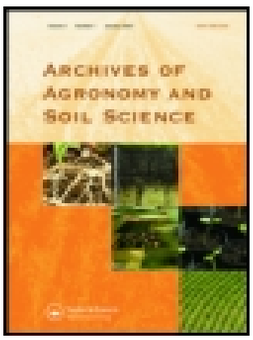

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Inhibitory effect of aqueous bean extract, *Phaseolus vulgaris* (fabaceae), on α-amylase of the cabbage aphid, *Brevicoryne brassicae*

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ABSTRACT

Brevicoryne brassicae is one of the most important pests of wide range of cruciferous host plants. In this study, the inhibitory effects of aqueous extracts of five cultivars of common bean (*Phaseolus vulgaris* L.), that includes white beans, pinto, red, cowpea and keshavarzi on the activity of the α-amylase enzyme of *B. brassicae* was studied. Aphids were reared under laboratory conditions with 25 ± 1°C, 60 ± 5% RH and 16 L:8D h photoperiods. According to obtained results, the cytotoxic effect of extracts of the highest concentration of 0.05 mg ml^{-1} and within 72 hours had the best performance and mortality. Also, all bean extracts had an inhibitory effect on insect α-amylase enzyme and red bean had the highest inhibitory effect (IC_{50} = 0.223). The effects of the bean inhibitor on α-amylase of *B. brassicae* showed a dose-dependent manner of inhibition, e.g., less inhibition of enzyme activity (around 10%) with a lower dose (0.25 mg protein) and high inhibition of enzyme activity (around 90%) when a high dose of inhibitor was used (1 mg protein). Based on the data presented in this study, it could be said that the bean extract has good inhibitory activity on *B. brassicae* α-amylase.

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α-amylase; *Brevicoryne brassicae*; *Phaseolus vulgaris*; proteinaceous inhibitor

Introduction

The cabbage aphid, *Brevicoryne brassicae* is distributed in many parts of the world and is present in most parts of Iran, especially in the central areas (Khanjani 2006; Duchovskienė et al. 2012). Different plants belonging to the crucifer family (Brassicaceae) act as a host for this aphid. Pest damage occurs on the cabbage leaves and transmits plant viruses (Clouston et al. 2016). Pesticide application has been a primary method of fighting and controlling aphids; nevertheless, unsystematic pesticide use has had adverse effects on the environment and non-target organisms (Saldo and Szpyrka 2009; Ahmad and Akhtar 2013).

There is an old view that aphids as plant sap feeders are not capable of digesting the ingested materials (McMurtry and Stanford 1960). This view has been questioned because of the existence of both biochemical and molecular evidence suggesting that this insect also uses a hydrolytic enzyme to digest the ingested materials (Foissac et al. 2002; Pyati et al. 2011). In addition to the free amino acids, phloem sap contains polymers such as lectins, proteins, and peptides that should be digested in the insect's alimentary canal before absorption (Kehr 2006). Regarding protein digestion, it is reported that aphids use proteinases, especially cysteine proteinase, in this process. Cysteine proteinase (cathepsin L) is the major protease in the gut of different species of aphids including

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Aphis gossypii, Acyrthosiphon pisum, and *Sitobion avenae* (Cristofoletti et al. 2003; Deraison et al. 2004; Pyati et al. 2011). In addition to cathepsin L, cathepsin B is reported to be present in the aphid gut (Rispe et al. 2007). However, regarding carbohydrate digestion in the gut, no studies have been undertaken. If digestion of food in the aphid gut happens, then the aphids, like the other species of chewing insects, are prone to enzyme inhibitors. Using enzyme inhibitors to control insect pest has already been demonstrated to be an important system for the insect pest control since these inhibitors have detrimental effects on the insect growth and development by interfering in food digestion (Confalonieri et al. 2002).

According to Say et al. (2013), α-amylase (α-1,4-glucan-4-glucanohydrolases; EC 3.2.1.1) catalyses hydrolysis of α-d-(1,4)-glucan linkages in glycogen and other related carbohydrates. Their activity causes the conversion of starch to maltose which is then hydrolysed to glucose by α-glucosidase. Many insects rely on different kinds of polysaccharides, so, α-amylase plays critical role in insect survival (Franco et al. 2000). The α-amylase activity has been described from different species of several insect orders including Orthoptera, Coleoptera, Hymenoptera, Diptera, Lepidoptera and Hemiptera (Oliveira-Neto et al. 2003; Kazzazi et al. 2005; Safaei-Khorram et al. 2010; Darvishzadeh and Bandani 2012; Darvishzadeh et al. 2014). The α-amylases are important enzymes involved in carbohydrate metabolism in insects, thus α-amylase inhibitors should be used in the control of the agricultural pest. For example, pea and azuki transgenic plants expressing α-amylase inhibitors from beans were completely resistant to *Bruchus pisorum* and *Callosobrouchus chinensis* which are two main pests of stored pulses (Carlini and Grossi-de-Sá 2002; Franco et al. 2002).

In the common bean (*Phaseolus vulgaris*), α-amylase inhibitor (a-AI) exists as at least two allelic variants that differ in their specificity towards α-amylase, despite their high degree of similarity (78% amino acid sequence identity). α-amylase inhibitor-1 (a-AI1) is the isoform found in cultivated beans (Pusztai and Bardocz 2014) and inhibits porcine pancreatic a-amylase (PPA), as well as the α-amylase of the cowpea weevil, *C. maculatus* and the azuki bean weevil, *C. chinensis*) (Yamaguchi et al. 2013), but not the amylase of bruchid (*Zabrotes subfasciatus*). α-amylase inhibitor-2 (a-AI2) is found in some wild bean accessions (Gupta et al. 2014). The growth of larvae of two seed feeding beetles, *C. maculates* and *C. chinensis*, is inhibited when the diet of the larvae contains relatively low levels of common bean a-AI (Fujii et al. 2012). Pea transgenic seeds containing up to 0.1–1.2% of a-AI1 were resistant to cowpea weevils and azuki bean weevils (Jaiwal and Singh 2013).

Also, bioassay results of a study using artificial diet showed that protease inhibitors in pea aphid (*A. pisum*), cotton aphid (*A. gossypii*), and peach potato aphid (*Myzus persicae*) (Rahbe et al. 2003; Ribeiro et al. 2006) can produce antimetabolic effects (detrimental effect on growth and development and reduced fecundity). The understanding of biochemistry and physiology of digestion is essential when developing methods of insect pest control using enzyme inhibitors.

Thus, the current study aimed to evaluate the inhibitory effects of aqueous extracts of common bean (*Phaseolus vulgaris* L.) five cultivars on the activity of the α-amylase enzyme of cabbage aphid. The knowledge thus achieved should lead to a better understanding of the digestive physiology of the aphid species which could be used to devise new management strategies for their control.

Materials and methods

Rearing of cabbage aphid

Aphid samples were collected in the fall of 2016 from a cauliflower field located at Shahed University, Tehran, Iran and all experiments were carried out during 2016–2017. These samples, along with pieces of the host plants, were later moved to the laboratory. Aphids were reared on the leaf of each cauliflower cultivar to three generations, 25 ± 2°C, 65 ± 5% relative humidity and 16/8 (Light/Dark) h photoperiods. The leaves were individually placed in clear plastic containers (5 × 13 × 15 cm) and were covered by netted lids, permitting the flow of air (Jahan et al. 2014).

Sample preparation and enzyme assays

Adult insects (50 individuals) were homogenized in a pre-cooled homogenizer (Sigma/Aldrich, UK) (teflon pestle with 0.1 mm clearance) in distilled water, then homogenates were put in the 1.5 ml centrifuge tubes and centrifuged at 15,000 × *g* at 4°C for 15 minutes. The supernatant was separated and kept at −20°C for subsequent analysis as an enzyme source.

Preparation of bean extract containing bean α-amylase inhibitor

10 g of each type of bean powder (including white beans, pinto, red, cowpea and keshavarzi) were mixed in 100 ml phosphate buffer and stirred at room temperature for 2 h. The resulting mixtures were centrifuged at 8000 × *g* for 8 h at 4°C. The supernatant collected was divided into two equal portions, one of which was kept at −80°C with no change, and the extract was used for insecticide experiments. Thirty percent and 40% ammonium salt was used to precipitate bean extract protein. In the experimental procedure, the precipitated proteins were dissolved in the appropriate amount of pH = 7 phosphate buffer and their effect on α-amylase inhibition was investigated (Barrett and Udani 2011).

Bioassay experiment

Liquids accessible *via* artificial membranes of stretched parafilm method was used to assay the insecticidal effect by placing 10 insects in small containers (25 × 32 mm) and covered with paraffin lid. Then, each of the different concentrations of bean extract (0.1, 0.5, 1.5, 2.5 and 5 mg ml^{-1}) was mixed with sucrose. The control treatment was also applied without adding bean extract and only by adding sucrose. The parafilm layer was then put on them again and returned to the container for a few minutes to allow the insects to attach to the parafilm and artificial food. The dishes were then placed in a germinator at 25 ± 1°C and dead and alive insects were counted every 24, 48 and 72 h. In this experiment, common bean (*Phaseolus vulgaris* L.) five cultivars, that includes white beans, pinto, red, cowpea and keshavarzi were selected for each of the five concentrations, in three replications and each replicate, 10 insects and control treatments. The experiment was also performed on two stages of fourth instar nymph and parthenogenetic adult.

Assays of α-amylase activity of Brevicoryne brassicae

α-Amylase activity was assayed by the dinitrosalicylic acid (DNS) procedure (Bernfeld 1955), using 1% soluble starch (Merck, Darmstad, Germany) solution as substrate as described by Bandani et al. (2009). For each sample, α-amylase activity was performed with three replicates. Maltose hydrate was used as a standard. The α-amylase enzyme assay was expressed in units of mg protein. By definition, each unit of α-amylase activity is the amount of enzyme that produces one micromole of maltose per minute at 25°C (Xu et al. 2014).

Evaluation of the inhibitory activity of bean extract on α-amylase

In order to evaluate the effect of protein extract of different beans on α-amylase enzyme activity of *B. brassicae*, concentrations of 1, 0.75, 0.5 and 0.25 mg of extract protein were used. For this purpose, the protein concentration of the extracts was first measured according to the Bradford (1976) method, and then the volume of the extracts was used in an enzymatic reaction solution to contain the indicated protein content. For this purpose, the protein concentration of the extracts was first measured according to the Bradford method (section 2.7), and then the volume of the extracts was used in an enzymatic reaction solution to contain the indicated protein content. Inhibitory percentages of bean extracts on the α-amylase enzyme of *B. brassicae* were compared to control.

Protein assay of bean extract

To measure protein bean extract, the Bradford (1976) method was used. In general, 100 µl of bean extract was mixed with one ml of Bradford reagent for 5 min. The data were then read using a spectrophotometer at 595 nm. Bovine serum albumin was used as a standard.

Statistical analysis

Data analysis was performed as a factorial experiment in a completely randomized design with three replications. Data were analysed by software SPSS (2006). The data were compared using Duncan's multiple range test ($\alpha < 0.05$).

Results

Effect of beans extract on mortality of Brevicoryne brassicae

Based on Table 1, the type of extract, concentration, impact time, interaction (extract type at impact) and interaction (concentration at impact) on the nymph of the cabbage aphid, *B. brassicae* were significant ($P < 0.01$). Also, the interaction (the type of extract at concentration) and interaction (the type of extract at the concentration at different times) were non-significant.

The results of the mean comparison of different treatments showed that mortality of the cabbage aphid nymphs was highest with 30% mortality from white bean and pinto bean extracts. Then other bean extracts rank next with 25% mortality (Figure 1). Also, the highest lethal effects on adult cabbage aphid, *B. brassicae* were related to cowpea and keshavarzi bean extracts with 30% mortality but no significant difference between them. Then, white bean and pinto bean extracts had the highest mortality with no significant difference. In the end, red beans had the least lethal effect.

Inhibitory activity of bean extract on α-amylase

Results of analysis of variance showed that the type of extract and concentration had a significant effect ($P < 0.05$) on the inhibition of cabbage aphid, *B. brassicae* α-amylase activity (Table 2). Since the protein fraction deposited with ammonium sulphate 40% lacked inhibitory activity, so in this section, only the activity of proteins deposited with 30% ammonium sulphate is expressed.

Evaluation of α-amylase activity in the presence of different concentrations of bean extracts indicated a concentration-dependent inhibition. In this study, concentrations of 0.25, 0.5, 0.75 and 1 mg of each extract were used. The results showed that cowpea at applied concentrations inhibited the insect α-amylase enzyme by 10%, 20%, 45% and 65% compared to control, respectively (Figure 2). The inhibitory percentages of pinto bean were 30%, 50%, 60% and 65%, respectively. In the case

Table 1. Results of analysis of variance of effect of five bean extracts at different concentrations and times on mortality of nymph and adult stages of the cabbage aphid, *B. brassicae.*

		nymph		adult	
Source of variance	df	Mean of Squares	F-value	Mean of Squares	F-value
Extract type (A)	4	449.44**	6.70	1522.41*	2.94
Concentration (B)	5	2492.22**	37.18	3686.89**	71.10
Impact time (C)	2	80,601.11**	1202.34	80,601.11**	1554.45
A × B	20	69.44^{ns}	1.04	44.85^{ns}	0.865
A × C	8	309.44**	4.62	108.52*	2.09
B × C	10	740.67**	11.05	1403.33**	27.06
A × B × C	40	36.78^{ns}	0.55	22.96^{ns}	0.44
Error	180	67.04		51.85	
Total	269	744.50		766.90	

* and ** show significant difference at α = 0.05, 0.01, respectively and ^{ns} non-significant

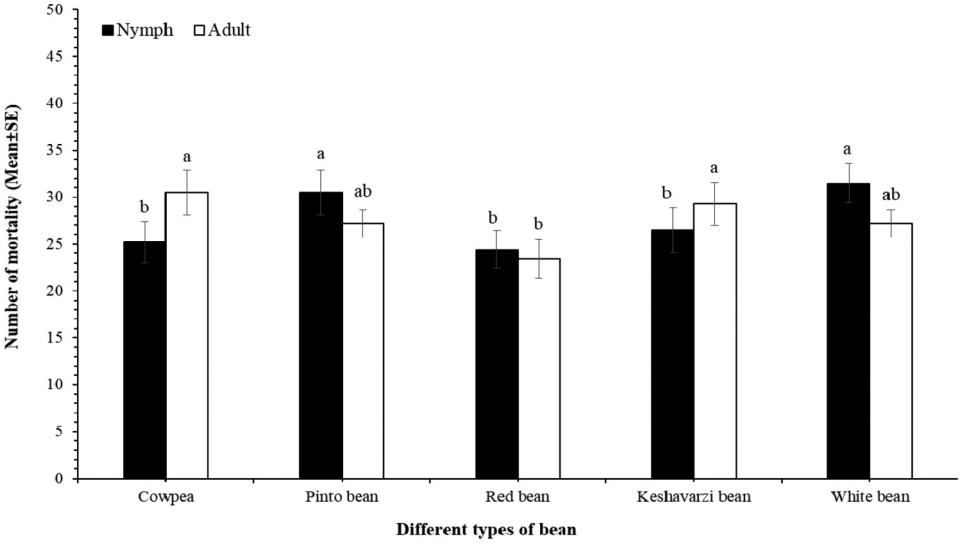

Figure 1. Mean comparison of mortality rate of nymph and adult of the cabbage aphid, *B. brassicae* under the effect of different types of bean extract (Duncan MRT test at $\alpha = 0.05$). The mean of the similar letters is not statistically significant.

Table 2. Analysis of variance of the effects of five types of bean extract at different concentrations on the inhibitory effect of α-amylase enzyme on the cabbage aphid, *B. brassicae*.

Source of variance	df	Sum of square	Mean of square	F-value	P-value
Extract type (A)	4	11,644.29	2911.07	16,484.14	0.001**
Concentration (B)	3	14,266.84	4755.61	26,928.98	0.001**
A × B	12	1412.31	117.69	666.44	0.001**
Error	40	7.06	0.18		
Total	59	27,330.50			

** show significant difference at α = 0.01.

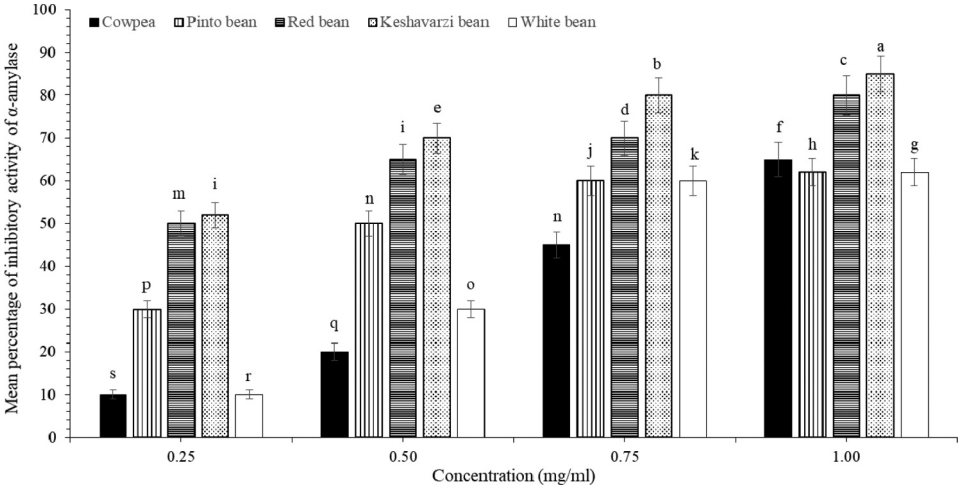

Figure 2. Mean comparison of inhibitory percentage of different bean extracts on the cabbage aphid, *B. brassicae* α-amylase in different concentrations (Duncan MRT test at $\alpha = 0.05$). The mean of the similar letters is not statistically significant.

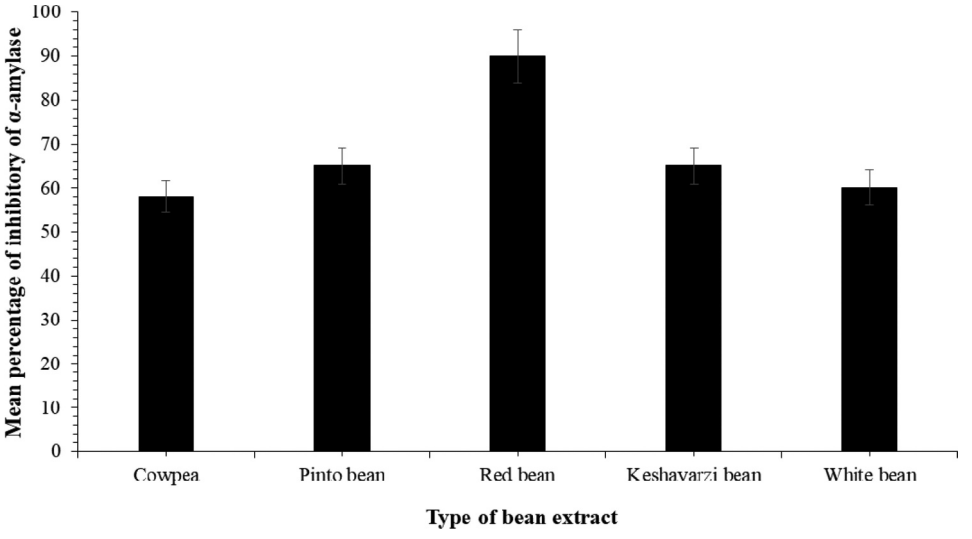

Figure 3. Mean comparison of inhibitory percentage of different bean extracts (1 mg protein) on the cabbage aphid, *B. brassicae* α-amylase (Duncan MRT test at $\alpha = 0.05$).

of white beans, it was 50%, 60%, 70% and 80% inhibited, respectively. Red beans were able to control 50%, 65%, 80% and 90% of insect α-amylase activity, respectively. Keshavarzi bean also inhibited by 15%, 35%, 50% and 65%, respectively. These experiments showed that by increasing the concentration of each extract, the percentage of inhibition of the cabbage aphid α-amylase increased.

A comparison between the percentages of inhibition of the α-amylase enzyme in different bean extracts showed that the most inhibitory effect was related to the red bean with 90% inhibition at 1 mg protein. The lowest inhibition was for cowpea with a concentration of 0.25 mg protein and 10% (Figure 3).

Discussion

In the last two decades, the specificity of α-amylase inhibitors has been widely explored, with some capable of acting only against insect α-amylases or mammalian enzymes (Franco et al. 2002). A number of inhibitors have received particular attention as attractive candidates for pest-control due to bifunctional properties. The combination of simultaneous biological activities, inhibiting serine proteinases and α-amylase, is useful and possible for these inhibitors (Srikanth and Chen 2016). Results of the present study showed that the type of extract and concentration had a significant effect on the inhibition of cabbage aphid, *B. brassicae* α-amylase activity. Other studies showed that wild common bean (*P. vulgaris*) seeds were highly showed α-amylase inhibition activity against the Mexican bean weevil, *Zabrotes subfasciatus* (Dayler et al. 2005). Previous studies showed that beans contain a group of lectins that have been shown to have α-amylase inhibitory properties (Franco et al. 2002). In beans, α-AI proteins have been identified, these proteins are the best-known proteins of the bean. According to the results of this study, the lectin fraction of bean extracts with 30% ammonium sulphate is probably given and the inhibitory activity is probably related to this family of proteins.

Measurement of α-amylase activity showed that the inhibition process was concentration-dependent. In this study, different concentrations of bean protein were used in concentrations of 0.25, 0.50, 0.75 and 1 mg ml^{-1} of protein. The mean levels of α-amylase inhibition using 1 mg concentration of different bean inhibitor proteins were 58%, 65%, 90%, 65% and 60% in cowpea, pinto, red, keshavarzi and red bean cultivars, respectively. Thus, it is believed that the results of

enzyme inhibition may be related to the predominant presence of the phenolic Gallic acid as well as the specificity of each bean species to inhibit the α-amylase activity (Telles et al. 2017). The results are consistent with those of other researchers, for example, Valencia et al. (2000) reported that the protease extract of *Amaranthus cruentus* at a concentration of 1.5 mg ml^{-1} caused a 5% inhibition of the α-amylase activity of the coffee berry borer, *Hypothenemus hampei* Ferrari. Whereas, *Amaranthus hybrid* protease inhibited the insect's α-amylase activity by about 40%.

Mehrabadi et al. (2009) investigated the effect of triticale (× *Triticosecale* Wittmack) amylase inhibitor on the sunn pest (*Eurygaster integriceps* Puton) amylase. They found that the effect of this inhibitor on α-amylase activity of *E. integriceps* was dose-dependent and at a low dose (0.5 mg) caused approximately 1% inhibition of the activity of *E. integriceps* α-amylase enzyme, whereas at high dose (1 mg) about 80% of the activity of the α-amylase enzyme was inhibited. Besides, the results of the study of Dastranj and Bandani (2012) on the inhibitory activity of triticale protein on the digestive α-amylase of the cotton bollworm, *Helicoverpa armigera* (Hübner) showed that the inhibitory process was concentration-dependent. The highest inhibition, about 70%, was observed at 2 µg of protein. In another study of Esmaeily and Bandani (2015) showed the proteinaceous extracts of mung bean, pea and two wheat cultivars against the white cabbage, *Pieris brassicae* midgut α-amylase enzyme was dose-dependent, i.e. with increasing dosage of extract, enzyme inhibition was greater. The effect of acidity on enzyme inhibition showed that the highest inhibition was observed in acidity 8, which is the optimum acidity for enzyme activity *in vitro*.

Conclusion

The current results showed that all the bean extracts had good inhibitory activity on cabbage aphid, *Brevicoryne brassicae* α-amylase varying from nearly 10% to 90% inhibition. Additional experimental studies by screening plants and plant extracts for insecticidal properties could lead to the discovery of new agents for an integrated pest management (IPM) based control of aphid's pests.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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