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Research article

Immunological Responses of *Sesamia cretica* to *Ferula ovina* Essential Oil

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Abstract

The current research was performed aiming to investigate the effects of *Ferula ovina* essential oil on the fourth instar larval hemogram of *Sesamia cretica.* Four main sorts of circulating hemocytes, including prohemocytes, plasmatocytes, granulocytes (GRs), and oenocytoides, were identified in the fourth instar larvae. Treatment of the larvae with the concentration of 1000 ppm of the essential oil led to an enhancement of the total hemocyte and GR count followed by a dose-dependent decrease at the concentrations of 2500 and 7000 ppm. Plasmatocyte numbers declined in all the treatments with more significant effects at increased doses. The greatest numbers of GRs, plasmatocytes, and total hemocytes were found after 48 h of treatment. The highest phenol-oxidase activity was recorded 12 h after treatment at the concentration of 2500 ppm. The highest effect on nodule formation was exerted by the concentration of 7000 ppm 12 h after treatment. The results of the present study clearly indicated that the treatment of larvae by the essential oil of *F. ovina* decreased the numbers of total and differential hemocyte counts although phenol-oxidase activity and the number of nodules showed no decline in the treated larvae. These results demonstrated that *Ferula ovina* essential oil has a significant effect on the immune ability of the studied insect and can be useful and usable for future research to practical management of this pest.

Key words: *Ferula ovina*, *Sesamia cretica*, hemogram, phenol-oxidase activity

Introduction

Chemical pesticides are currently utilized to control pests in highly infested plantations; however, unfavorable effects like destruction of ozone layer, contamination to the environment, toxicity in the organisms non-targeted at, resistance to pests, residues of pesticides, and direct toxicity in users are inevitable (Isman 2006). Plant essential oils ordinarily demonstrate a wide spectrum of activities, such as insecticidal, repellent, anti-feedant, oviposition deterrent, and growth-regulatory effects against pests. Some of the oils and their constituents are mainly used as spices and seasonings in foods and drinks, while their application in pest control would be exempt from pesticide registration.

Sesamia cretica Lederer (Lepidoptera: Noctuidae) may cause a severe damage to maize plantations, particularly when infestation occurs shortly after the plant emergence (Semeada 1985, 1988). High-population densities of *S. cretica* have been recorded in several regions of Iran (Seraj 2001). However, no information is available on the effects of *F. ovina* essential oils on hemocytes and cellular immunity in *S. cretica*.

Insect immune system helps prevent pathogens from entering insect bodies (Nappi and Christensen 2005). Hemocytes in insect hemolymph are engaged in cellular and humoral immunity. Encapsulation, nodule formation, and phagocytosis are among cellular immune responses. On the other hand, humoral immune responses comprise the induced production of antimicrobial peptides, cell adhesion molecules, pro-phenol oxidase (pro-PO), lysozymes, and lectins (Hoffmann 2003; Kanost et al. 2004). As stocked pre-enzymes in insect hemolymph, POs are activated by infection or wounding and thus act as a part of the innate immune response (Kanost and Gorman 2008). Furthermore, recent researches have reported POs as important defense tools employed against various pathogens (Cerenius and Soderhall 2004; Khosravi et al. 2014). It has been shown that cellular response in insects is a major obstacle to the process of infection (Hoffmann 1995, 2003). Depending on the stages of physiological development, innate variability is observed among hemocytes within a species (Sanjayan et al. 1996; Beetz et al. 2008). Prohemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs), and oenocytoides (OEs) are considered as the most common types of hemocytes. Nodulation is a cellular process whereby hemocytes recognize a foreign material and insulate it within the hemocoel (Mullen and Goldsworthy 2003).

Ferula ovina Boiss is an herbaceous plant of Umbrellaceae family. Other species in the genus are known to produce compounds

with insecticidal, repellent, and anti-feedant properties. No information is available on the hemocytes and innate cellular responses of *S. cretica* against *F. ovina* essential oils. The current investigation was conducted to study the effects of *F. ovina* essential oil on the cellular immunity of these insects by assessing their total hemocyte counts (THCs), differential hemocyte counts, nodule formation, and PO activity in larval stage. So, significant effect on the immune ability of the studied insect can be used as an introduction to further studies in the field of practical management and immune response in this great pest.

Materials and Methods

Rearing Insects

Pink borer larvae were collected from the maize fields of Varamin, south east of Tehran, Iran, and reared on maize seedlings at 25 ± 1 °C, a relative humidity of 65 ± 5%, and a photoperiod of 16 h light:8 h darkness (L:D 16 h:8 h). Fresh maize stems were daily supplied for egg laying. The hatched larvae were fed on the fresh stems of maize until they reached the fourth instar.

Extraction of the Plant Essential Oil

Ferula spp. are known to accumulate their secondary metabolites mostly in their roots. Hence, *F. ovina* roots were collected from Binalood mountain (north-east of Iran), dried in the shade with proper ventilation, and kept at 24 °C. Using a modified apparatus of clevenger type, essential oil extraction from 50 g of the air-dried material in 750 ml distilled water was carried out by hydrodistillation for 4 h (Negahban et al. 2007). To remove water after extraction, anhydrous sodium sulfate was utilized. The resultant oil was kept in 1.5-ml micro-tubes at 4 °C.

Determination of Hemocyte Types

A drop of hemolymph was examined by cutting one of the prolegs of fourth instar larvae *of S. cretica*. The drop was allowed to dry in the dark at 0°C for 10 min and stained with Giemsa (diluted 1:9 in distilled water) for 20–40 min (Brayner et al. 2005). To fix the hemocytes, the slides were immersed in a saturated lithium carbonate solution for 5 s and quickly rinsed in distilled water (Brayner et al. 2005). Then, the hemocytes were observed under a brightfield microscope.

The Effect of *F. ovina* Essential Oil on the Number of Circulating Hemocytes

The fourth instar larvae were injected with 1 µl of each concentration (1000, 2500, and 7000 ppm) of *F. ovina* oils. Using a Hamilton syringe, the oils were applied to the mesosternum of the larvae after being diluted in acetone (Burkard, England). The controls received only 1 µl of acetone. Hemolymph was collected from the control and oil-treated larvae 3, 12, 24, and 48 h after the treatment. Immediate dilution of the collected hemolymph was done in an anticoagulant solution (0.01M EDTA, 0.1 M glucose, 0.062 M NaCl, 0.026 M citric acid, and pH = 4.6) as described by Azambuja et al. (1991) at 1:9 (anticoagulant: hemolymph) proportion. The total hemocyte, GR, and PL numbers were counted by using a hemocytometer. The bloods of five larvae were used in each treatment (five larvae for per concentration in each time) that each larvae was used as a replicate.

The Effect of *F. ovina* Essential Oil on Nodule Formation

The numbers of nodules formed in the hemocoel were counted 3, 12, 24, and 48 h after treatment. The oil-treated larvae were chilled in ice, the hemolymph was collected in a micro-tube containing the anticoagulant solution, and a drop of the mixture was placed on the hemocytometer slide for counting the nodules (Franssens et al. 2006). One larvae was used in each replication and the experiment was done with five replicates for each treatment.

The Effects of *F. ovina* Essential Oils on PO Activity

To evaluate the PO activity in the *S. cretica* larvae treated with the essential oil, the hemolymph was collected after 3, 12, 24, and 48 h as mentioned above, mixed with the anticoagulant buffer (0.01M EDTA, 0.1 M glucose, 0.062 M NaCl, 0.026 M citric acid, and pH = 4.6), kept overnight at −20 °C, and then centrifuged at 13,000 rpm for 15 min. The pelletized cells were homogenized in 100 µl Tris-HCl (pH7) and centrifuged at 13,000 rpm for 15 min. The supernatant containing hemocyte lysate was applied for the PO assay. The samples (25 µl) were transferred to each ELISA plate well containing 70 µl Tris-HCl buffer and pre-incubated at 30 °C for 3 min, which was followed by the addition of 40 µl of 10 mM L-DOPA solution as the substrate. The samples were incubated for an additional 5 min at 30 °C and their PO activities (ΔA_{405nm}/min 0.001). One unit of PO activity represents the amount of enzyme required to produce an increase in absorbance of 0.01 min^{-1} (Dularay and Lackie 1985) were measured at 492 nm by a spectrophotometer (Leonard et al. 1985).

Statistical Analysis

Experiments were performed on a completely randomized design using one-way analysis of variance (ANOVA). Data normaility was tested and was analyzed with variance analysis using SAS software (SAS 1996) and the means were compared by Tukey's test at 5% level.

Results

Determination of Hemocyte Types

Brightfield microscopy observations of the stained larval hemolymph revealed the presence of four morphologically distinct types of hemocytes. The smallest rounded cells with a large central nucleus and thin cytoplasm (Fig. 1a), large cells with an irregular shape and a central nucleus (Fig. 1b), oval-shaped ones with a large nucleus and highly granular cytoplasm (Fig. 1c), and circular cells with a large peripheral nucleus (Fig. 1d) were identified as PRs, PLs, GRs, and OEs, respectively.

The Effect of *F. ovina* Essential Oil on the Hemocyte Counts

Treatment of the fourth instar larvae of *S. cretica* with the different concentrations of the essential oil resulted a significant different in the number of hemocytes. THC was variously affected by the essential oil after treatments following a decrease until 12 h and an increase by 24–48 h. The highest number of THC was observed after 48 h (Fig. 2, $F = 7.14$, $df = 3$, and $P < 0.0001$). Similar results obtained for GRs and GRs showed increasing at the concentration of 1000 ppm and decreased at the concentrations of 2500 and 7000 ppm. In terms of differential hemocyte count, the highest number of GRs was counted 48 h after the treatment (Fig. 3a, $F = 4.06$, $df = 3$, and $P < 0.0001$). Also, a dose-dependent reduction in the PL numbers was observed. The treatment with the essential oil exerted

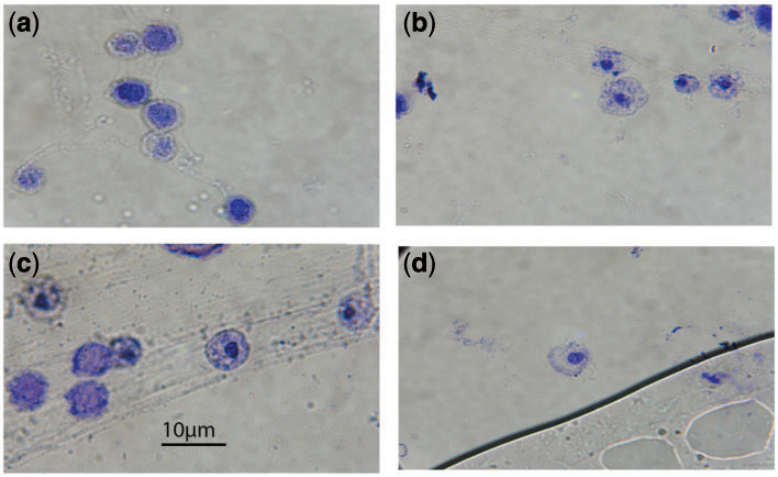

Fig.1 *Sesamia cretica* hemocytes stained with Giemsa, (a) PRs, (b) a PL, (c) a GR, and (d) an oenocytoid.

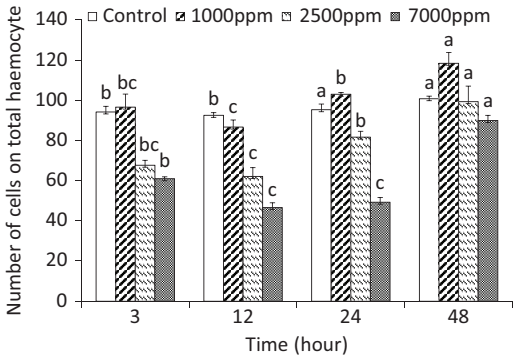

Fig. 2 Effects of essential oil of *Ferula ovina* on THC in *Sesamia cretica* larvae. *Columns capped with the same letter are not significantly different at $p < 0.05$ according to Tukey's test.

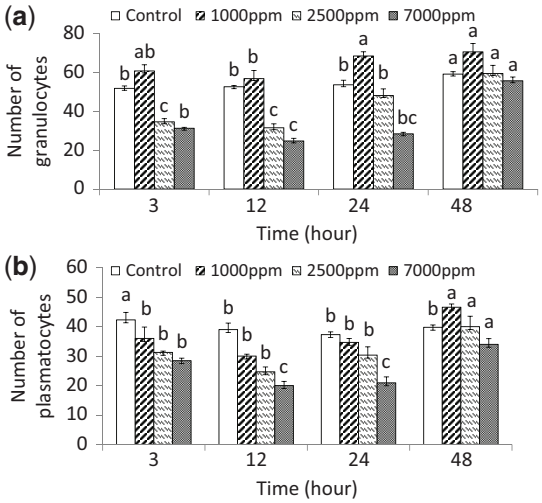

Fig. 3 Effects of different concentrations of *Ferula ovina* essential oil on the number of GRs (a) and PLs (b) in *Sesamia cretica* larvae. *Columns capped with the same letter are not significantly different at $p < 0.05$ according to Tukey's test.

its maximal effect on PLs after 48 h (Fig. 3b, $F = 9$, d$f = 3$, and $P < 0.0001$). Plasmatocytes and GRs are the important hemocytes in cellular immune responses to pathogens (Strand 2008). So at the present study, the number of these cells has been evaluated and reported.

The Effect of *F. ovina* Essential Oil on Nodule Formation

Treatment of the larvae with the essential oil exerted its maximal effect on nodule formation at a concentration of 2500 ppm and 12 h after the treatment (Fig. 4, $F = 174.25$, d$f = 3$, and $P < 0.0001$). These findings are correspondence with the lower number of PLs and GRs after 12 h injection with concentration of 2500 ppm and after 24 h injection with a concentration of 7000 ppm. Hence, it could be concluded that higher production of nodules is due to the involvement of these hemocytes in nodule formation.

PO Activity

The highest PO activity was observed after treatment with the essential oil at a concentration of 7000 ppm and 12 h after the treatment (Fig. 5, $F = 20$, d$f = 3$, and $P < 0.0001$). Results of the current study are carried out to melanin deposition to complete of nodule formation process.

Discussion

The study of the fourth instar larvae of *S. cretica* revealed the presence of four hemocyte types, namely PRs, PLs, GRs, and OEs, all of which were easily distinguishable in the smears using Giemsa staining. Similar results were found by Zibaee and Malagoli (2014) on the *Chilo suppressalis*, Giglio et al. (2008) on the *Carabus lefebvrei*, and Nakahara et al. (2009) on the *Bombyx mori* where brightfield microscopy demonstrated the presence of four hemocyte types (prohemocytes, PLs, OEs, and GRs). Composition of hemocyte population might differ depending on the developmental stages and insect taxa (Ribeiro and Brehélin 2006; Giglio et al. 2008). As reported, the most abundant hemocyte type in Lepidoptera larvae has been GR (Strand 2008). It has been shown that GRs, PLs, and OEs play multiple roles in silkworm immunity (Nakahara et al. 2009). GRs, PLs, and OEs are engaged in phagocytosis, nodule formation, and PO activity, respectively. The use of the plant essential oil was conducted to determine its effect on hemocytes and defense responses of

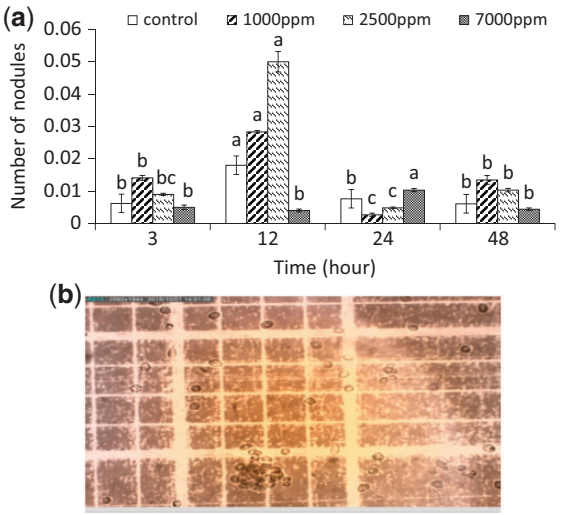

Fig. 4 Effects of different concentrations of *Ferula ovina* essential oil on nodule formation in *Sesamia cretica* larvae (a). An image of observed nodules (b). *Columns capped with the same letter are not significantly different at $p < 0.05$ according to Tukey's test.

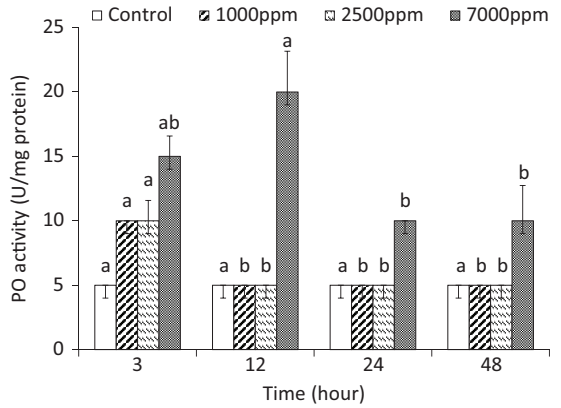

Fig. 5 Effects of different concentrations of *Ferula ovina* essential oil on phenol-oxidase activity in *Sesamia cretica* larvae. *Columns capped with the same letter are not significantly different at $p < 0.05$ according to Tukey's test.

S. cretica larvae. In this study, the THC, GRs, and PLs during treatment initially increased, then decreased with increasing concentration. Reductions in THC, PLs and GRs of insects during essential oil treatment have been recorded previously, e.g., *Cyrtacanthacris tatarica* THCs have been reported to undergo an overall reduction of 31–41% caused by Azadirachtin (Peter and Ananthakrishnan 1995). Zibaee and Bandani (2010) reported that the treatment of *Eurygaster integriceps* with *Artemisia annua* extract could reduce hemocyte total number as it circulated in the hemolymph, which might have resulted from its toxic effects on the immune cells. THC of *Ephestia kuehniella* significantly decreased by increasing *Ferula gummosa* oil concentration (Ghasemi et al. 2013). Also, the present study shows that the number of hemocytes increased with increasing exposure time. Similar studies have been reported fluctuation of hemocyte numbers in the immune challenged insects such as *Acorus calamus* essential oil was found to cause a decline in PLs and enhancement in GRs in *Spodoptera litura* at 500 and 1000 ppm 24–72 h after treatment (Sharma et al. 2008). *Xanthogaleruca luteola* Mull treated by *A. annua* THC revealed a reduction 6 and 12 h and increased 24 and 48 h after the injection (Kohan and Sendi 2013). *Spodoptera litura* treated by Azadirachtin represented an enhancement in hemocyte number 24 h after the treatment (Sharma et al. 2003). These fluctuations could be attributed to taking part of hemocytes in nodule formation after treatment. The highest numbers of nodules were observed at a concentration of 2500 ppm and 12 h after treatment. There is no efficient study on the effect of essential oils on insects. However, different results have been showed by Zibaee and Bandani (2010) that *A. annua* extract decreased nodule formation in *Eurygaster integriceps*. Our study shows the negative effect of highest concentration of *F. ovina* essential oil on phenoloxidase activity of *S. cretica*. Similar results have been showed by Zibaee and Bandani (2010) reported that increasing of concentration of *A. annua* extract has reduction effect on the POs activity in *E. integriceps*. These results are carried out to melanin deposition to complete nodule formation process. Insect defenses against external agents through two cellular and humoral pathways (Schmid 2005). Several studies indicated that entrance of an invader in insect hemocoel induced two pathways and led to phagocytosis, nodule formation, encapsulation, and synthesis of antimicrobial peptides (Beckage 2008; Borges et al. 2008). Nappi and Christensen (2005) reported humoral responses including production of proPO ascade and antibacterial peptide induced after nodule formation for killing of exogenous agents. The present study shows increasing of nodule formation followed with the enhancement of PO. According to the past study, it is anticipatable that encapsulation and antibacterial peptides will induce after nodule formation and PO, although this needs more investigations. These results are not sufficient to increase the insect immune response against pathogenic microbes due to the immune system of insects that is very wide and immune responses also vary depending on the type of invader. The results of this study can serve as an introduction to the immune system *S. cretica* used by other researchers. It is concluded that the botanical treatment used in this study can alter the immunological responses of insect species, thus providing a promising candidate for the physiological control of *S. cretica*.

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References Cited

Azambuja, P., E. S. Garcia, and N. A. Ratcliffe. 1991. Aspects of classification of Hemiptera haemocytes from six triatomine species. Mem. Inst. Oswaldo Cruz. 86: 1–10.

Beckage, N. E. 2008. Insect immunology, 348 pp. Academic Press, New York.

Beetz, S., T. K. Holthusen, J. Koolman, and T. Trenczek. 2008. Correlation of haemocyte counts with different developmental parameters during the last larval instar of the tobacco hornworm, *Manduca sexta*. Arch. Insect Biochem. Physiol. 67: 63–75.

Borges, A. R., P. N. Santos, A. F. Furtado, and R. C. Figueiredo. 2008. Phagocytosis of latex beads and bacteria by hemocytes of the triatomine bug *Rhodnius prolixus* (Hemiptera: Reduviidae). Micron 39: 486–494.

Brayner, F. A., H. R. C. Araujo, M. G. Cavalcanti, L. C. Alves, and C. A. Peixoto. 2005. Ultrastructural characterization of the haemocytes of *Culex quinquefasciatus* (Diptera: Culicidae). Micron 36: 359–367.

Cerenius, L., and K. Soderhall. 2004. The prophenol-oxidase-activating system in invertebrates. Immunological Rev. 198: 116–126.

Dularay, B., and A. M. Lackie. 1985. Haemocytic encapsulation and the prophenoloxidase activaion pathway in the locust *Schistocerca gregaria*. J. Insect. Physiol. 15: 827–834.

Franssens, V., G. Smagghe, G. Simonet, I. Claeys, B. Breugelmans, A. DeLoof, and J. Vanden Broeck. 2006. 20-Hydroxy ecdysone and juvenile hormone regulate the laminarin-induced nodulation reaction in larvae of the flesh fly, *Neobellieria bullata*. Dev. Comp. Immunol. 30: 735–740.

Ghasemi, V., A. Khoshnood Yazdi, F. Zaker Tavallaie, and J. J. Sendi. 2013. Effect of essential oils from *Callistemon viminalis* and *Ferula gummosa* on toxicity and on the haemocyte profile of *Ephestia kuehniella* (Lep.: Pyralidae). Arch. Phytopathol. Plant Prot. 47: 268–278.

Giglio, A., S. Battistella, F. F. Talarico, T. Z. Brandmayr, and P. G. Giulianini. 2008. Circulating haemocytes from larvae and adults of *Carabus* (*Chaetocarabus*) *lefebvrei* Dejean (Coleoptera: Carabidae): cell types and their role in phagocytosis after *in vivo* artificial non-self-challenge. Micron 39: 552–558.

Hoffmann, J. A. 1995. Innate immunity of insects. Curr. Opin. Immunol. 7: 4–10.

Hoffmann, J. A. 2003. The immune response of *Drosophila*. Nature 426: 33–38.

Isman, M. B. 2006. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. Ann. Rev. Entomol. 51: 45–66.

Kanost, M. R., H. Jiang, and X. Q. Yu. 2004. Innate immune responses of a lepidopteran insect, *Manduca sexta*. Immunological Rev. 198: 97–105.

Kanost, M. R., and M. J. Gorman. 2008. Phenol-oxidases in insect immunity, pp. 69–96. *In* Insect immunology. Academic Press/Elsevier, San Diego.

Khosravi, R., J. J. Sendi, A. Zibaee, and M. A. Shokrgozar. 2014. Immune reactions of the lesser mulberry pyralid, *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae) to the entomopathogenic fungus, *Beauveria bassiana* (Bals.-Criv.) Vuill and two developmental hormones. Invertebrate Surviv. J. 11: 11–21.

Kohan, R., and J. J. Sendi. 2013. Immune responses of elm leaf beetle *Xanthogaleruca luteola* Mull. (Col:Chrysomellidae) to *Beaveria bassiana* and *Artemisia annua* essential oil. SOAJ Entomol. Studi. 2: 16–25.

Leonard, C., S. Kenneth, and N. A. Ratcliffe. 1985. Studies on pro phenoloxidase and protease activity of *Blaberua craniifer* haemocytes. Insect Biochem. 15: 803–810.

Mullen, L., and G. Goldsworthy. 2003. Changes in lipophorins are related to the activation of phenol-oxidase in the haemolymph of *Locusta migratoria* in response to injection of immunogens. Insect Biochem. Mol. Biol. 33: 661–670.

Nakahara, Y., S. Shimura, C. Ueno, Y. Kanamori, K. Mita, M. Kiuchi, and M. Kamimura. 2009. Purification and characterization of silkworm haemocytes by flow cytometry. Dev. Comp. Immunol. 33: 439–448.

Nappi, A. J., and B. M. Christensen. 2005. Melanogenesis and associated cytotoxic reactions: applications to insect innate immunity. Insect Biochem. Mol. Biol. 35: 443–459.

Negahban, M., S. Moharramipour, and F. Sefidkon. 2007. Fumigant toxicity of essential oil from *Artemisia sieberi* Besser against three stored-product insects. J. Stored Prod. Res. 43: 123–128.

Peter, A. J., and T. N. Ananthakrishnan. 1995. Impact of azadirachtin on the haemolymph of *Cyrtacanthacris tatarica* L. (Acrididae, Orthoptera). J. Entomol. Res. 19: 285–290.

Ribeiro, C., and M. Brehélin. 2006. Insect haemocytes: what type of cell is that? J. Insect Physiol. 52: 417–429.

Sanjayan, K. P., T. Ravikumar, and S. Albert. 1996. Changes in the haemocyte profile of *Spilostethus hospes* (Fab.) (Heteroptera: Lygaeidae) in relation to eclosion, sex and mating. J. Biosci. 21: 781–788.

SAS Institute. 1996. SAS/STAT users guide, release 9.1. SAS Institute Inc, Cary.

Semeada, A. M. 1985. Relative susceptibility of certain maize germplasm to infestation with the greater sugar-cane borer *Sesamia cretica* Led. (Lepidoptera, Noctuidae). MS thesis, Faculty of Agriculture, Cairo University.

Semeada, A. M. 1988. Management of *Sesamia cretica* Led. population in maize fields (Lepidoptera: Noctuidae). PhD thesis, Faculty of Agriculture, Cairo University.

Seraj, A. A. 2001. Damage and assessment of losses caused on sugar-cane borer by *Sesamia cretica* Led. (Lepidoptera, Noctuidae) in Khuzestan region. J. Water Soil Sci. 5: 169–180.

Sharma, P. R., O. P. Sharma, and B. P. Saxena .2003. Effect of neem gold on haemocytes of the tobacco armyworm, *Spodoptera littura* (Fabricius) (Lepidoptera: Noctuidae). Curr Sci. 84: 690–695.

Sharma, P. R., O. P. Sharma, and B. P. Saxena. 2008. Effect of sweet flag rhizome oil (*Acorus calamus*) on hemogram and ultrastructure of haemocytes of the tobacco armyworm, *Spodoptera litura* (Lepidoptera: Noctuidae). Micron 39: 544–551.

Schmid, H. P. 2005. Evolutionary ecology of insect immune defenses. Annu. Rev. Entomol. 50: 529–551.

Strand, M. 2008. The insect cellular immune response. Insect Sci. 15: 1–14.

Zibaee, A., and A. R. Bandani. 2010. A study on the toxicity of the medicinal plant, *Artemisia annua* L. (Astracea) extracts the Sunn pest, *Eurygaster integriceps* Puton (Heteroptera: Scutelleridae). J. Plant Prot. Res. 50: 48–54.

Zibaee, A., and D. Malagoli. 2014. Immune response of *Chilo suppressalis* Walker (Lepidoptera: Crambidae) larvae to different entomopathogenic fungi. B. Entomol. Res. 104: 155–163.