

Research Article

## Effect of quercetin on some digestive enzyme activity via crustacean cardioactive peptide (CCAP) content of the midgut of the diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae)

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**Abstract:** Efficacy of quercetin on  $\alpha$ -amylase, lipase and protease activities via crustacean cardioactive peptide (CCAP) content of the midgut of the diamondback moth, *Plutella xylostella* (L.) was investigated. Fresh cabbage leaf discs were dipped in quercetin solution at different concentrations (100, 500 and 1000ppm) for 10 seconds. Third instar larvae of *P. xylostella* were fed on leaf dipped in quercetin solutions for 5 days.  $\alpha$ -Amylase, lipase and protease activities were evaluated for 5 days. Quercetin significantly decreased lipase, protease and  $\alpha$ -amylase activities in the midgut. The results of competitive ELISA showed that different concentration of quercetin had no effect on short neuropeptide F, tachykinin-4 and allatostatin content of the midgut, but it was shown that quercetin (500 and 1000ppm) decreased CCAP content of the midgut. Moreover, incubation of dissected midgut with CCAP increased  $\alpha$ -amylase, lipase and protease activities. The injection of CCAP into the hemocoel clearly increased  $\alpha$ -amylase, lipase and protease activities. Here, for the first time, it was confirmed that feeding on leaf dipped in quercetin, decreases CCAP content in the midgut of *P. xylostella*, that itself led to decrease of  $\alpha$ -amylase, protease and lipase activities.

**Keywords:** *Plutella xylostella*, Quercetin, Crustacean cardioactive peptide,  $\alpha$ -Amylase, Protease

### Introduction

The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is one of the most important pests of crucifers in many countries. Using insecticides still is the main strategy for controlling this pest (Liu *et al.*, 2012). Some the diamondback moth, populations have shown resistance to many

insecticide classes including organophosphates, carbamates, pyrethroids and neonicotinoids (Grzywacz *et al.*, 2010). Moreover, using insecticides has more problems such as environmental pollution, effects on non-target organisms. These drawbacks have led researchers to find alternatives for controlling pests (Hasheminia *et al.*, 2011).

Plants produce many kinds of secondary metabolites that are involved in interactions between plants and insects (Simmonds, 2003). Some of these secondary metabolites are antifedant. Flavonoids are very

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important family of such compounds (Felgines *et al.* 2000). Quercetin is a natural flavonoid that is found in almost all vegetables and fruits including red and common onion, blueberry and cranberry (Lesjak *et al.*, 2018).

Goławska *et al.* (2014) showed that adding naringenin and quercetin to the liquid artificial diet significantly decreased fecundity, increased the pre-reproductive period and also mortality of adult *Acyrtosiphon pisum* (Harris). In another study, it was shown that the quercetin and rutin increased the mortality rate of third instar through the adult stages of *Anticarsia gemmatalis* Hübner (Peres *et al.*, 2017). Napal *et al.* (2010) indicated that 48 hr-feeding on *Cucurbita maxima* leaves treated with the quercetin increased the mortality rates of *Epilachna paenulata* (Germar) in larval stage.

Neuropeptides are present in the nervous system at all levels of organization and they are the most diverse signaling substances, not only structurally but also functionally. They have many physiological functions including locomotor activity, ecdysis behavior, circadian rhythm and regulation of feeding (Nässel. 2002). The regulation of feeding in insects is very complex, and involves the interaction of different mechanisms (Audsley and Weaver, 2009). One of these mechanisms is the release, either centrally or locally, of neuropeptides including allatostatin-A (Matsui *et al.*, 2013), tachykinin-4 (Mikani, 2016), short neuropeptide F (Mikani *et al.*, 2015) and crustacean cardioactive peptide (CCAP) (Sakai *et al.*, 2006).

Although some researches can be found on effects of quercetin on insect feeding behaviors, its effect on digestive enzyme activities and its probable mechanisms in *P. xylostella* are not fully understood. In the present study, the effect of quercetin on mortality, digestive enzyme activities and CCAP level in the midgut of *P. xylostella* were evaluated.

## Materials and Methods

### Insect rearing

*P. xylostella* were obtained from cauliflower, *Brassica oleracea* L. farm located in Dezful (32°22'57"N 48°24'07"E), Iran in 2016. In order to obtain eggs, adults were reared on cabbage leaves in plastic cages (50 × 50 × 50 cm). 10% sugar solution was used for adult feeding. Larvae were reared on cabbage at 25 ± 2 °C, 65 ± 5% relative humidity (RH) and 16:8 (L: D) photoperiod. 3<sup>rd</sup> instar larvae were used in all experiments.

### Survival rate

In order to determination of mortality, the leaf disc-dip method (Tabashnik *et al.*, 1987) was used. Fresh cabbage leaf discs (5 cm diameter) were washed with distilled water containing 0.1% Triton X-100 and dried at room temperature (RT). The leaf discs were dipped in quercetin (Sigma Chemical Co. Inc. U. S. A) solution at different concentrations (100, 500 and 1000ppm) for 10 seconds. The control leaf discs were dipped in ethanol for the same time. Then the leaf discs were placed in plastic boxes (7 cm diameter), ten, third instar larvae were moved into each box. Experiment was repeated five times and the mortality was recorded 1, 2, 3, 4 and 5 days after treatment.

### Protease, $\alpha$ -amylase and lipase assays

Protease activity of the midgut was measured followed by Elpidina *et al.* (2001) method. After dissection of the midgut in Tris-HCl (50mM, pH 11), it was incubated in the same buffer (at RT), for 30min. Enzyme was released into the buffer. 300 $\mu$ L of enzyme samples and the same volume of 0.5% (v/v) azocasein solution in Tris-HCl (pH 7.4) were incubated for 30min at 37 °C. Finally by adding 800 $\mu$ L of 20% trichloroacetic acid (TCA) and cooling on ice for 10min, the reaction was stopped. The solution was centrifuged (4000g, 15min at 4 °C) and the supernatant was moved into the new 1.5mL tube. The absorbance of the supernatant was read at 335nm using microplate reader (Biotek, USA).

The midgut of *P. xylostella* was dissected in Tris-HCl (50mM, pH 9.5). It was incubated in the same buffer for 30min. Enzyme was released into the buffer was measured using  $\alpha$ -amylase measuring kit (Kikkoman Corp., Chiba, Japan) according to the method of Mikani *et al.* (2012). Finally the absorbance was measured at 400nm using microplate reader (Biotek, USA).

In order to measure lipase activity, the midgut was dissected in phosphate-buffer saline (PBS; 145mM NaCl, 1.45mM  $\text{NaH}_2\text{PO}_4$ , 8.55mM  $\text{Na}_2\text{HPO}_4$ , pH 7.5). It was incubated in the same buffer for 30min at RT that led to releasing enzyme into the buffer. Lipase activity was measured using a lipase measuring kit (Quantichrom TM Lipase Assay Kit, BioAssay System, USA) according to the method of Mikani *et al.* (2012). Reading at 412nm by the microplate reader (Biotek, USA) was used to measure to the enzyme activity.

Protein concentration of each sample was measured using BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). Bovine serum albumin was used as a standard.

### Competitive ELISA

Competitive ELISA was carried out as described earlier (Mikani *et al.*, 2012). Rabbit anti-CCAP (PFCNAFTGCamide) antibody (Genemed Synthesis, South San Francisco, CA, USA) was used as a primary antibody. The suitable concentration for primary antibody was 1: 12000 [CCAP 1: 12000 2% skimmed milk in Tris-buffered saline (TBS; 2.6mM KCl, 25mM Tris-HCl, 135mM NaCl, pH 7.6)].

### Immunohistochemistry (IHC)

The experiment was done as described by Mikani *et al.* (2012). Preabsorption test confirmed the CCAP-antiserum specificity.

### Morphometric analysis

The CCAP-ir cells were counted using the point-counting method as described before (Lucocq, 1992). Immunostained midgut of 3<sup>rd</sup>

instar larva of *P. xylostella* was photographed and 30 sections were randomly chosen from 300 sections. A grid lattice of 1000 (40 × 25) points was put on each image of IHC, and the points that covered CCAP-ir cells of the midgut were counted.

### Effects of CCAP on lipase, $\alpha$ -amylase and protease activities

The midgut of *P. xylostella* was dissected in Tris-HCl (pH 11 and 9.5 for protease and  $\alpha$ -amylase and protease assay respectively, 50mM) at RT in the presence or absence of CCAP. In the case of lipase assay, the midgut was dissected in PBS. The enzymes released into each buffer were measured.

### CCAP injection into the hemolymph

Different concentrations of CCAP in 2 $\mu$ L of PBS was injected into *P. xylostella* hemolymph using a Hamilton syringe (Hamilton, USA.) followed by sealing with adhesive (Toagosei, Japan). After 9 hours, the effect of CCAP on enzymes (lipase,  $\alpha$ -amylase activities and protease) activities was examined. In the case of control 2 $\mu$ L of PBS was injected.

### Statistical analysis

$p < 0.05$  was considered the level of significant difference between means by one-way ANOVA (Fishers LSD). For survival rate Tukey's test was used.

### Results

#### Effect of quercetin on mortality

Quercetin had concentration-dependent impact on mortality of *P. xylostella*. The mortality percentages were 31 and 41.2%, five days after feeding on leaf discs dipped in quercetin solutions (500 and 1000ppm respectively) (Fig. 1).

#### Effects of quercetin on lipase, $\alpha$ -amylase and protease activities

Feeding for 4 days on leaf dipped in quercetin solutions (500 and 1000ppm), decreased lipase,  $\alpha$ -amylase and protease activities in 3<sup>rd</sup>

instar larvae of *P. xylostella*.  $\alpha$ -Amylase activity decreased from 150mU in control to 72 and 55.2mU in insects fed on treated leaf with 500 and 1000ppm quercetin, 5 days after treatment respectively. Interestingly, protease activity also showed sharp decreasing from 80mU in control to 40.7 and 40.2mU in insects that fed on treated leaf with 500 or 1000ppm quercetin respectively. Lipase activity that was 98.4mU in control, decreased to 62.5 and 44.8mU in larvae fed on leaf dipped in 500 and 1000ppm quercetin, respectively (Fig. 2).

#### Effect of quercetin on short neuropeptide F (sNPF), tachykinin-4, allatostatin and CCAP in the midgut

The results of competitive ELISA showed that different concentration of quercetin had no effect on sNPF (Fig. 3A), tachykinin-4 and allatostatin content of the midgut. But it

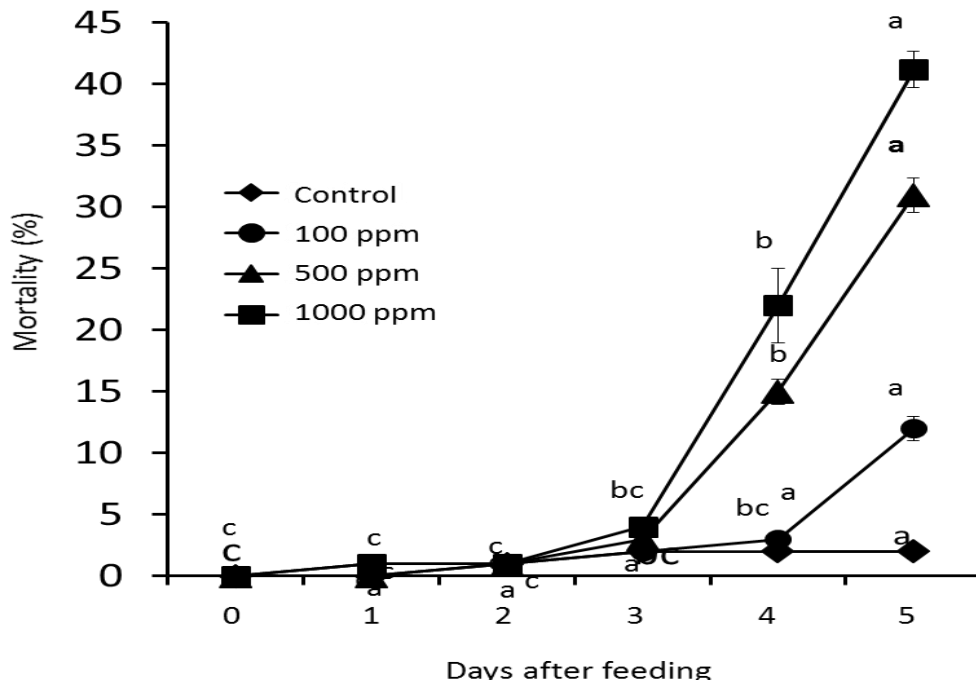
was shown that quercetin (500 and 1000ppm) decreased CCAP content of the midgut (Fig. 3).

In the next step, IHC confirmed the occurrence of CCAP-ir cells in the midgut (Fig. 4). Moreover the effect of quercetin on the number of CCAP-ir cells in the midgut was determined. IHC results showed that the number of CCAP-ir cells was decreased clearly in anterior, median and posterior of the midgut (Fig. 5).

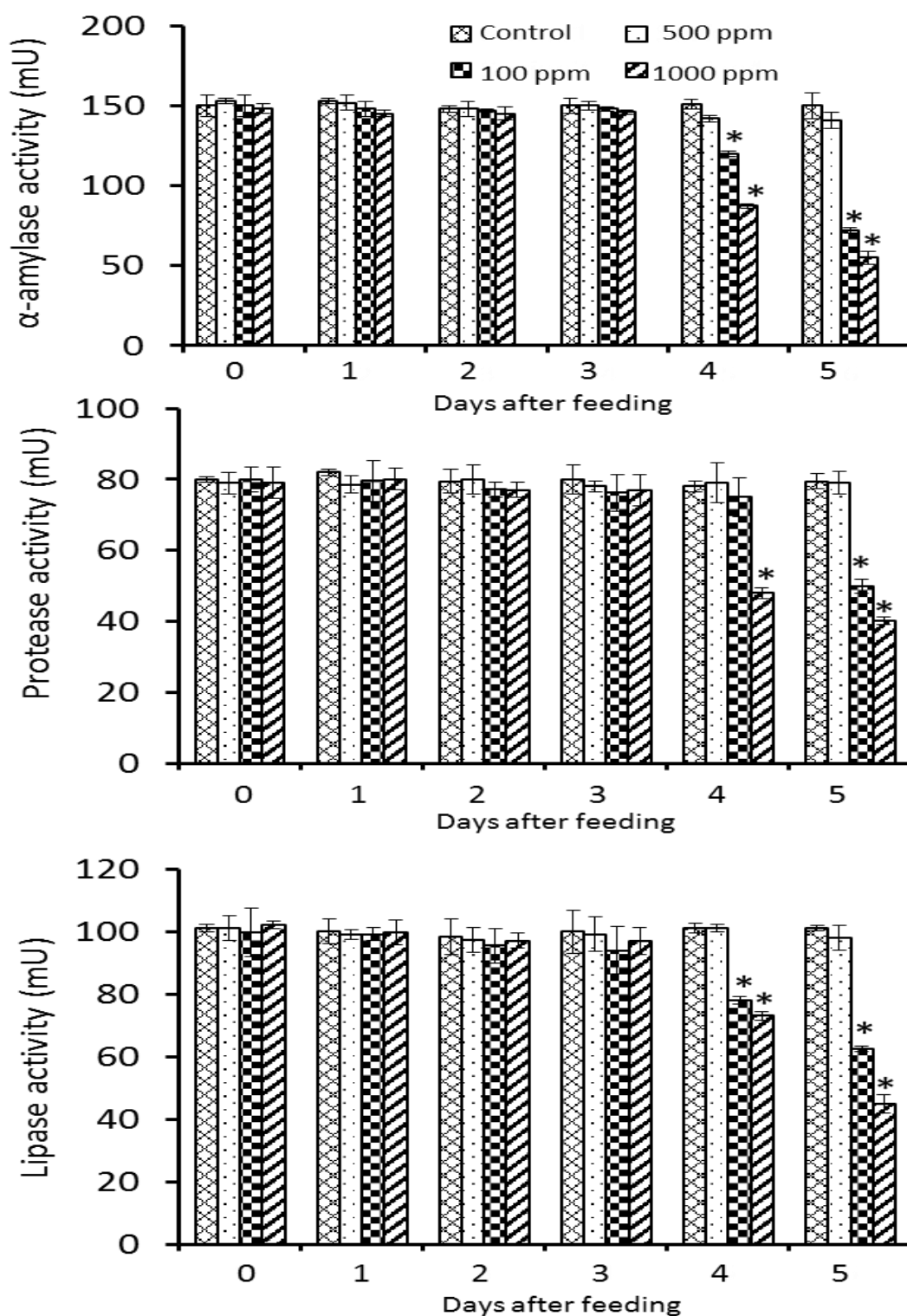
#### Effects of CCAP on $\alpha$ -amylase, protease and lipase activities, *In vitro* and *In vivo*

Incubation of the midgut of *P. xylostella* in buffer containing CCAP increased  $\alpha$ -amylase, protease and lipase activities (Fig. 6).

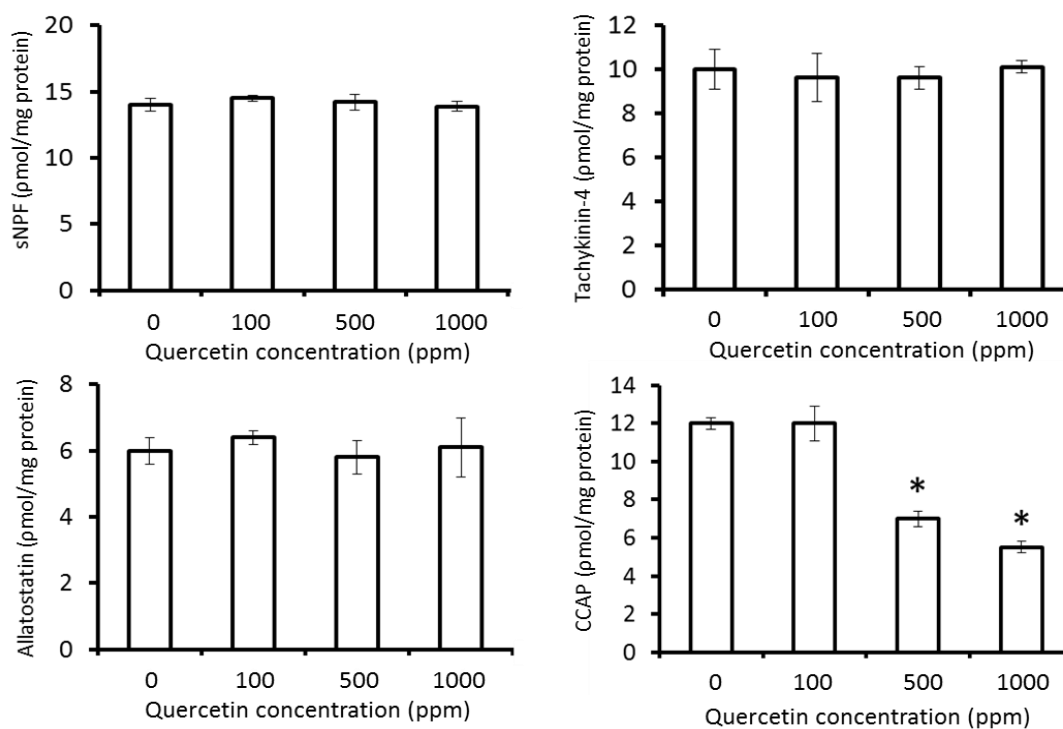
Moreover, injection of different concentration of quercetin into the hemocoel of 3<sup>rd</sup> instar larvae, increased  $\alpha$ -amylase, protease and lipase activities, *in vivo* (Fig. 7).



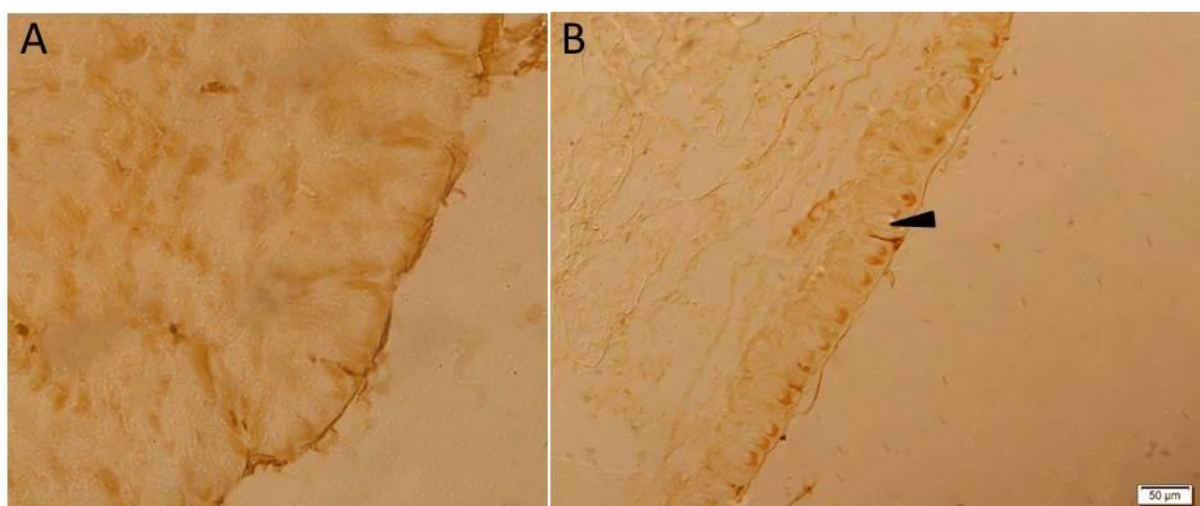
**Figure 1** Mortality (%) of *Plutella xylostella*, for 5 days after feeding on leaf dipped in different concentration of quercetin (100, 500 and 1000ppm) for 10 seconds. Each point shows the mean  $\pm$  SEM. \* indicates significance at  $p < 0.05$  (Tukey's test).



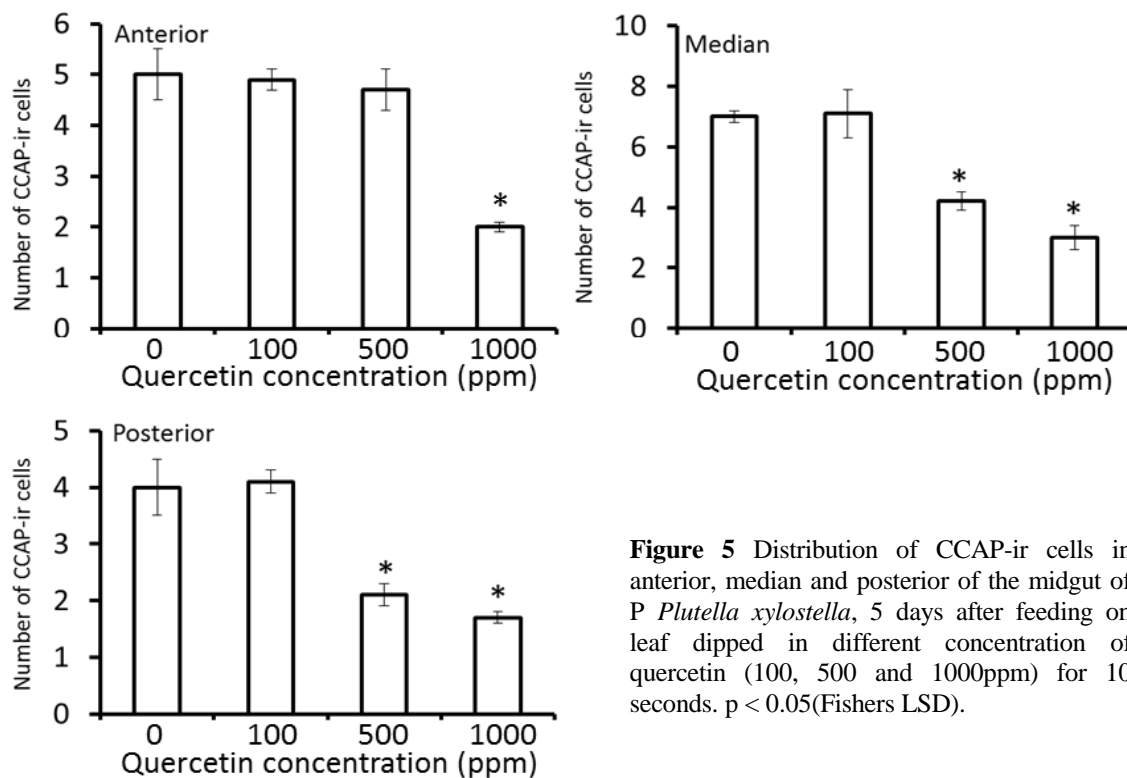
**Figure 2** α-Amylase, protease and lipase activities in *Plutella xylostella*, 5 days after feeding on leaf dipped in different concentration of quercetin (100, 500 and 1000ppm) for 10 seconds. Each point represents the mean  $\pm$  SEM. \* indicates significance at  $p < 0.05$  (Fishers LSD).



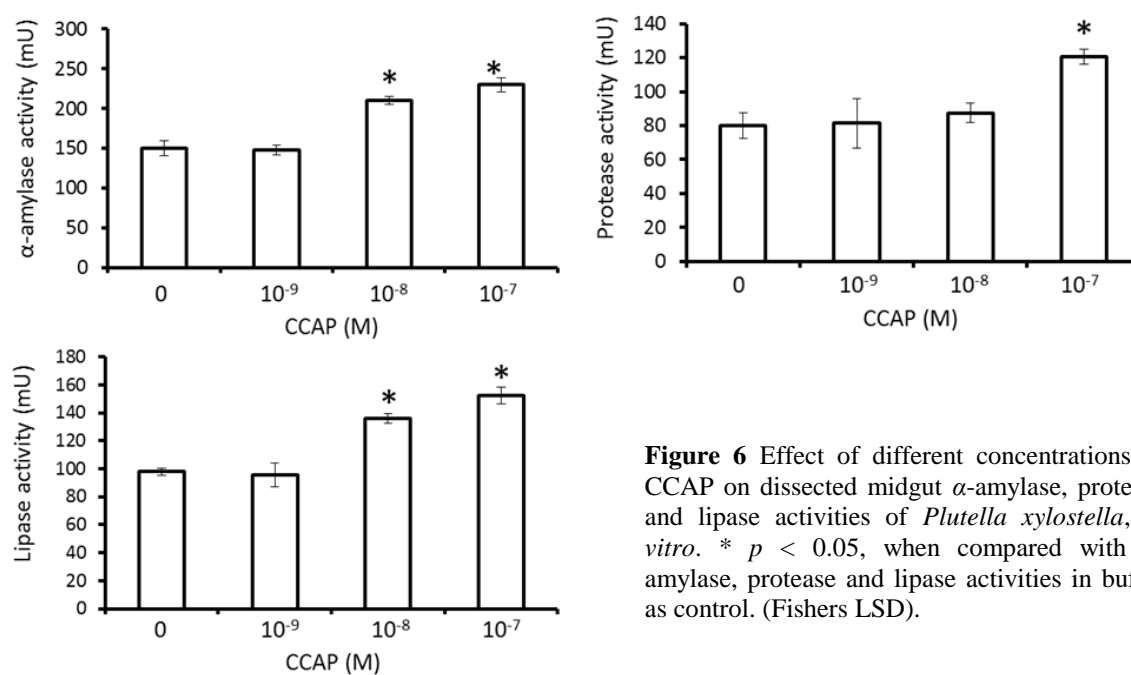
**Figure 3** Effect of different concentration of quercetin on short neuropeptide F (sNPF), tachykinin-4, allatostatin and CCAP content of the midgut of *Plutella xylostella*, 5 days after feeding on leaf dipped in different concentration of quercetin (100, 500 and 1000ppm) for 10 seconds, using competitive ELISA. Each point represents the mean  $\pm$  SEM. \*indicates significance at  $p < 0.05$  (Fishers LSD).



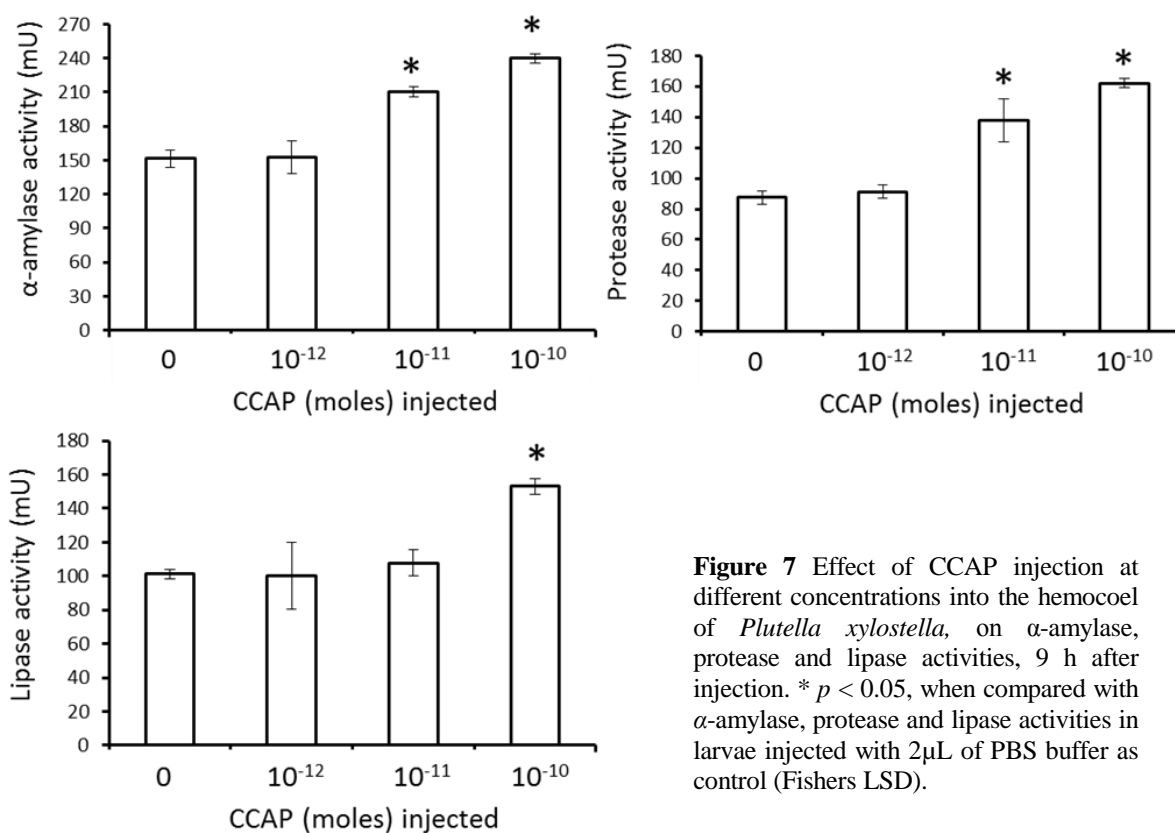
**Figure 4** CCAP-ir cells in the midgut epithelium of 3th instar larvae of *Plutella xylostella*. A, Control (preabsorption test). B, CCAP-ir cells in the midgut epithelium. Arrowhead shows the CCAP-ir cell. Scale bars shows 50 $\mu$ m.



**Figure 5** Distribution of CCAP-ir cells in anterior, median and posterior of the midgut of *P. xylostella*, 5 days after feeding on leaf dipped in different concentration of quercetin (100, 500 and 1000ppm) for 10 seconds.  $p < 0.05$ (Fishers LSD).



**Figure 6** Effect of different concentrations of CCAP on dissected midgut  $\alpha$ -amylase, protease and lipase activities of *Plutella xylostella*, in vitro. \*  $p < 0.05$ , when compared with  $\alpha$ -amylase, protease and lipase activities in buffer as control. (Fishers LSD).



**Figure 7** Effect of CCAP injection at different concentrations into the hemocoel of *Plutella xylostella*, on  $\alpha$ -amylase, protease and lipase activities, 9 h after injection. \*  $p < 0.05$ , when compared with  $\alpha$ -amylase, protease and lipase activities in larvae injected with 2  $\mu$ L of PBS buffer as control (Fishers LSD).

## Discussion

Effects of flavonoids on insect growth, fecundity and mortality has been proved previously (Alonso *et al.*, 2002). Here, also it was confirmed that the mortality was up to 41.2%, five days after feeding on leaf dipped in quercetin solutions (Fig. 1). Previously, it was shown that after adding quercetin to diet of *A. pisum*, the mortality increased clearly (Goławska *et al.*, 2014). Another study showed that quercetin influenced the biology of *A. gemmatilis* and increased the length of life cycle and mortality of the caterpillar (Peres *et al.*, 2017). In another study, the pea aphid, *A. pisum*, feeding behaviors on sucrose-agarose gels were affected by quercetin. It reduced aphid ingestion. The high concentrations of quercetin in phloem of broad bean *Vicia faba* L. might reduce insect activities because of ingestion of phloem sap (Goławska *et al.*, 2014). Norris (1977) showed that quercetin has

an oxidised C-ring that inhibited feeding in *Scolytus multistriatus* (Marsham).

Our results indicated that quercetin decreased  $\alpha$ -amylase, protease and lipase activity in the midgut (Fig. 2). These data support the hypothesis that the mode of insecticidal activity of flavonoids is related to their effect on the feeding of insect (Simmonds 2001).

Neuropeptides play important roles in the regulation of many physiological functions in insects (Nässel, 2002). There are several neuropeptides that have the ability to affect feeding behaviors. sNPF inhibits  $\alpha$ -amylase, protease and lipase activities in *Periplaneta americana* (L) (Mikani *et al.*, 2012). In contrast, allatostatin and Tachykinin-4 increased  $\alpha$ -amylase and protease activities in the midgut of *P. americana* (Matsui *et al.*, 2013; Mikani *et al.*, 2016). Moreover, allatostatin increased protease and  $\alpha$ -amylase activities in 3<sup>rd</sup> instar larvae of *Spodoptera*



*littoralis* (Boisd) (Nakhaie Bahrami *et al.*, 2018). Here, the results suggested that quercetin did not have effect on neuropeptides mentioned above but interestingly it affected CCAP content in the midgut (Fig. 3). IHC, one of the most reliable methods for analyzing cellular expression of individual neuropeptide (Nässel. 2002), showed clearly that quercetin decreased CCAP content of the midgut (Fig. 5). Previously, it was shown that CCAP increases  $\alpha$ -amylase and protease activities in *P. americana* (Sakai *et al.*, 2006) and *Helicoverpa armigera* (Hübner) (Mohammadi Gisour *et al.*, 2017). Moreover, CCAP increased  $\alpha$ -amylase, protease and lipase activities *in vitro* (Fig. 6) and *in vivo* (Fig. 7). Detection of increasing digestive enzyme activity in the midgut after injection of CCAP indicates a CCAP-mediated regulatory mechanism in the diamondback moth digestion in which  $\alpha$ -amylase, lipase and protease activities are up-regulated in response to increasing CCAP.

In conclusion, the results presented here suggest that the mortality may be a consequence of feeding on leaf dipped in quercetin. In other words, feeding on food containing quercetin caused CCAP content of the midgut to decrease which itself led to decreased  $\alpha$ -amylase, protease and lipase activities.

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اثر کوئرستین بر میزان فعالیت آنزیم‌های گوارشی از طریق نوروپپتید سی‌سی‌ای پی  
(Crustacean cardioactive peptide) در معده میانی شب‌پره پشت‌الماسی  
*Plutella xylostella* (Lepidoptera: Plutellidae)

اعظم میکانی

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**چکیده:** در این پژوهش اثر کوئرستین (Quercetin) بر میزان فعالیت آلفا‌امیلاز، پروتئاز و لیپاز از طریق تأثیر بر نوروپپتید سی‌سی‌ای پی (Crustacean cardioactive peptide) در شب‌پره پشت‌الماسی *Plutella xylostella* مورد مطالعه قرار گرفت. برگ‌های تازه کلم در محلول کوئرستین (غلظت‌های ۱۰۰، ۵۰۰ و ۱۰۰۰ پی‌پی‌ام) به مدت ۱۰ ثانیه غوطه‌ور شدند. لارو سن سه شب‌پره پشت‌الماسی از این برگ‌ها به مدت ۵ روز تغذیه کردند. فعالیت این آنزیم‌ها در مدت ۵ روز سنجش شد. کوئرستین میزان فعالیت این آنزیم‌ها را کاهش داد. نتایج الی‌ای رقابتی نشان داد که کوئرستین هیچ اثری بر نوروپپتید اف کوچک، تاکیکینین ۴ و آلتوستاتین نداشت ولی غلظت سی‌سی‌ای پی را در معده میانی کاهش داد. از طرفی قرار دادن معده میانی در بافر حاوی سی‌سی‌ای پی فعالیت آلفا‌امیلاز، پروتئاز و لیپاز را افزایش داد. تزریق این نوروپپتید نیز نتیجه مشابهی روی میزان فعالیت این آنزیم‌ها نشان داد. در اینجا برای اولین بار نشان داده شد که تغذیه از برگ آلوده به کوئرستین میزان سی‌سی‌ای پی معده میانی را کاهش داده که این امر به نوبه خود منجر به کاهش فعالیت آلفا‌امیلاز، پروتئاز و لیپاز می‌شود.

**واژگان کلیدی:** *Plutella xylostella*، کوئرستین، Crustacean cardioactive peptide، آلفا‌امیلاز، پروتئاز