolite/s.

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بررسی تأثیر عصاره‌های پروتئینی استخراج شده از دانه‌های داتوره، یولاف وحشی و تاج‌خروس بر فعالیت آنزیم آلفاآمیلاز سوسک برگخوار نارون *Xanthogalerucella luteola* (Muller) (Col.: Chrysomelidae)

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چکیده: سوسک برگخوان نارون *Xanthogalerucella luteola* (Muller) (Col.: Chrysomelidae) یکی از آفات مهم و خسارت‌زای نارون در سراسر جهان است. هدف از مطالعه حاضر بررسی تأثیر مهارکنندگی پروتئین‌های استخراج شده از ۳ بذر علف هرز شامل داتوره *Datura stramonium* L.، تاج‌خروس *Amaranthus retroflexus* L. و یولاف وحشی *Avena fatua* L. روی آلفاآمیلاز سوسک برگخوار نارون با روش رنگ‌سنجی و هم‌چنین سنجش در ژل الکتروفورز می‌باشد. تأثیر ۵ غلظت از هر کدام از مهارکننده‌های استخراج شده از دانه‌ها بر فعالیت آلفاآمیلاز گوارشی لاروها بررسی شد. نتایج وابسته به غلظت بودن مهارکنندگی آنزیم را نشان داد. در بیش‌ترین غلظت پروتئین استخراج شده (۱۲ میکروگرم پروتئین) از دانه‌های تاج‌خروس، یولاف وحشی و داتوره به‌ترتیب ۷۱، ۷۹ و ۳۱ درصد از فعالیت آنزیم مهار شد درحالی‌که در کم‌ترین غلظت (۰/۷۵ میکروگرم پروتئین)، میزان مهار آنزیم به‌ترتیب ۱۵، ۳۶ و ۵ درصد بود. بنابراین مهارکننده یولاف وحشی باعث بیش‌ترین میزان مهار آنزیم شد. این نتایج در ژل الکتروفورز نیز به اثبات رسیدند. همه مهارکننده‌های استخراج شده از دانه‌ها در اسیدیته بهینه ۶ بیش‌ترین میزان مهار را نشان دادند. بنابراین می‌توان نتیجه گرفت که مهارکننده استخراج شده از یولاف وحشی توانایی خوبی برای تحقیق بیش‌تر به‌منظور دستیابی به تصویر روشنی از رابطه بین ترکیبات فعال و ساختار و عملکرد آن‌ها دارد.

واژگان کلیدی: سوسک برگخوار نارون، عصاره پروتئینی دانه‌ها، آلفاآمیلاز، اسدیته، سنجش در ژل الکتروفورز