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Impacts of pyriproxyfen, flufenoxuron and acetone extract of *Melia azedarach* fruits on the haemogram of the black cutworm, *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae)

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ABSTRACT

The black cutworm, *Agrotis ipsilon*, is a serious agricultural insect pest worldwide. In Egypt it has acquired resistance against most of the applied conventional pesticides. Therefore, the present study aimed to investigate the effect of pyriproxyfen, flufenoxuron, and acetone extract of *Melia azedarach* fruits on the total haemocyte count (THC), percentage of haemocyte viability and percentage of each type of haemocyte (differential haemocyte count; DHC) in *A. ipsilon* last instars. For this purpose newly molted 4th instars were treated with the LC_{50} of these compounds based on the leaf-dip method. THC was conducted with Neubauer haemocytometer. Flufenoxuron significantly increased both the THC and their viability compared to the untreated control. There are five types of haemocytes in *A. ipsilon* larvae: prohaemocytes, plasmatocytes, granulocytes, spherulocytes and oenocytoids. Both pyriproxyfen and flufenoxuron significantly increased the number of granulocytes and oenocytoids compared to the control. Also, granulocytes were significantly increased in larvae treated with acetone extract of *M. azedarach* fruits. On the contrary, this extract significantly decreased the number of spherulocytes and oenocytoids compared to the control. Plasmatocytes were significantly decreased in pyriproxyfen–treated larvae. In conclusion, flufenoxuron and acetone extract of *M. azedarach* fruits may stimulate the cellular immune system of *A. ipsilon* larvae via increasing the number of phagocytic granulocytes, in addition to increasing the haemocyte viability with flufenoxuron. On the other hand, pyriproxyfen may act as immunosuppressant to *A. ipsilon* where it suppresses the number of phagocytic plasmatocytes although this compound had no significant effect on haemocyte viability.

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Introduction

The black cutworm, *Agrotis ipsilon*, is an insect pest with great agricultural importance worldwide. In Egypt it infests cotton (*Gossypium barbadense*) seedlings and cause severe damage to many economic winter crops, for example, clover (*Trifolium alexandrinum*), wheat (*Triticum aestivum*) and bean (*Vicia faba*) (El-Kady *et al.*, 1990; Abo El-Ghar *et al.*, 1994).

The control measure of *A. ipsilon* in Egypt depends mainly on the application of conventional insecticides particularly organophosphates.

However, these chemicals cause several hazardous effects, for example, environmental pollution, negative impacts on beneficial insects, outbreak of secondary pests and development of resistance in the non-target pests (Vattikonda and Sangam, 2017). Thus, it is necessary to search for safe and effective alternative control measures with new modes of action. Among these measures are the insect growth regulators (IGRs) and insecticides of plant origin. IGRs and botanical insecticides are selective compounds with minimum effects on natural enemies and the environment; thus they can be classified as biorational insecticides (Ishaaya *et al.,* 2005; Horowitz *et al.,* 2009). Reducing the risks associated with insect pest management tactics by using selective insecticides along with resistance management to maintain their effectiveness during a prolonged period is of utmost agricultural importance (Horowitz and Ishaaya 2004).

Pyriproxyfen which belongs to a class of IGRs called juvenile hormone analogs is pyridine-based insecticide acting against several insect species (Mirhaghparast *et al.,* 2015). It exhibits reasonable field stability and high potency on agricultural insect pests (Ishaaya and Degheele, 1982). Pyriproxyfen mimics the action of juvenile hormones on a number of physiological events; however it is a potent inhibitor of embryonic development, metamorphosis, and adult formation (Ishaaya and Horowitz, 1992a, 1995; Ishaaya *et al.,* 1994). It is one of the most important components in insecticide resistance management programs in cotton fields worldwide (Ishaaya and Horowitz, 1992b; Horowitz and Ishaaya 1994; Dennehy and Williams 1997).

Flufenoxuron is a benzoylurea insecticide (BPU) belonging to a class of IGRs called chitin synthesis inhibitors (CSIs) that act on different insect orders by inhibiting chitin formation; thereby causing abnormal endocuticular deposition and abortive molting (Mulder and Gijswijt, 1973; Ishaaya and Casida, 1974; Post *et al.,* 1974). Recently the molecular mode of action of BPUs has been elucidated by Douris *et al.,* (2016) who uncovered a mutation (I1042M) in the chitin synthase 1 (*CHS1*) gene of BPU-resistant *Plutella xylostella* at the same position as the I1017F mutation reported in spider mites that confers resistance to etoxazole; an oxazoline acaricide which has been proposed to interfere with chitin biosynthesis or cuticle formation (Nauen and Smagghe 2006). The potential target of BPUs is sulfonylurea receptors in epidermal cells that mediate chitin vesicle transport (Abo El-Ghar *et al.,* 2004; Matsumura, 2010). BPUs affect generally the larval stages of insects which synthesize chitin during the molting process. Hence, the adults of predators and parasitoids are not affected. BPUs are therefore considered important components in integrated pest management (IPM) programs (Ishaaya and Degheele, 1982).

Azadirachtin, a tetranortriterpenoid, is a well-known IGR of plant origin isolated from the seeds of the Indian Neem tree, *Azadirachta indica* and the fruits of the Chinaberry tree, *Melia azedarach* (family Meliaceae) (Rembold *et al.,* 1982). It has two profound effects on insects: first it acts as an antifeedant; and second it inhibits the synthesis and release of ecdysteroids from the prothoracic gland resulting in incomplete ecdysis in immature insects and sterility in adult females (Schmutterer, 2002; Isman, 2006). Azadirachtin showed insecticidal activity to nearly 550 insect species (Debashri and Tamal, 2012; Hummel *et al.,* 2012). It seems to be selective, non-mutagenic, readily degradable, with low toxicity to non-target and beneficial organisms and causes minimal disruption to ecosystem (Sundaram, 1996, Raizada *et al.,* 2001).

In contrast to vertebrates, insects lack an adaptive (acquired) immune system; thus it is innate immune system which is classified as cellular and humeral responses. Cellular immunity is a haemocyte-mediated reaction which includes three different ways to control foreign invaders: phagocytosis, nodule formation and encapsulation (Strand and Pech, 1995; Gillespie *et al.,* 1997; Irving *et al.,* 2005). The close similarities of insect haemocytes to vertebrate blood cells enable their use as a tractable

model for immunological research (Strand, 2008). The insect haemogram and the peculiar haemocyte combination in each developmental stage are important and serve as indicators of environmental adaptability (Sharma *et al.,* 2008). Synthetic insecticides, botanical insecticides and IGRs intervene in the intermediary metabolism and immune capability of insects (Ayyad *et al.,* 2001; Pandey *et al.,* 2008; Rahimi *et al.,* 2013; Kurt and Kayiş, 2015; Haszcz, 2016). Haemocytes, via a change in cell number, are frequently used to demonstrate the cytogenetic damage induced by toxic xenobiotics (Wessel *et al.,* 2007).

Therefore, the present study aimed to investigate the total haemocyte count (THC), percentage of haemocyte viability and percentage of each type of haemocyte (differential haemocyte count; DHC) in *A. ipsilon* last instars that survived treatment of newly molted 4th instars with the LC_{50} of pyriproxyfen, flufenoxuron and acetone extract of *M. azedarach* fruits. The present study sheds the light on the importance of studying the immune response of insect to any control agent before reaching a definite evaluation concerning its success in pest control.

Materials and methods

Insects

The stock colony of *A. ipsilon* was originally obtained from the Plant Protection Research Institute, Agricultural Research Center, Ministry of Agriculture. They were maintained at 25 ± 2 °C, 70 ± 5% RH and 12:12 h (L:D) photoperiod. Larvae were fed castor bean leaves (*Ricinus communis*) and moths were fed 15% sucrose solution.

Pyriproxyfen, flufenoxuron and acetone extract of M. azedarach fruits

Pyriproxyfen and flufenoxuron (97% technical grade) were supplied by Chemos GmbH & Co. KG, Germany. *M. azedarach* fruits were collected from Salhiya, Sharkia governorate. They were dried at room temperature for three weeks and then extracted by acetone as a solvent. In brief, 150 g of the dried sample were soaked in acetone (1: 4) (w/v) in a tightly sealed flask (2 l) for one week. The solution was stirred using a magnetic stirrer and the crude extract was extracted using Soxhlet extractor. The extract was evaporated in a rotary vacuum apparatus. The collected stock extract was lyophilized and stored at 4°C until use.

Bioassay

Pyriproxyfen and flufenoxuron were dissolved in distilled water, while acetone extract of *M. azedarach* fruits was dissolved in acetone. Six aqueous concentrations of pyriproxyfen (25, 50, 100, 200, 300 & 400 mg/l), flufenoxuron (1.0, 2.5, 5.0, 7.5, 10.0 & 12.5 mg/l) and acetone extract of *M. azedarach* fruits (5×10^3, 10×10^3, 25×10^3, 50×10^3, 75×10^3 & 100×10^3 mg/l) were prepared. Castor bean leaves (*R. communis*) were dipped for 10 sec in each concentration and then air-dried at room temperature. Discs (5 cm in diameter) of treated leaves were offered for 48 h to newly molted 4th instars of *A. ipsilon* (35.0 ± 1.5 mg) (El-Kady *et al.,* 1990; Abo El-Ghar *et al.,* 1994) that were starved first for 2 h. They were then fed fresh, untreated leaves until pupation. Larvae that were fed castor bean leaves dipped in distilled water were used as the control for both pyriproxyfen and flufenoxuron, while those fed castor bean leaves dipped in acetone were used as the control for *M. azedarach* fruit extract. Each concentration was replicated four times (25 larvae/replicate) with different insect batches at different times. The percentage of mortality was determined and corrected by Abbott's formula (Abbott, 1925). The LC_{50} values were estimated by probit analysis (Finney, 1971).

Collection of haemolymph sample

A. ipsilon late last instars were first treated with the LC_{50} of pyriproxyfen (150 mg/l) and flufenoxuron (5 mg/l) and acetone extract of *M. azedarach* fruits (60×10^3 mg/l) as described above. One of the prolegs was then removed by a sterile fine scissor and the haemolymph was collected in cold tubes coated with crystals of phenylthiourea to prevent melanization. Each treatment was replicated four times (10 larvae/replicate) with different insect batches at different times. A parallel control was run.

Total haemocyte count and viability

Haemolymph from pooled sample (10 late last instars), treated first with the LC_{50} of pyriproxyfen, flufenoxuron and acetone extract of *M. azedarach* fruits, was taken up directly by Thoma white-blood cell diluting pipette to the 0.1 mark. A parallel control of untreated insects was run. Diluting solution [trypan blue (0.4%) in Tauber-Yeager's solution, pH 6.7- 6.8] (Tauber and Yeager, 1936) was taken up to 11th mark on the pipette. The mixture was hand shaken for three min and then dispensed to both chambers of Neubauer haemocytometer (DHC-No1). After about one min, the total number of blood cells in 64 squares of the four corners was recognized as viable and dead cells. Dead haemocytes were stained with trypan blue; whereas viable cells were not (Horohov and Dunn, 1982). Cells within the lines and at the left and bottom boundary lines of the four corner squares were counted. The total number of cells was multiplied by a factor of 250 to give the number of cells/mm^3 of haemolymph. This procedure was replicated 10 times for each treatment. The number of circulating haemocytes per cubic millimeter was calculated by the formula suggested by Jones (1962). The percentage of viability was calculated according to the equation given by Horohov and Dunn (1982).

Different types of haemocytes

One drop of fresh haemolymph of larvae treated first with the LC_{50} of pyriproxyfen, flufenoxuron and acetone extract of *M. azedarach* fruits was smeared on a clean glass slide, air-dried and then fixed for 2 min with ethanol. A parallel control of untreated insects was conducted. Blood films were then stained with Giemsa stain freshly prepared by mixing stock Giemsa with distilled water (1: 10) (v/v) for 15 min. After a brief wash in distilled water, slides were dipped for about 30 sec in tap water. Blood smears were air-dried for 24 h, mounted in Canada balsam, and then examined under a light microscope (100 × magnifications; Nikon, Nippon Kogaku, Tokyo, Japan). Different types of haemocytes were classified according to methods described by Jones (1964)

Statistical analysis

Data obtained were given as mean ± standard error (SE). They were analyzed by Student's *t*-test between treated and control group. Significance level was set at $P < 0.05$.

Results and discussion

Flufenoxuron significantly ($P < 0.05$) increased both the THC and their viability of *A. ipsilon* last instars compared to the untreated control (Table 1). Similarly, the CSIs, flufenoxuron and novaluron, significantly increased the THC of *Spodoptera littoralis* and *Pectinophora gossypiella* last instars (Abdel-Aal, 2002; Ghoneim *et al.,* 2015). The promoting effect of flufenoxuron in the present study may be a response to detoxification by *A. ipsilon* larvae. Patton (1961) implicated haemocytes of insects in the detoxification of toxic xenobiotics. In disagreement with our results, several CSIs decreased the THC of other lepidopteran species (Abdel-Aziz and Awad, 2010; Abdel-Aal *et al.,* 2011; Zhu *et al.,* 2012; Ghoneim *et al.,* 2015).

***Table 1**.* Total haemocyte count (THC) and percentage of haemocyte viability of *A. ipsilon* last instars treated with the LC_{50} of pyriproxyfen, flufenoxuron and acetone extract of *M. azedarach* fruit extract

Treatment	THC ± SE (cell /mm^3) × 10^3	Haemocyte viability ± SE (%)
Control	30.75 ± 0.34	71.28 ± 1.12
Pyriproxyfen	27.38 ± 0.23	79.51 ± 1.36
Flufenoxuron	44.75 ± 0.32*	88.13 ± 1.23*
M. azedarch	35.83 ± 0.35	78.52 ± 1.58

*Significant at $P < 0.05$ compared to the untreated control in Student's *t*-test between treated and control group. Values are given as mean ± SE.

Table 2. Differential haemocyte count (DHC) of *A. ipsilo*n last instars treated with the LC_{50} of pyriproxyfen, flufenoxuron and acetone extract of *M. azedarach* fruit extract

Treatment	DHC ± SE (%)				
	Prohaemocytes	Plasmatocytes	Granulocytes	Spherulocytes	Oenocytoids
Control	11.70 ± 0.40	61.40 ± 0.67	8.20 ± 0.39	14.10 ± 0.38	4.60 ± 0.31
Pyriproxyfen	14.20 ± 0.42	40.20 ± 0.59*	26.60 ± 0.79*	12.20 ± 0.47	6.80 ± 0.42*
Flufenoxuron	16.20 ± 0.36	45.70 ± 0.42	11.60 ± 0.48*	15.10 ± 0.98	11.40 ± 0.37*
M. azedarch	7.30 ± 0.30	67.70 ± 1.03	18.30 ± 1.35*	4.50 ± 0.40*	2.20 ± 0.25*

*Significant at $P < 0.05$ compared to the untreated control in Student's *t*-test between treated and control group. Values are given as mean ± SE.

Abdel-Aal (2002) reported that pyriproxyfen had no effect on the THC of *S. littoralis* larvae. This result is in agreement with the result obtained in the current study. Mirhaghparast et al. (2015) found that the THC of *Chilo suppressalis* 4th instars increased after 1–3 h of treatment with 72 µg/ml of pyriproxyfen, whereas the same dose decreased the THC after 6–24 h. Rahimi et al. (2013) reported an increase in the THC of *Ephestia kuehniella* after 24 h of treatment with pyriproxyfen.

Ayyad et al. (2001) recorded a significant increase in the THC of *Parasarcophaga surcoufi* last instars after 40 h of injection with the LD_{30} and LD_{70} of azadirachtin by about 2.58 and 4.58 folds of the control, respectively. On the contrary, a significant decrease in the THC after treatment with the LD_{50} of azadirachtin was recoded in *Spodoptera litura* (Ayyangar and Rao, 1990; Sharma *et al.,* 2003), *Helicoverpa armigera* (Padmaja and Rao, 2000), *Danaus chrysippus* (Pandey *et al.,* 2008), and *S. littoralis* (Shaurub *et al.,* 2014). These results disagree with our results where acetone extract of *M. azedarach* fruits insignificantly changed the THC in *A. ipsilon* larvae.

We identified five types of haemocytes in *A. ipsilon* larvae: prohaemocytes, plasmatocytes, granulocytes, spherulocytes, and oenocytoids. These results are in agreement with the results of

several authors for several lepidopteran species (Abdel-Aal, 2002; Lavine and Strand, 2002; Kurt and Kayiş, 2015; Ghoneim *et al.,* 2015; Haszcz, 2016). However, Manogem et al. (2016) identified eight distinct classes of haemocytes in *Spodoptera mauritia* larvae: plasmatocytes, granulocytes, prohaemocytes, spherulocytes, adipohaemocytes, oenocytoids, vermicytes and podocytes. Ghoneim et al. (2017) reported six types of haemocytes in *P. gossypiella* larvae: prohaemocytes, plasmatocytes, granulocytes, spherulocytes, oenocytoids and adipohaemocytes.

Both pyriproxyfen and flufenoxuron significantly ($P < 0.05$) increased the number of granulocytes and oenocytoids in *A. ipsilon* larvae compared to the untreated control. Plasmatocytes were significantly ($P < 0.05$) decreased in pyriproxyfen–treated larvae (Table 2). Abdel-Aal (2002) reported a significant increase in the number of prohaemocytes and spherulocytes in *S. littoralis* last instars treated with the LC_{50} of pyriproxyfen. He also reported that flufenoxuron caused a significant increase in spherulocytes and a significant decrease in granulocytes. Ghoneim et al. (2015) found that the effect of the LC_{50} of the CSIs, novaluron and cyromazine, on the DHC in the last instars of *S. litura* was significant during the first or second half of the instar.

Granulocytes in *A. ipsilon* larvae were significantly ($P < 0.05$) increased due to treatment with acetone extract of *M. azedarach* fruits. On the contrary, this extract significantly ($P < 0.05$) decreased the number of spherulocytes and oenocytoids (Table 2). Haszcz (2016) reported that Neem essential oil inhibited plasmatocyte production in *Galleria mellonella*. Ayyad et al. (2001) found an increase in the oenocytoids of *P. surcoufi* last instars after 40 h of injection with the LD_{30} of azadirachtin and a decrease in the plasmatocytes and granulocytes after 10 h of injection with the same dose.

The alteration in the number of some haemocyte types may be due to transformation of some haemocyte types into other types for the phagocytic function, or combating against biotic and abiotic foreign invaders and apoptotic bodies (Hernandez *et al.,* 1999; De Silva *et al.,* 2000). Prohaemocytes are progenitor stem cells which can differentiate into other haemocyte types (Yamashita and Iwabuchi, 2001; Lavine and Strand, 2002).

In conclusion, flufenoxuron may stimulate the cellular immune system of *A. ipsilon* larvae via increasing the number of viable haemocytes and granulocytes. The latter type of haemocytes is implicated in phagocytosis (Tojo *et al.,* 2000; Nardi *et al.,* 2001; Costa *et al.,* 2005). This conclusion is applied to acetone extract of *M. azedarach* fruits where this extract increases also the number of granulocytes. Therefore, flufenoxuron and acetone extract of *M. azedarach* fruits are not recommended as control agents to *A. ipsilon*. On the other hand, although pyriproxyfen, does not affect the viability of haemocytes of *A. ipsilon*, this pesticide significantly decreases the number of plasmatocytes which are known to play also a significant role in phagocytosis (Tojo *et al.,* 2000; Ling and Yu, 2006). Thus, pyriproxyfen may act as immunosuppressant to *A. ipsilon* and consequently it is recommended as a control agent to this pest.

Conflict of interest

All the authors declare that there is no conflict of interest.

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