

Research Article

Effect of kaempferol on ecdysteroid titer and oocyte size via tachykinin-4 in cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae)

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Abstract: The cotton bollworm, *Helicoverpa armigera* (Hübner) is an important pest of many crops. Here, effects of kaempferol on ecdysteroid titer in the ovaries, hemolymph and oocyte size via tachykinin-4 content of *H. armigera* were studied. Third instar larvae of *H. armigera* were fed on artificial diet containing different concentrations of kaempferol for 8 days. Kaempferol had insecticidal activity after 6 days. The highest level of larval mortality was approximately 36% in larvae fed diet containing 10 µg/g of kaempferol for 8 days. Competitive ELISA showed that tachykinin-4 titer decreased clearly in the adult female moth's brain and hemolymph which fed on diet containing 5 and 10 µg/g kaempferol during their larval stage for 8 days. It also decreased ecdysteroid in the ovary and hemolymph of adult female. Moreover, oocyte size was significantly decreased. On the other hand, the injection of tachykinin-4 into the hemocoel not only increased ecdysteroid titer in the ovary but also approximately 1.4 fold increases in oocyte size was observed. In conclusion, feeding on food containing kaempferol decreased tachykinin-4 level in the brain and hemolymph that itself led to decreasing ecdysteroid titer in the ovary and hemolymph. Finally the decrease in ecdysteroid titer resulted in smaller oocytes.

Keywords: Kaempferol, *Helicoverpa armigera*, Tachykinin-4, Ecdysteroid level, Oocyte size

Introduction

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is known as an important polyphagous pest causing damage to many crops. Resistance of *H. armigera* to many insecticides has been reported; thus, it seems necessary to look for alternative methods to control this pest (Talekar *et al.*, 2006). Nowadays, problems

associated with the use of synthetic insecticides such as harmful effects on non-target organisms, insect resistance, and pollution of underground water and the environment, have led to the application of more friendly alternatives to synthetic pesticides (Hasheminia *et al.*, 2011). In this sense, plant extracts which can interfere with reproduction, pheromone or growth of the insects are used (Copping and Menn, 2000).

Flavonoids that include about 4000 chemical structures play an important role in interaction between plant and the environment. They can be found in vegetables, fruits and grains. It has been

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proved that flavonoids are important in plant defense against many pests including insects (War *et al.*, 2013). Two flavonoids, rutin hydrate and quercetin dehydrate, showed insecticidal activity against *Eriosoma lanigerum* (Hausmann) (Atteyat *et al.*, 2012). In another study, it was shown that pinocembrin and quercetin, affected the feeding behavior, survival, and development of *Spodoptera frugiperda* (Smith) (Napal and Palacios, 2015). Kaempferol is a flavonoid that can be found in a large variety of plants including tea, beans, tomatoes, strawberries and grapes (Calderón-Montaña *et al.*, 2011). The leaves extract of *Ginkgo biloba* containing flavonoids including kaempferol, illustrated insecticidal activity against *Nilaparvata lugens* (Stal) (Ding *et al.*, 2013). Kaempferol isolated from *Ricinus communis* caused high mortality in *Callosobruchus chinensis* (Linnaeus) (Upasani *et al.*, 2003). *Spodoptera litura* (Fabricius), larvae fed on kaempferol-treated diets illustrated significant reduction in serine protease activity (Su *et al.*, 2018).

Previously, we showed that feeding on artificial diet containing caffeic acid dropped allatostatin concentration that is an important neuropeptide, in the midgut of *Spodoptera littoralis* (Boisduval) which itself decreased α -amylase and protease activities (Nakhaie Bahrami *et al.*, 2018). Neuropeptides are known as chemicals that are produced and also released by neurons and involved in many physiological functions (Burbach 2011) such as ecdysis, feeding behavior, locomotory activity, circadian rhythm and reproduction (Nässel and Winther, 2010). Van Wielendaele *et al.* (2013) indicated that injection of neuropeptide F (NPF) into the hemolymph of *Schistocerca gregaria* (Forsskal) increased ecdysteroids titer in ovaries and hemolymph. It also increased oocyte size. The role of ecdysteroid in reproductive system in insects is well known. Vitellogenins are taken up by oocytes during vitellogenesis in insects. Juvenile hormone and ecdysteroids play an

important role in regulation of vitellogenesis (Elgendy *et al.*, 2013). Ecdysteroids are synthesized by the ovaries and have a role in the growing of oocyte during the vitellogenic period that explains correlations of their synthesis with oocyte growth (Tawfik *et al.*, 1997). Insect tachykinin-related peptide (TRP) acts not only as a neuromodulator but also as an endocrine signaling peptide. It was originally identified in *Locusta migratoria* (Linnaeus) (Schoofs *et al.*, 1990). It plays a wide range of roles in stimulatory responses in visceral muscles (Zhao *et al.*, 2017), lipid metabolism, olfactory information processing (Gui *et al.*, 2017) and locomotory activity (Zhao *et al.*, 2017).

In the present study, the effect of Kaempferol on toxicity, oocyte size and ecdysteroid level in the ovary and hemolymph via tachykinin-4 in *Helicoverpa armigera* was considered.

Materials and Methods

Insect rearing

H. armigera were obtained from cotton field in Gorgan province (36°50'19"N 54°26'05"E), Iran. Insects were reared on artificial diets (Shorey and Hale, 1965) at 25 ± 1 °C, 65 ± 5% RH and a photoperiod of 16:8 (L: D) h. Third instar larvae were fed on artificial diets containing three different concentrations of kaempferol (1, 5 and 10 µg/g) for 8 days. Newly emerged adult females were used in all of the experiments.

Survival rate

Third instar larvae were fed on artificial diet containing 1, 5 and 10 µg/g of kaempferol (Sigma, USA). In the case of control, only ethanol (kaempferol solvent) was mixed with diet. The effect of kaempferol on third instar larvae of *H. armigera* survival rate was analyzed on alternate days during 8 days of study. Five replications were tested and each replication consisted of ten larvae.

Competitive ELISA

Competitive ELISA was carried out as previously described (Mikani *et al.*, 2015). Briefly, the brain of third instar larvae of *H. armigera* that were fed on artificial diet containing 1, 5 and 10 µg/gr of kaempferol (treatments) or normal diet (control) for 8 days were dissected, homogenized in tris-buffered saline (TBS; 25mM Tris-HCl, 2.6mM KCl, 135 mM NaCl, pH 7.6) and centrifuged (4000 ×g, 15min, 4 °C). The supernatant was used as sample to measure tachykinin-4 content of the brain. Hemolymph samples were extracted using Hamilton syringe (Hamilton, USA.) and kept at -80 °C for further experiment. After preparing tachykinin-4- BSA conjugate using dimethyl suberimidate (Sigma-Aldrich, USA), the 96 well plate was coated with tachykinin-4-BSA (0.6 µg/ml per well) in 0.05M sodium carbonate-bicarbonate buffer (pH 9.0) for 3h followed by blocking with 250 µl of skimmed milk (2% in TBS) at RT for 1h. Fifty microliters of the sample (supernatant of centrifugation or hemolymph) or standard peptide solutions (0.01-100 nmol/well) was added to each well. Subsequently, antibody (tachykinin-4 1:11000 TBS with 2% skimmed milk) was poured into all wells and the 96-well plate was kept at 4 °C, overnight. After rinsing with TBS- Tween-20 0.5% (3 times), secondary antibody (100 µL containing goat immunoglobulin anti-rabbit IgG labeled with alkaline phosphatase at 1:1000 TBS) was added to each well and the plate was shaken for 1 hat RT, and then washed three times. 100 µl of substrate solution [1mg/ml p-nitrophenylphosphate disodium salt hexahydrate (Sigma, USA) in 10mM diethanolamine buffer (Sigma-Aldrich, USA), pH 9.5] was poured into each well. It was shaken at RT for 1 h. In order to stop the reaction, 50 µl NaOH (4M) was added to each well and the absorbance was measured at 405nm, by a microplate reader (Bio Tek, USA).

Tachykinin-4 injection into the hemolymph

Different amounts of tachykinin-4 (10^{-14} , 10^{-13} , 10^{-12} and 10^{-11} moles) in 2 µL of phosphate-buffer saline (PBS; 1.45mM NaH₂PO₄, 145mM NaCl, 8.55mM Na₂HPO₄, pH 7.5) were injected daily, for 6 days, into adult females of *H. armigera* using a Hamilton syringe and the puncture was sealed using instant adhesive (Toagosei, Japan). After 6 days, the effects of tachykinin-4 on ecdysteroid level in the hemolymph, ovaries and oocyte size were examined, also 2 µL of PBS was injected, for 6 days, as control.

Ecdysteroid quantification in hemolymph and ovary

Ecdysteroid level was quantified by Enzyme Immunoassay (EIA) using Hackney *et al.* (2012) method. The assay is based on competition between 20-Hydroxyecdysone and a 20-Hydroxyecdysone-acetylcholinesterase (AChE) conjugate (20E Tracer) for a limited number of 20E-specific rabbit antiserum binding sites. Two microliter hemolymph was collected from newly adult females, using Hamilton syringe (Hamilton Company, Nevada, USA). Complete ovaries were dissected. The hemolymph and ovaries were preserved in 100 µl methanol separately, followed by drying using a Speedvac. They were resuspended in 100ml of EIA buffer (1mM EDTA, 0.4 M NaCl, 0.1% BSA in 0.1M phosphate buffer). The EIA plate was incubated with 50 µl samples, 50 µl 20E AChE tracer, 100 µl EIA buffer and 50 µl 20E EIA antiserum for 20h at 4 °C. The plate was washed with washing buffer and 200 µL of Ellmann reagent (Cayman Chemicals, Inc., USA) was added to each well. Later, 5 µl of tracer was added to the total activity wells. Optimum development was obtained by using an orbital shaker equipped with a large flat cover to allow the plates to develop in the dark. This assay typically develops in 90-120 minutes. Finally, the absorbance was read at 412nm using a microplate reader (Epoch, Biotek, USA). 20-hydroxyecdysone was used as a standard.

Measuring oocyte size

For determining the effect of kaempferol on oocyte size of newly emerged female adult, the length and width of the basal oocytes were measured using a graduated slide under BX50F4 microscope (Olympus, Japan). The size was calculated using a Loeb et al (Loeb et al., 1984) formula.

$$\text{Oocyte size} = 4/\pi (ab^2)$$

Where, a is the radius and b is the short dimension of oocyte.

Statistical analysis

Differences were considered as significant at $p < 0.05$ between means using one-way ANOVA (Fishers LSD). Student's t test was used for pairwise comparison of the means.

Results

Effect of Kaempferol on mortality of *H. armigera*

Kaempferol showed concentration dependent insecticidal activity against 3rd instar larvae that

were fed diet containing 1, 5 and 10 $\mu\text{g/g}$ of kaempferol for 8 days. There was a significant difference between the mortality in each treatment and control from day 6. The highest level of larval mortality during 8 days was observed in larvae fed diet containing 10 $\mu\text{g/g}$ of kaempferol (36%) (Fig. 1).

Effect of kaempferol on tachykinin-4 content in the brain and hemolymph of adult female *H. armigera*

Competitive ELISA showed that tachykinin-4 titer decreased noticeably in the brain (Fig. 2A) and hemolymph (Fig. 2B) of adult female moth which fed on diet containing 5 and 10 $\mu\text{g/g}$ kaempferol during their larval stage for 8 days. Tachykinin-4 titer was 7.1 pmol/mg protein in the brain of control insect which decreased to 4.2, and 2 pmol/mg protein in the brain of females fed on food containing 5 and 10 $\mu\text{g/g}$ kaempferol during their larval stage for 10 days (Fig. 2A). The same effect was observed in tachykinin-4 level in the hemolymph (Fig. 2B).

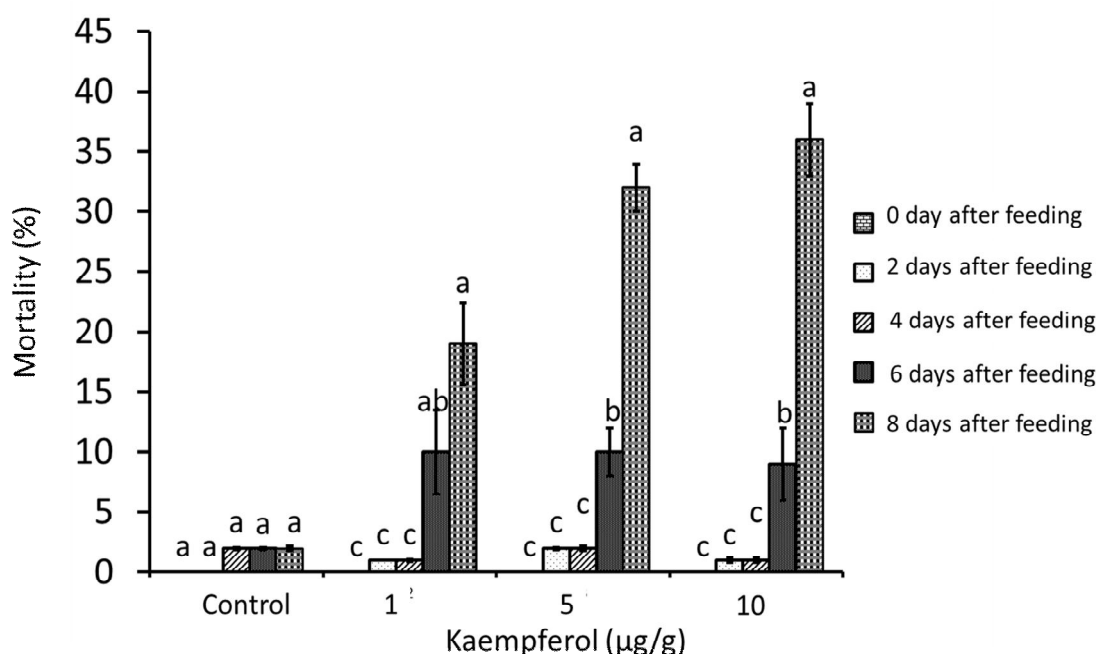


Figure 1 Mortality (%) of *Helicoverpa armigera* fed on artificial diet containing 1, 5 or 10 $\mu\text{g/g}$ of kaempferol for 8 days during larval stage. Each point is the mean \pm SEM. Means with the same letters are not significantly different (Fishers LSD, $P < 0.05$).

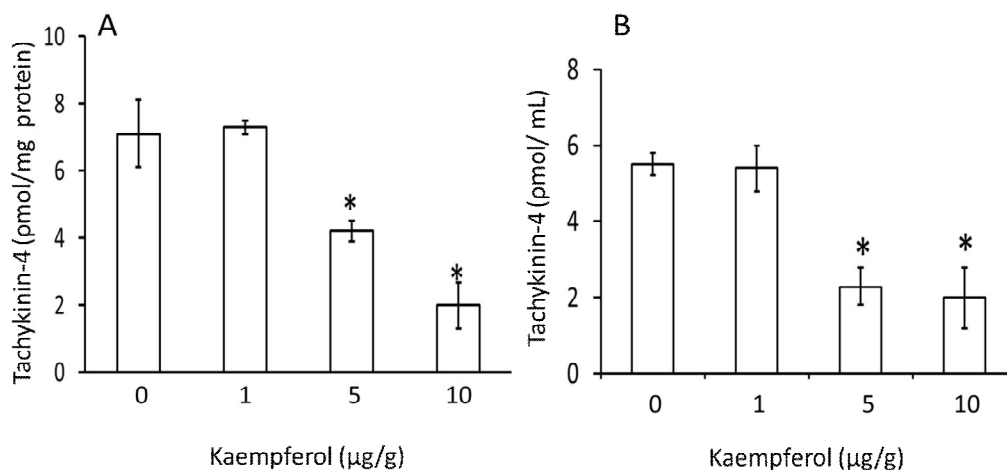


Figure 2 Tachykinin-4-ir titer detection using competitive ELISA in the (A) brain and (B) hemolymph of adult female *Helicoverpa armigera* fed on artificial diet containing 1, 5 or 10 µg/g of kaempferol for 8 days during larval stage. *p < 0.05, when compared with adult female fed normal diet for 8 days during larval stage as control (LSD test).

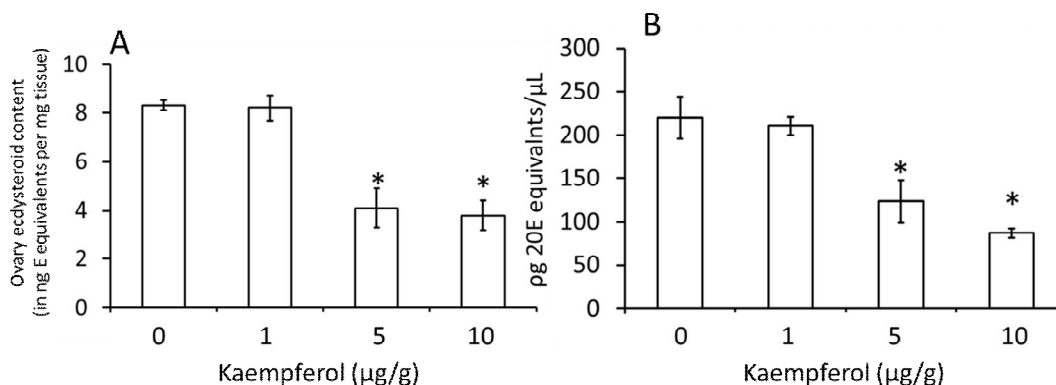


Figure 3 Ecdysteroid titer in the (A) ovary and (B) hemolymph of adult female *Helicoverpa armigera* fed on artificial diet contacting 1, 5 or 10 µg/g of kaempferol during larval stage for 8 days. * indicates significance at p < 0.05 (Fishers LSD).

Ecdysteroid titer in the ovary and hemolymph of newly adult female *H. armigera* after feeding on artificial diet containing kaempferol during larval stage

Feeding on artificial diet containing kaempferol for 8 days during larval stage, resulted in decreased ecdysteroid titer in the ovary and hemolymph of newly emerged adult female. Ecdysteroid titer in ovary decreased from 8.3 in control to 4.1 and 3.8 ng E equivalents per mg tissue in adult female that fed on artificial diet containing 5 or 10 µg/g kaempferol during their larval stage for 8 days respectively (Fig. 3A). Ecdysteroids titer in the hemolymph of newly

emerged adult female of *H. armigera* fed on diet containing kaempferol decreased from 220.3 in control to 123.4 and 87.3 pg 20E equivalents/ µL when the insect fed on artificial diet containing 5 or 10 µg/g of kaempferol respectively (Fig. 3B).

Oocyte size of newly emerged adult female *H. armigera* after feeding on artificial diet containing kaempferol during larval stage

Feeding on artificial diet containing kaempferol for 8 days during larval stage, resulted in significantly smaller oocytes in newly emerged adult female. The mean oocyte size for insect

fed on diet containing 10 μ g/g kaempferol was nearly half the size of the control (Fig. 4).

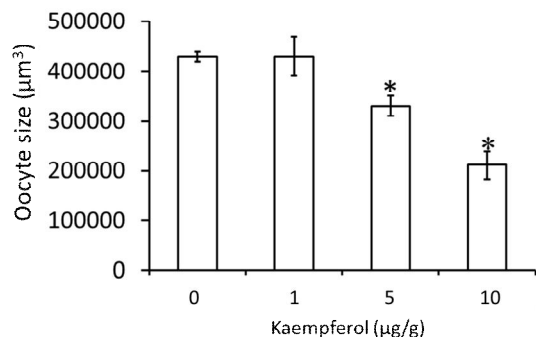


Figure 4 Effect of feeding on artificial diet containing different concentration of kaempferol for 8 days during larval stage on oocyte size of newly emerged adult female of *Helicoverpa armigera*. * indicates significance at $p < 0.05$ (Fishers LSD).

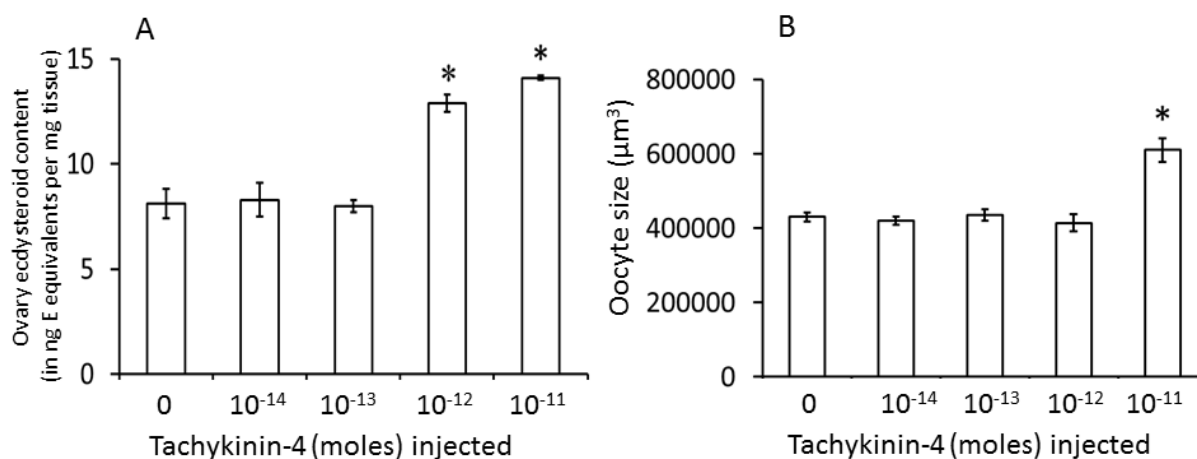


Figure 5 Change in (A) ecdysteroid titer and (B) oocyte size of adult female, 9h after injection of different amounts of tachykinin-4 into the hemocoel. Each point represents the mean \pm S. E. M of 10 preparations. * indicates significance at $p < 0.05$ (Fishers LSD). Buffer (PBS) was injected into the hemocoel of adult female insect as control (LSD test).

Discussion

Flavonoids as important plant secondary metabolites, play important roles in plant physiology and biochemistry. They participate in interactions between plants and other organisms. Sharma and Norris (1991) demonstrated the effect of flavonoids including glyceolin, coumestrol and daidzein on *Trichoplusia ni*

Effect of Tachykinin-4 injection at different concentrations into the hemocoel of *H. armigera*, on ecdysteroid titer and oocyte size

The injection of tachykinin-4 equal or greater than 10^{-11} moles into the hemocoel clearly soared ecdysteroid titer in the ovary. It was 8.1 in control which increased to 12.9 and 14.1 ng E equivalents per mg tissue in adult female injected with 10^{-12} and 10^{-11} mole tachykinin-4 respectively (Fig. 5A). Approximately, 1.4 fold increases in oocyte size was observed after injection of 10^{-11} moles tachykinin into the hemocoel (Fig. 5B).

(Hübner). Moreover, kaempferol-3, 7-diglucoside showed deterrent effects on *Mamestra configurata* (Walker) (Onyilagha *et al.* 2004). Kaempferol and quercetin are two major flavonoids present in *R. communis* leaf extract and they showed strong insecticidal activity against *C. chinensis* (Upasani *et al.*, 2003). In addition, high mortality was observed in *S. litura* larvae fed diet containing kaempferol

(Su *et al.*, 2018). In current study, it was also confirmed that mortality (%) significantly increased in the third instar larvae of *H. armigera* fed food containing kaempferol.

Several flavonoids are reported to have effect on reproductive system. Treatment of melon fruit fly, *Bactrocera cucurbitae* (Coquillett) with quercetin decreased egg hatching (Sharma and Sohal, 2013). Moreover, Biochanin A, that is a phytochemical, reduced fecundity of the Formosan subterranean termite, *Coptotermes formosanus* (Shiraki) (Boue and Raina, 2003). Here we showed that kaempferol decreased ecdysteroid titer in the ovary and hemolymph clearly. Here, it was shown that decreasing ecdysteroid level by kaempferol, led to decreasing oocyte size. On the other side, competitive ELISA showed that feeding on artificial diet containing kaempferol decreased tachykinin-4 in the brain and hemolymph.

Several neuropeptides are reported to affect reproductive physiology. Schoofs and coworkers (2001) showed that neuropeptide F (NPF) increased oocyte size in adult female *S. gregaria*. Moreover, Van Wielendaele *et al.* (2013) indicated that treatment with NPF dsRNA decreased ecdysteroids titer in ovaries and hemolymph. It also decreased oocyte size. Tachykinins are multifunctional peptides that affect many biological activities including stimulation of contractions in oviduct (Schoofs *et al.*, 1993) and hindgut contractions (Sliwowska *et al.*, 2001). Here, for the first time, it was confirmed that injection of tachykinin-4 increased ecdysteroid titer in the ovary. Moreover, it increased oocyte size. It may be possible that tachykinin-4 has direct effect on ecdysteroid titers and oocyte growth. Another possibility is occurrence of tachykinin-4 receptor in ovaries that is affected by tachykinin-4 present in the hemolymph (Van Wielendaele *et al.*, 2013). As we showed previously, tachykinin-4 increases food intake in *Periplaneta americana* (Linnaeus) (Mikani, 2016). It may be possible that the peptide has the same effect on food intake in *H. armigera*. This feeding-regulation activity of the peptide can increase acquisition of energy that in turn

speeds up maturation of the reproductive system. It led to increase of ecdysteroid titer and larger oocyte size.

As a result, feeding on food containing kaempferol caused a decrease in tachykinin-4 level of the brain and hemolymph that itself led to decreased ecdysteroid titer in the ovary and hemolymph. Finally the decrease in ecdysteroid titer resulted in smaller oocytes.

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اثر کیمپفرول بر روی میزان اکدیستروئید و اندازه اووسیت از طریق تأثیر بر میزان تاکیکینین-۴ در کرم غوزه پنبه، *Helicoverpa armigera*

اعظم میکانی

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چکیده: اثر کیمپفرول بر روی میزان اکدیستروئید در تخمدان‌ها و همولنف و نیز اندازه اووسیت از طریق تأثیر بر روی میزان تاکیکینین-۴ در کرم غوزه پنبه مورد مطالعه قرار گرفت. لارو سن سه کرم غوزه پنبه از غذای مصنوعی حاوی غلظت‌های مختلف کیمپفرول به مدت هشت روز تغذیه شد. فعالیت حشره‌کشی از روز ششم مشاهده شد. نتایج الی‌زای رقابتی نشان داد که میزان تاکیکینین-۴ در مغز و همولنف حشره ماده‌ای که ۸ روز در دوران لاروی از غذای مصنوعی حاوی کیمپفرول به غلظت ۵ یا ۱۰ میکروگرم بر گرم تغذیه کرده بود کاهش یافت. تغذیه از این غذا، میزان اکدیستروئید را در تخمدان‌ها و همولنف حشره ماده را نیز کاهش داد. ضمناً اندازه اووسیت نیز به طرز چشم‌گیری کوچک شد. از طرف دیگر، تزریق تاکیکینین-۴ به همولنف حشره کامل نه‌تنها میزان اکدیستروئید را در تخمدان‌ها افزایش داد بلکه اووسیت‌ها نیز در مقایسه با شاهد ۱.۴ برابر بزرگتر بودند. در کل، تغذیه از غذای مصنوعی حاوی کیمپفرول میزان تاکیکینین-۴ را در مغز و همولنف کاهش داد که این امر منجر به کم شدن میزان اکدیستروئید در تخمدان‌ها و همولنف و نیز کاهش اندازه اووسیت‌ها شد.

واژگان کلیدی: کیمپفرول، تاکیکینین-۴، میزان اکدیستروئید، اندازه اووسیت