ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Effects of Novaluron and Cyromazine, chitin synthesis inhibitors, on the larval hemogram of *Spodoptera littoralis* (Boisd.)(Lepidoptera: Noctuidae).

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Manuscript Info

Manuscript History:

Received: 22 November 2014
Final Accepted: 11 December 2014
Published Online: January 2015

Key words:

cytopathology, differential hemocyte count, hemocyte, granulocyte, oenocytoid, plasmatocytes, prohemocyte, spherulocytes, total hemocyte count

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Abstract

The present study was carried out aiming to investigate the effect of two chitin synthesis inhibitors (CSIs), Novaluron and Cyromazine, on the larval hemogram of *S. littoralis*. Five main types of circulating hemocytes, *viz*., Prohemocytes (PRs), Plasmatocytes (PLs), Granulocytes (GRs), Spherulocytes (SPs) and Oenocytoides (OEs), had been identified in last instar (6^{th}) larvae. The most important diagnostic characteristics of each type were described. After treatment of the newly moulted penultimate instar larvae with LC_{50} of Novaluron or Cyromazine, the successfully moulted last instar larvae were used to investigate the most important hematological responses. Novaluron remarkably enhanced the production of hemocytes at two limits of larval instar. On the other hand, Cyromazine exerted an inhibitory action on THC during the majority of larval instar, with few exceptions. Novaluron treatment resulted in slightly increasing PRs and PLs during the first half of instar but slightly decreasing during the second half. GRs were prohibited but SPs had been enhanced by Novaluron along most larval duration, with few exceptions. Cyromazine exerted a prominent prohibiting action on PRs and GRs but remarkably stimulated PLs along the larval life. Cyromazine exhibited contradictory effects on SPs, depending on the larval age. During the second half of larval instar, OEs were enhanced by both CSIs. The present CSIs exhibited some destructive cytopathological effects on all hemocyte types, morphologically and intercellulary, except Cyromazine against OEs.

INTRODUCTION

The Egyptian cotton leaf worm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) is distributed throughout the world but it is native to Africa (Shonouda and Osmam, 2000). It is a serious or major pest of cultivated crops primarily in tropical and subtropical regions, in Africa, Southern Europe, Middle East and Asia Minor (Brown and Dewhurst, 1975) and the Mediterranean area (Hosny et al., 1986; Bayoumi et al. 1998, Salama et. al., 1990; Azab et al., 2001; El-Aswad et al. 2003; Pineda et al., 2007). It is one of the most destructive pests of several crops such as cotton, *Gossypium hirsutum*, peanut, *Arachis hypogaea*, soybean, *Glycine max* and vegetables (El-Khawas and Abd El-Gawad, 2002). In addition to cotton, it infests more than 29 other crops and vegetables of economic importance (Magd El-din and El-Gengaihi, 2000). In **Egypt**, *S. littoralis* is a major polyphagous key pest attacking several economically important Egyptian crops (Raslan, 2002; El-Aswad et al., 2003). In addition to its direct damage reducing photosynthetic area, its larval presence, feeding marks and excrement residues reduce marketability of vegetables and ornamentals (Pluschkell et al., 1998). Natural defoliations by *S. littoralis* larvae (20-

70%) can result in reductions in yield < or = 50% (Russell et. al., 1993). When large numbers of the pest are present complete crop loss is possible (Khalil, 1988; El-Khawas and Abd El-Gawad, 2002; Korrat et. al., 2012).

Although some farmers in Egypt laboriously and pick the egg batches to control *S. littoralis* population, several synthetic insecticides have been used. Over the past three decades, the intensive use of broad-spectrum insecticides against *S. littoralis* has led to the development of resistance against many registered pesticides, detrimental effects on the natural enemies, pollinators and all other non-target insects, and serious toxicological problems to humans and the environment (Miles and Lysandrou, 2002; Abo-El Ghar et al., 2005; Aydin and Gurkan, 2006; Davies et al., 2007; Costa et al., 2008; Relyea, 2009; Mosallanejad and Smagghe, 2009). Therefore, much safer insecticides need to be developed in order to decrease the burden on the environment by using less chemical in comparison to conventional insecticides (Dahi et al., 2009; Hussain, 2012).

At present, using insect growth regulators (IGRs) is considered as the possible alternative way of synthetic insecticides for controlling this pest (Raslan, 2002). IGRs are bio-rational compounds that are species-specific and highly selective in action (Staal, 1975) and act by disrupting the normal development of several insect species (Henrick et al., 1973). The effects of IGRs, more precisely, the chitin synthesis inhibitors (CSIs) which interfere with chitin biosynthesis have been worked out on a number of insect species. The results revealed that there is a wide range of responses and susceptibility with respect to different insect species against a CSI (Hajjar and Casida, 1978; Gijswijt et al., 1979). CSIs interfere with chitin biosynthesis in insects preventing the moulting process or producing an imperfect cuticle (Hammock and Quistad, 1981). Thus, they are effective suppressors of development for the entire life cycle of insects (Verloop and Ferrell, 1977). These compounds, also, affect the hormonal balance resulting in physiological disturbances (DeLoach et al., 1981). Novaluron (Rimon, Chemtura Corporation, Middlebury, CT) is a relatively new benzoylphenyl urea compound with good activity against the Colorado potato beetle (Cutler et al., 2005a,b, 2007; Alyokhin et al., 2009) and low mammalian toxicity (Barazani, 2001; Ishaaya and Horowitz, 2002). Its residues tend to dissipate with half-life of 2.08 days and the safe use of it on possibly various crops in Egypt was established (Malhat et al., 2014). Cyromazine (Trigard) is an IGR commonly used to control immature houseflies on poultry farms (Miller and Corley, 1980; Awad and Mulla, 1984). The effects of cyromazine were closer to that of CSIs rather than that of juvenile hormone analogues (Darriet et al., 2008). Cyromazine was used in the pest control because it is harmless to parasitoids (Beitia et al., 1991; Schuster, 1994).

The insect pests may be controlled by disturbing their physiological activities, i.e. feeding, molting, reproductive and immune systems (Pandey et al., 2012**)**. Insects lack an acquired immune system like of the higher animals but have a well-developed innate response. The cellular defense of insects refers to haemocyte-mediated immune responses like phagocytosis, nodulation and encapsulation (Schmidt et al., 2001; Lavine and Strand, 2002). The primary functions of the insect haemocytes are: coagulation, phagocytosis, encapsulation, detoxification and storage and distribution of nutritive materials (for reviews, see: Garcia and Rosales, 2002; Zhou et al., 2004; Ling and Yu, 2006; Ribeiro and Brehelin, 2006; Siddiqui and Al-Khalifa, 2012a). Knowledge of normal haemocytes of an insect is necessary to physiologists, toxicologists and biochemists, as alterations in structure, types and number of cells reflects changes in physiological and biochemical processes (Qamar and Jamal, 2009; Berger and Jurčová, 2012). Among the environmental factors affecting insect hemocytes, morphologically and functionally, are insecticides (Zibaee, 2011). The present work was carried out aiming to investigate the influences of two CSIs, viz., Novaluron and Cyromazine, on the larval hemogram of *S. littoralis*.

MATERIALS AND METHODS:

1. Experimental insect.

A sample of *S. littoralis* pupae was kindly obtained from the culture of susceptible strain maintained for several generations in Plant Protection Research Institute, Agricultural Research Center, Doqqi, Giza, Egypt. In laboratory of Entomology, Faculty of Science, Al-Azhar University, Cairo, a culture was reared under laboratory controlled conditions (27 ± 2^{o}C, 65 ± 5% R.H., photoperiod 14 h L and 10 h D). Rearing procedure was carried out according to Ghoneim (1985) and improved by Bakr et al. (2010). Egg patches were kept in Petri dishes until hatching. The hatched larvae were transferred into glass containers containing a layer of dry saw dust and tightly covered with muslin cloth secured with rubber bands. Larvae were provided daily with fresh castor bean leaves *Ricinus communis*. The resulting pupae were then collected and placed in clean jars provided with a layer of moistened saw dust. All jars had been kept in suitable cages provided with branches of fresh Tafla plant, *Nerium oleader*, as oviposition sites. The emerged adults were provided with 10% honey solution on a cotton wick as a food source. Moths were allowed to lay eggs on branches, the egg patches were collected daily and transferred into Petri dishes for another generation.

2. Tested compounds and larval treatments.

The CSIs used in the present study are: Novaluron [1-[chloro-4-(1,1,2-trifluoromethoxyethoxy) phenyl] -3- (2,6-difluorobenzoyl) urea] and Cyromazine [N-cyclopropyl-1,3,5-triazine-2,4,6-triamine] were supplied by Sigma-Aldrich Chemicals (https://www.sigmaaldrich.com).

In a preliminary experiment, the newly moulted penultimate instar larvae were treated with a concentration range 10.0-0.0001 ppm of Novaluron, and 200.0-0.001 ppm of Cyromazine. LC_{50} values were calculated in 2.71 and 74.44 ppm of these CSIs, respectively. After treatment of these larvae with LC_{50} of each compound, the successfully moulted last instar larvae were used to investigate the effects of these CSIs on some of the most important hematological parameters.

3. Haematological characterization.

3.1. Collection of haemolymph.

For conducting the hematological investigation, haemolymph was collected from the treated and control 6th (last) instar larvae (of different ages: 0-, 2-, 4-, and 6-day old). The haemolymph was obtained by amputation of one or two prothoracic legs, before coxa of the larva using fine scissors. Gentle pressure was done on the thorax for obtaining haemolymph drops by non-heparinized capillary tube. Three replicates were used and the haemolymph from two individuals was never mixed.

3.2. Total haemocyte count.

The haemolymph was collected into thoma-white blood cell diluting pipette to the mark (0.5). Diluting solution (Na Cl 4.65 gm, K Cl 0.15 gm, $CaCl_2$ 0.11 gm, Crystal violet 0.05 gm and acetic acid 1.25 ml / liter distilled water) was taken up to the mark (11) on the pipette (dilution is 20 times). The first three drops were discharged to avoid errors. The mixture was dispended to the chamber of counting slide. After three minutes, the total numbers of cells recognized in 64 squares of the four corners were counted. If the cells clumped or uneven distributed, the preparation was discarded. The number of haemocytes per cubic millimeter was calculated according to the formula of Jones **(1962)** as follows:

$$\frac{\text{Number of haemocyte counted per champer X dilution X depth factor}}{\text{Number of 1 mm squares counted}}$$

Where the depth factor is usually 10.

3.3. Differential haemocyte counts.

Stained haemolymph preparations were carried out, according to Arnold and Hinks **(**1979**).** The haemolymph was smeared on clean glass slides, allowed to dry for 1 minute, and fixed for 2 minutes with drops of absolute methyl alcohol. Fixed cells were stained with Giemsa's solution (diluted 1 : 20 in distilled water) for 20 minutes, washed several times with tap water, and dipped in distilled water. The stained smears were air-dried and mounted in DPX with slip cover. The haemocytes were viewed under light microscope at a magnification 10 X 40 = 400 and 100 cells per slide were examined. The cell shape, cytoplasmic ratio, cytoplasmic inclusions and shape of nucleus were used for classification of haemocytes using the classification scheme of Brehelin and Zachary (1986). The percentages of haemocyte types were calculated by the formula:

$$\frac{\text{Number of each haemocyte type}}{\text{Total number of haemocytes examined}} \text{ X } 100$$

3.4. Haemocyte deformations.

For recording of the haemocyte deformities caused by CSIs, photomicrographs were obtained by using a light microscope provided with a camera at a magnification 10 X 40 = 400.

4. Statistical analysis of data:

Data obtained were analyzed by the Student's *t*-distribution, and refined by Bessel correction (Moroney, 1956) for the test significance of difference between means.

RESULTS:

1. Identification and description of circulating hemocytes in larvae of *S. littoralis*.

Depending on the cell shape, cytoplasmic ratio, cytoplasmic inclusions and shape of nucleus, the free hemocytes in last (6^{th}) instar larvae of *S. littoralis*, in the present study, had been identified into five main types, *viz*., Prohemocytes (PRs), Plasmatocytes (PLs), Granulocytes (GRs), Spherulocytes (SPs) and Oenocytoids (OEs). It is interesting to give the most important diagnostic characteristics of each type as follows.

PRs can be described as variable in size (3-7 µm wide and 6-8 µm long). They were observed as nearly ovoid, round or spherical in shape. Abundant cytoplasm was deeply stained and contained few organelles. Large nucleus was centrally located and occupied most of the cell volume (see Plate 1).

PLs were found polymorphic and appeared as spindle-, oval- or spherical-shaped cells. When spherical, they were 13-26 µm in diameter. When oval, they were 26-34 µm long and 15-30 µm wide. Cytoplasm was basophilic (faintly stained) and rich in organelles such as a moderate amount of rough endoplasmic reticulum, scattered chromatin masses and several tapering projections. Large nucleus (occupying 40-50% of the cell volume) was observed as round, elongate or spherical and centric or eccentric in position with a distinct nucleolus. Many PLs had been seen in haemolymph of last instar larvae of S. littoralis as dense cells with pale nuclei and punctate chromatin granules. They were seen singles, in pairs or occasionally in small clusters of 4-8 cells (see Plate 2).

GRs appeared as spherical-, oval- or spindle-shaped cells of variable size (8-17 µm long and 9-23 µm wide). Cytoplasm was basophilic (deeply stained) and contained an enormous number of spherical, ovoid, elongate or irregularly polygonal acidophilic granules. Nucleus was spherical to ovoid and might be centric or eccentric occupying 58.3-66.6% of the cell volume. Nucleus contained, also, scattered chromatin masses and nucleolus. Some GRs had surface with projections mainly phillopodial (see Plate 3).

SPs were distinguished as basophilic or acidophilic cells of variable size (8-20 µm wide and 7-24 µm long) and round or oval in shape. They were characterized by several cytoplasmic inclusions and intracytoplasmic spherules taking up almost all the cytoplasm. Nucleus appeared small, centric or eccentric in position, mostly deformed by the spherules. The cellular surface was homogenous but exhibited cytoplasmic protrusion corresponding to the spherules (see Plate 4).

OEs were seen as the largest hemocyte type, spherical (22-35.5 µm in diameter) or oval (18.7-25 µm long and 26.5-35.6 µm wide) in shape. When stained with Geimsa stain, cytoplasm was observed homogenous basophilic. It contained darkly stained small eccentric nucleus and scarce organelles including round acidophilic granules (see Plate 5).

2. Effects of CSIs on total hemocyte count (THC).

2.1. Effect of Novaluron.

According to data distributed in Table (1), Novaluron remarkably promoted the production of hemocytes at the two limits of last larval instar since THC was pronouncedly increased with 13.54 and 24.18% in haemolymph of 0- and 6-day old larvae, respectively. In contrast, production of hemocytes was prohibited during the middle duration of instar since THC significantly was reduced in 22.18 and 21.54%, in 2- and 4-day old larvae, respectively.

2.2. Effect of Cyromazine.

After treatment of penultimate instar larvae with LC_{50} of Cyromazine, THC data in haemolymph of last instar larvae were assorted in Table (1). Depending on these data, Cyromazine exerted an extended inhibitory action on the hemocyte production during the majority of life because THC was drastically descended (5.08, 24.55 and 6.67% reductions in 0-, 2- and 4-day old larvae, respectively). A reverse effect was exhibited on 6-day old larvae since the hemocyte production was stimulated as obviously shown in increasing THC (14268.47±27.26 compared to 11650.00±45.83 cell/mm^3 in control larvae).

3. Effects of CSIs on differential hemocyte count (DHC).

3.1. Effect of Novaluron.

Data listed in Table (2) clearly reveal that Novaluron exhibited diverse effects on DHC, depending on the hemocyte type and larval age. With regard to PRs, slightly increasing population had been determined in haemolymph during the first half of instar (12.97 and 31.92% increments in 0- and 2-day old larvae, respectively) while slightly decreasing population was recorded during the second half (8.36 and 34.54% reductions in 4- and 6-day old larvae, respectively). A similar trend of effect was detected for PLs, viz., increasing count was estimated during first half of the instar (25.82 and 4.58% increments in 0- and 2-day old larvae, respectively) but regressed count was measured during the second half (25.00 and 26.20% reductions in 4- and 6-day old larvae, respectively). According to data arranged in the same table, production of GRs was slightly or remarkably prohibited along most larval duration (18.56, 48.79 and 63.01% reductions in 0-, 2- and 4-day old larvae, respectively) but insignificantly promoted at the end of instar (6.98% increment). Production of SPs had been affected in a reverse trend, i.e., their population was drastically suppressed only at the beginning of instar (26.54% reduction) but insignificantly or pronouncedly enhanced along most larval duration (8.82, 66.31 and 48.28% increments in 2-, 4- and 6-day old larvae, respectively). In respect of OEs, production of the hemocyte population suffered a serious inhibitory effect of Novaluron only in the newly moulted last instar larvae (24.81% reduction) but no effect was exhibited on 2-day old larvae. Moreover, the population production was enhanced along the second half of life (33.00 and 24.81% increments in 4- and 6-day old larvae, respectively).

3.2. Effect of Cyromazine.

As exiguously shown by data of Table (3), Cyromazine exerted a prominent prohibiting action on the production of PRs since their count was slightly or tremendously dropped in haemolymph throughout the larval life (25.75, 45.43, 37.50 and 34.54% reductions in 0-, 2-, 4- and 6-day old larvae, respectively). Depending on data of the same table, a reverse effect of Cyromazine was exhibited on the production of PLs because obviously increasing count had been determined (42.75, 19.07, 11.36 and 33.57% increments in 0-, 2-, 4- and 6-day old larvae, respectively). Regarding the GRs, Cyromazine exhibited a prevalent inhibitory effect on the population production since their recorded count was unexceptionally decreased (39.50, 58.52, 71.75 and 72.09% reductions in 0-, 2-, 4- and 6-day old larvae, respectively). On the other hand, Cyromazine exhibited a contradictory effect on the population production of SPs, depending on the larval age. According to data of the previously mentioned table, SPs count was slightly decreased in haemolymph at the two limits of larval life (26.54 and 17.24% reductions in 0- and 6-day old larvae, respectively) but insignificantly or elaborately increased during the middle duration of instar (8.82 and 27.34% increments in 2- and 4-day old larvae, respectively). With an exception of unaffected OEs population in haemolymph of 2-day old larvae, Cyromazine considerably promoted the production of such hemocytes in larvae of other ages (24.81, 33.00 and 24.81% increments in 0-, 4- and 6-day old larvae, respectively).

4. Affected qualitative hemocyte profile by CSIs.

To shed some light on the cytopathological effects of CSIs on PRs, photomicrographs presented in Plates 1 and 6 clearly show some morphological deformities and affected intracellular constituents. Novaluron and Cyromazine treatments resulted in darkly stained PRs with destroyed membranes and extruded cytoplasmic contents.

With regard to PLs, Novaluron treatment led to some morphological disorders such as cell membrane rupture and hemocyte microaggregation. Also, a number of vacuoles and extruded cytoplasmic contents had been observed (Plate 2). Cyromazine caused cell membrane rupture, cytoplasm lysis and cytoplasmic extrusion (Plate 7). In connection with GRs, photomicrographs obviously demonstrate some cells with destroyed cell membrane, lysed and vacuolated cytoplasm as responses to the disruptive effects of both Novaluron and Cyromazine (Plates 3 and 8). As easily seen in Plates (4 and 9), SPs had been seriously affected by Novaluron and Cyromazine since the plasma membrane of some cells was destroyed and vacuoles were formed in cytoplasm (appeared in continuous or separated vacuoles, in case of Cyromazine). Only Novaluron could affect OEs as evidently demonstrated in Plate (5). As easily seen, some OEs appeared with degenerated darkly stained nucleus and destroyed cell membrane (Plate 5B) as well as other appeared with lysed cytoplasm and granulated nucleus (Plate 5C).

Table 1: THC (cell/mm^3) in last instar larvae of *S. littoralis* as affected by LC_{50} values of Novaluron and Cyromazine.

CSI		Larval age			
		0-day old	2-day old	4-day old	6-day old
Novaluron	mean±SD	11183.33±60.07 d	7133.33±37.65 d	7650.00±19.64 d	14466.67±33.82 d
	Change (%)	+13.54	-22.18	-21.54	+24.18
Cyromazine	mean±SD	9350.00±59.39 c	6916.67±18.29 d	9100.00±48.11 d	14268.47±27.26
	Change (%)	-5.08	-24.55	-6.67	+22.84
Control (mean±SD)		09850.00±27.64	9166.67±50.08	9750.24±55.83	11650.00±45.83

Mean ± SD followed with the same letter (a): insignificantly different (P >0.01), (b): significantly different (P<0.05), (c): highly significantly different (P<0.01), (d): very highly significantly different (P<0.001).

Table 2: DHC (%) in last instar larvae of *S. littoralis* as affected by LC_{50} of Novaluron.

Haemocyte type		Larval age			
		0-day old	2-day old	4-day old	6-day old
PRs	mean ± SD	11.67±1.12 a	9.67±1.53 a	7.33±0.44 a	6.33±1.67 a
	Change (%)	+12.97	+31.92	-8.38	-34.54
	Control (mean ± SD)	10.33±2.08	7.33±1.53	8.00±1.00	9.67±1.53
PLs	mean ± SD	52.00±3.61 b	45.67±2.08 a	33.00±4.00 b	33.67±2.52 c
	Change (%)	+25.82	+4.58	-25.00	-26.28
	Control (mean ± SD)	41.33±2.52	43.67±1.53	44.00±1.73	45.67±2.08
GRs	mean ± SD	11.67±2.52 a	7.00±2.00 b	5.67±1.53 c	15.33±2.08 a
	Change (%)	-18.56	-48.79	-63.01	+6.98
	Control (mean ± SD)	14.33±1.53	13.67±1.53	15.33±2.08	14.33±1.16
SPs	mean ± SD	24.00±2.00 b	37.00±1.00 a	52.67±3.22 c	43.00±3.61 c
	Change (%)	-26.54	+8.82	+66.31	+48.28
	Control (mean ± SD)	32.67±3.06	34.00±2.00	31.67±4.04	29.00±1.73
OEs	mean ± SD	1.00±0.01 d	1.33±0.08 a	1.33±0.08 c	1.66±0.01 d
	Change (%)	-24.81	0.00	+33.00	+24.81
	Control (mean ± SD)	1.33±0.05	1.33±0.05	1.00±0.01	1.33±0.05

a, b, c, d: See footnote of Table (1). PRs: Prohemocytes, PLs: Plasmatocytes, GRs: Granulocytes, SPs: Spherulocytes, OEs: Oenocytoides.

Table 3: DHC (%) in last instar larvae of *S. littoralis* as affected by LC_{50} of Cyromazine.

Haemocyte type		Larval age			
		0-day old	2-day old	4-day old	6-day old
PRs	mean ± SD	7.67±1.35 a	4.00±1.00 b	5.00±0.73 b	6.33±0.53 b
	Change (%)	-25.75	-45.43	-37.50	-34.54
	Control (mean ± SD)	10.33±2.08	7.33±1.53	8.00±1.00	9.67±1.53
PLs	mean ± SD	59.00±6.25 b	52.00±3.61 b	49.00±3.00 a	61.00±4.00 c
	Change (%)	+42.75	+19.07	+11.36	+33.57
	Control (mean ± SD)	41.33±2.52	43.67±1.53	44.00±1.73	45.67±2.08
GRs	mean ± SD	8.67±0.36 b	5.67±0.75 c	4.33±0.09 c	7.00±0.92 c
	Change (%)	-39.50	-58.52	-71.75	-72.09
	Control (mean ± SD)	14.33±1.53	13.67±1.53	15.33±2.08	14.33±1.16
SPs	mean ± SD	24.00±7.21 a	37.00±4.00 a	40.33±2.89 b	24.00±3.50 a
	Change (%)	-26.54	+8.82	+27.34	-17.24
	Control (mean ± SD)	32.67±3.06	34.00±2.00	31.67±4.04	29.00±1.73
OEs	mean ± SD	1.66±0.09 c	1.33±0.05 a	1.33±0.05 d	1.66±0.07 c
	Change (%)	+24.81	0.00	+33.00	+24.81
	Control (mean ± SD)	1.33±0.05	1.33±0.05	1.00±0.01	1.33±0.05

a, b, c, d: See footnote of Table (1). PRs, PLs, GRs, SPs, OEs: See footnote of Table (2).

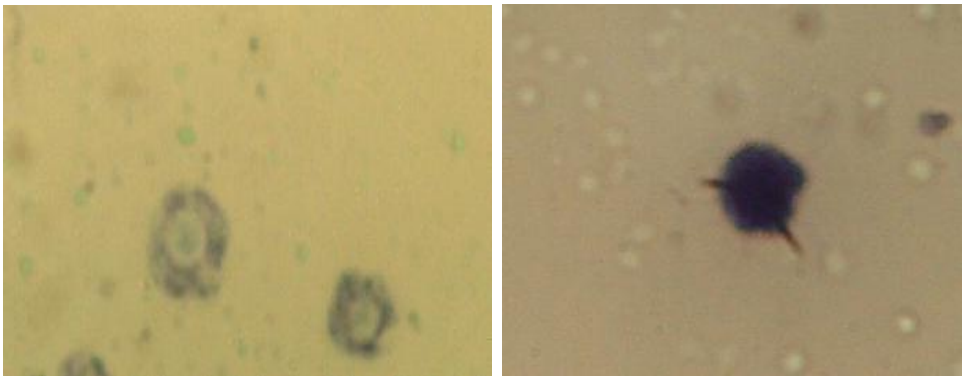

Plate 1: Photomicrographs of PRs of *S. littoralis* last instar larvae (Geimsa stain, 400x). (A): Normal cell. (B): Hemocyte deformation by LC_{50} of Novaluron, darkly stained with destroyed membrane and extruded cytoplasmic contents.

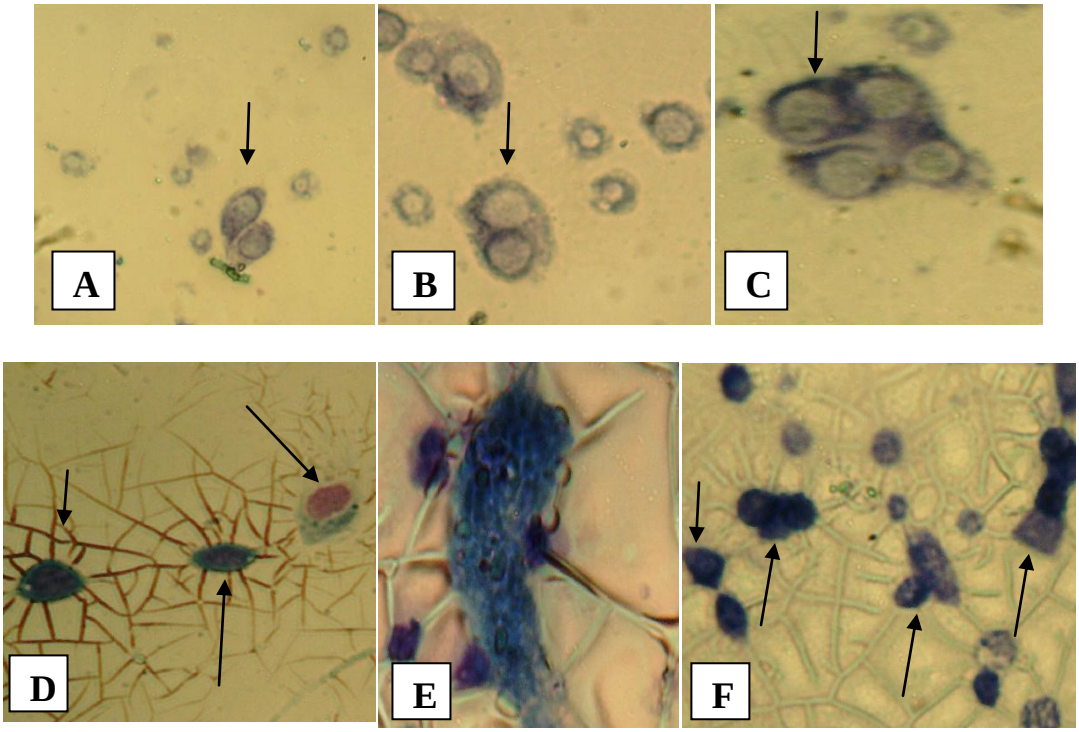

Plate 2: Photomicrographs of PLs of *S. littoralis* last instar larvae (Geimsa stain, 400x). (A): Normal spindle-shaped PLs. (B): Normal oval-shaped PLs in pairs. (C): Normal oval-shaped PLs in cluster of four cells. Hemocyte deformities by LC_{50} of Novaluron: (D); Lysed PL (at right) and two vacuolated PLs (at left). (E): Haemocytic microaggregation of PLs with destroyed cell membranes and lysed cytoplasm. (F): PLs with destroyed cell membranes and extruded cytoplasmic contents.

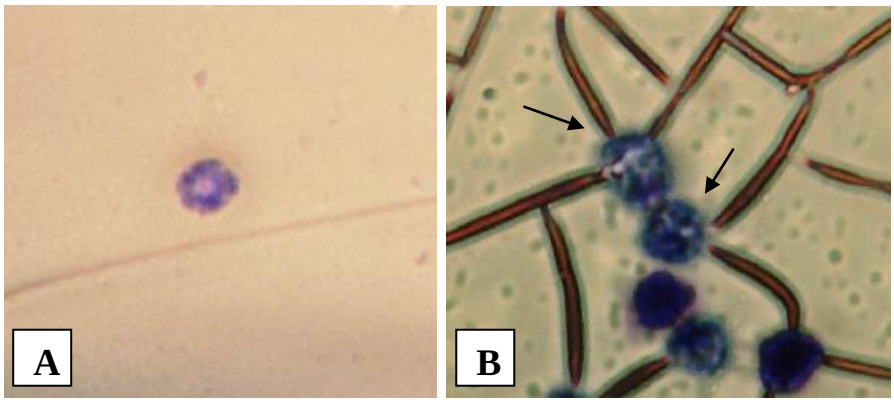

Plate 3: Photomicrographs of GRs of *S. littoralis* last instar larvae (Geimsa stain, 400x). (A): Normal cell. (B): Hemocyte deformation by LC_{50} of Novaluron, showing destroyed cell membranes and vacuolated cytoplasm.

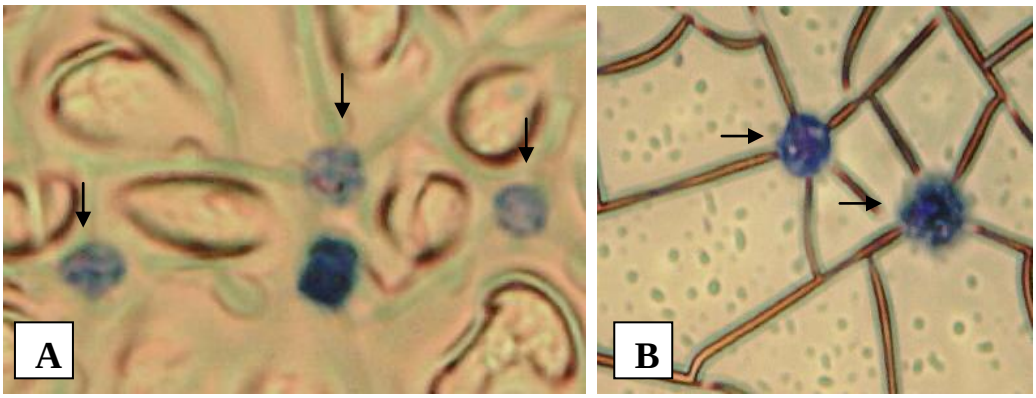

Plate 4: Photomicrographs of SPs of *S. littoralis* last instar larvae (Geimsa stain, 400x). (A): Normal cell. (B): Hemocyte deformation by LC_{50} of Novaluron, showing destroyed cell membranes and vacuolated cytoplasm.

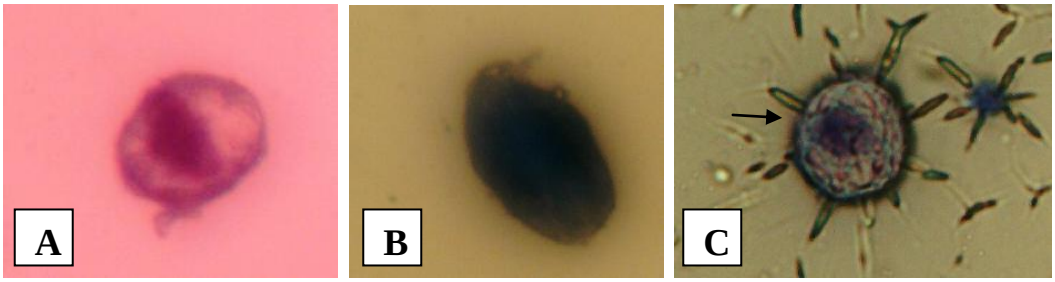

Plate 5: Photomicrographs of OEs of *S. littoralis* last instar larvae (Geimsa stain, 400x). (A): Normal cell. Hemocyte deformation by LC_{50} of Novaluron: (B): OE with degenerated darkly stained nucleus and cytoplasm as well as destroyed cell membrane. (C): OE with lysed cytoplasm and granulated nucleus.

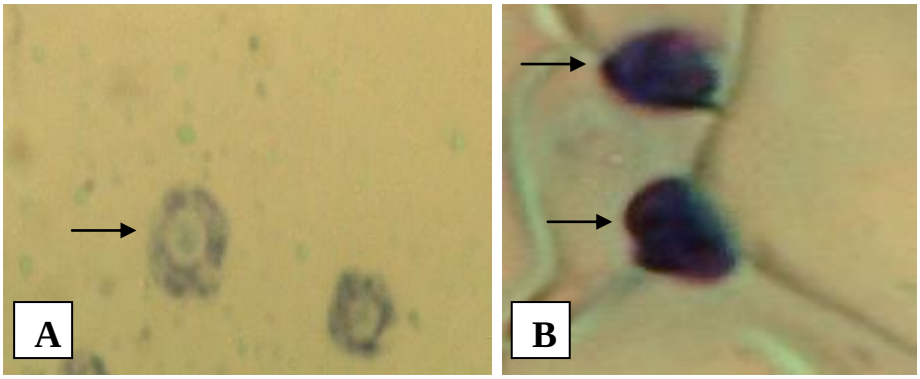

Plate 6: Photomicrographs of PRs of *S. littoralis* last instar larvae (Geimsa stain, 400x). (A): Normal cell. (B): Hemocyte deformation by LC_{50} of Cyromazine, darkly stained with destroyed membrane and extruded cytoplasmic contents.

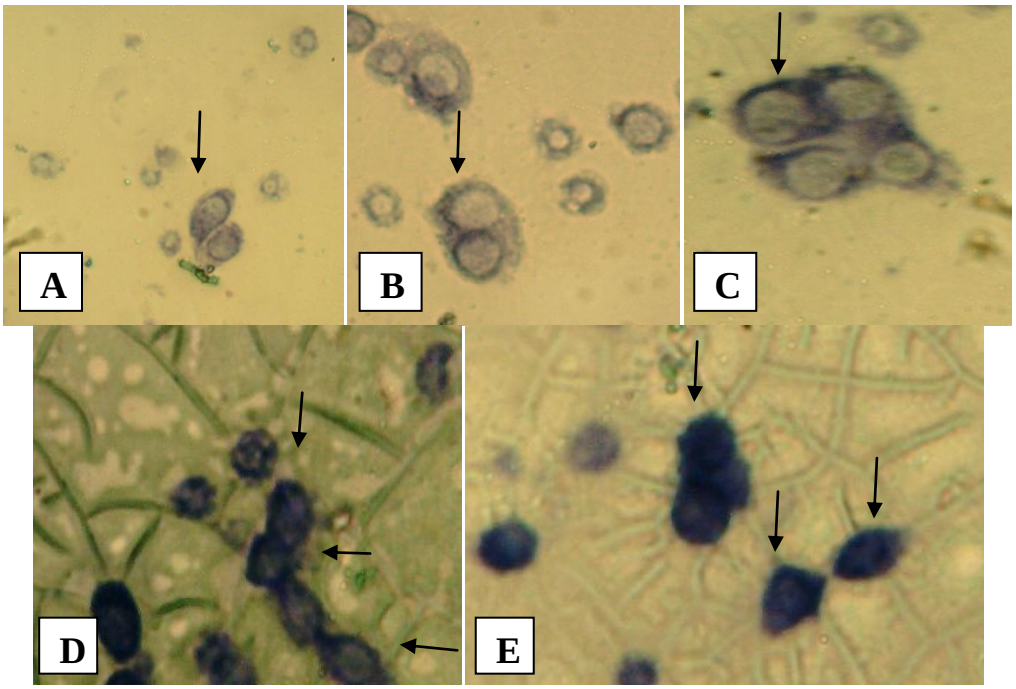

Plate 7: Photomicrographs of PLs of *S. littoralis* last instar larvae (Geimsa stain, 400x). (A): Normal spindle-shaped PLs. (B): Normal oval-shaped PLs in pairs. (C): Normal oval-shaped PLs in cluster of four cells. Hemocyte deformities by LC_{50} of Cyromazine: (D): PLs with destroyed cell membranes and lysed cytoplasm. (E): PLs with destroyed cell membranes and extruded cytoplasmic contents.

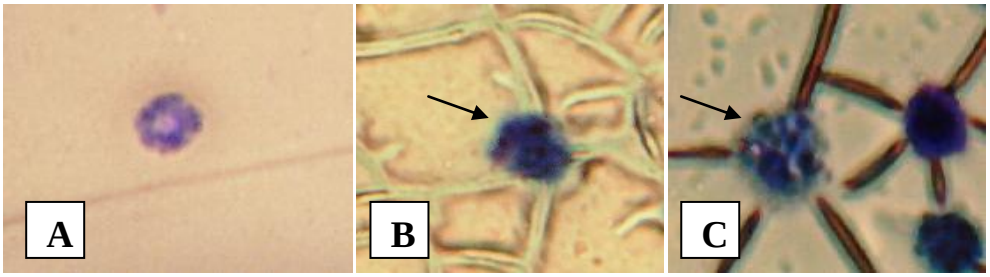

Plate 8: Photomicrographs of GRs of *S. littoralis* last instar larvae (Geimsa stain, 400x). (A): Normal cell. Hemocyte deformities by LC_{50} of Cyromazine, (B): GR with destroyed cell membrane and lysed cytoplasm, (C): GR with destroyed cell membrane and vacuolated cytoplasm.

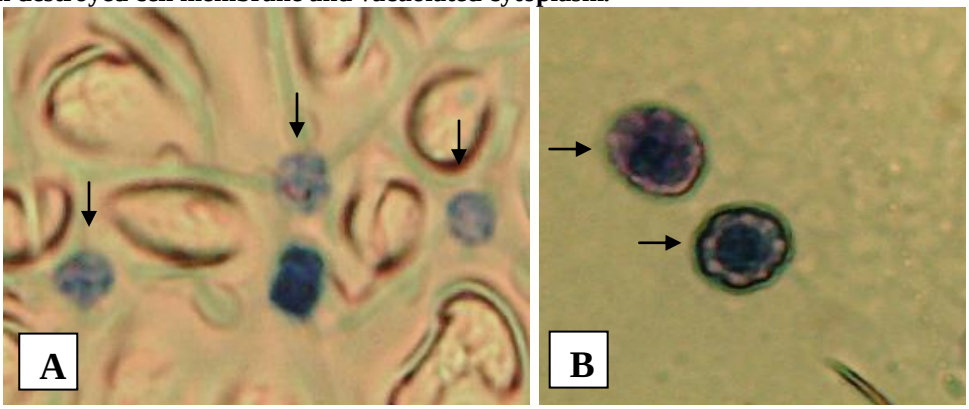

Plate 9: Photomicrographs of SPs of *S. littoralis* last instar larvae (Geimsa stain, 400x). (A): Normal cell. (B): Hemocyte deformation by LC_{50} of Cyromazine, showing destroyed cell membranes and cytoplasm with continuous vacuole (upper cell) or separated vacuoles (lower cell).

DISCUSSION:

Haemocytes have been studied mostly in Lepidoptera, Hymenoptera, Coleoptera and Diptera (Osman et al., 1984; Gupta, 1985; Gurwattan et al., 1991; Miller and Stanley, 2000; Ayaad et al., 2001; Rizk et al., 2001; Lavine and Strand, 2002; El-Sheikh, 2002; Gelbic et al., 2006; Zohry, 2006; Ribeiro and Brehelin, 2006; Annuradha and Anuadurai, 2008) as well as Dictyoptera (Chiang et al., 1988), Heteroptera (Sanjayan et al., 1996), Hemiptera (Georges and Ambrose, 2004) and Orthoptera (Barakat et al., 2002; Tanani, 2010).

1. Circulating hemocyte types in *Spodoptera littoralis*.

Classified categories of haemocyte types range from four to seven (Gupta, **1979**) or between three and nine (Wigglesworth, 1959; Jones, 1962; Arnold, 1972, 1974; Al-Khalifa and Siddiqui, 1985). Otherwise, the available literature shows that the most common types are PRs, GRs and OEs as described from different species in various orders (Ahmad, 1992; Fenoglio and Gervaso, 1993; Joshi and Lambdin, 1996; Hernandez et al., 1999; De Silva et al., 2000; Siddiqui and Al-Khalifa, 2012b). There is confusion between various haemocyte types such as PRs and PLs as well as GRs and adipohaemocytes (ADs) (Jones, 1967; Nruwirth, 1973). As reported in the literature, also, seven types of hemocytes have been described in various insects (Gupta, 1985; Brehélin and Zachary, 1986). Six types of hemocytes were identified in *Diatraea saccharalis* (Falleiros et al., 2003) and *Papilio demoleus* (Jalali and Salehi, 2008). Five distinct classes of haemocytes were identified in different insect species, such as *Manduca sexta* (David and Peter, 1982; Miller and Stanely, 2000), *Poekilocerus bufonius* (Al-Robai et al., 2002), *Spodoptera litura* (Sharma et al., 2003), *Ostrinia furnacolis* (Jian et al., 2003) and *Bombyx mori* (Han et al., 1998; Ling et al., 2003a; Tan et al., 2013; Liu et al., 2013). Four types of haemocytes were identified in some other insect species (Hoffmann, 1967; Akai and Sato, 1973; Osman et al., 1984; Mahmood and Yousaf, 1985; Masconi et al., 1989; Peter and Ananthakrishnan, 1995; Gelbic et al., 2006). Three types were characterized in some insects such as *Schistocerca gregaria* (Tanani, 2010). Only two types could be identified in *Drosophila* spp. (Lavine and Strand, 2002) and *Melanoplus sanguinipes* (Gurwattan et al., 1991; Meranpuri et al., 1991). However, Sendi and Salehi (2010) identified only two major hemocyte types in *P. demoleus* basing on their role in immunity, i.e., PLs and GRs.

In *S. littoralis*, morphological characteristics of hemocytes were studied by some investigators (Harapaz et al., 1969; Stettler et al., 1998). Only four hemocyte types were identified without GRs (Osman et al., 1984), cytocytes (Abdel-Rahman et al., 2000) or OEs (Gelbic et al., 2006). In the present study, five main hemocyte types could be identified in the haemolymph of last instar larvae of *S. littoralis*: PRs, PLs, GRs, SPs and OEs. Thus, the present result disagrees with many reported records in various insect species but consistently agrees with Zohry (2006) and Hassan et al. (2013) who characterized the same types in the same insect. However, the diverse results might be attributed to the differences in insect species or even its developmental stage, several technical difficulties for identification and the characters adopted by other workers (George and Ambrose, 2004; Ribeiro and Brehelin, 2006). In addition, hemocyte classification, types and morphology are often influenced by some factors affecting the haemolymph physical properties or biochemical composition (Carrel et al., 1990), physiological condition of the insect (Chapman, 1998) and the insect developmental stages. Therefore, the hemocyte classification has been recommended to be revised several times in the same species (Gupta, 1979; Dean et al., 2004; Ribeiro and Brehelin, 2006; Wood and Jacinto, 2007; Qamar and Jamal, 2009; Siddiqui and Al-Khalifa, 2012a, b).

2. Total haemocyte population in *S. littoralis* as affected by Novaluron and Cyromazine.

The total hemocyte count (THC) has been found to be quite variable depending upon the insect species, developmental stage, physiological state and the technique followed (Romosen and Stofolano, 1998). It may be important to mention that the brain endocrine complex is involved in haemocyte accumulation following some initial stimulus (Nappi, 1974). Jones (1967) suggested that ecdysteroids can regulate the number of haemocytes. In addition, hormones, synthetic pesticides and insect growth regulators (IGRs) intervene in the intermediary metabolism and immune capability of insects as observed in changes in hemocyte number, differentiation and phagocytosis (Qamar and Jamal, 2009).

In the current work on *S. littoralis*, chitin synthesis inhibitor (CSI) Novaluron remarkably enhanced the hemocyte production at two limits of the larval instar since THC was pronouncedly increased but prohibited during the middle duration. On the other hand, Cyromazine exerted an inhibitory action during the majority of larval instar, with few exceptions. The enhanced THC by Novaluron is in agreement with some of reported results for the same insect species by CSIs diflubenzuaron (Osman et al., 1984), flufenxuron and chlorfluazuron (Bakr et al., 2007), teflubenzuron (Abdel-Al et al., 2011), hexaflumuron (Zhu et al., 2012) and some compounds derived from urea waste (Hassan et al., 2013). Also, similar THC enhancement was reported for other insect species, such as

Periplaneta americana as response to several foreign particles (Ryan and Nicholas, 1972); *S. litura* by ecdysone (Rao et al., 1984); *Gryllus bimaculatus* (Mahmoud and Yousuf, 1985), Acanthaspis pedestris (Ambrose and George, 1996), *S. gregaria* (Al-Hariri and Suhail, 2001), *Rhynocoris kumarii* (George and Ambrose, 2004), *Dysdercus cingulatus* (Haq et al., 2005), and *Leptinotarsa decemlineata* (Dubovskiy et al., 2014) by some insecticides. After injection of the *B. mori* larvae with 20-ecdysone, hemocyte density significantly increased at approximately 12-18 h post injection. However, the application of IGR methoprene to the injected larvae kept the hemocyte level stable without an obvious change (Ling et al., 2003b). THC was increased in *Coccinella septempunctata* after treatment with spinosad (Suhail et al., 2007) and *Eurygaster integriceps* by the ecdysone agonist methoxyfenozide (Zibaee et al., 2012).

On the other hand, prohibited THC by Cyromazine, in the present study, corresponds to similar results reported for the same insect after treatment with flufenoxuron (Bakr et al., 2007) or certain concentration levels of some compounds derived from urea waste (Hassan et al., 2013); as well as decreased THC in other insects by various insecticides and IGRs, such as *R. kumarii* by the insecticide endosulfan (George and Ambrose, 2004); *S. gregaria* by some conventional insecticides, spinosad and proclaim (Halawa et al., 2007); C. septempunctata by abamectin (Suhail et al., 2007); *P. demoleus* by IGR methoprene (Sendi and Salehi, 2010); *Mythima separata* by IGR hydroprene (Wang et al., 1993), *D. cingulatus* by β–ecdysone and the phytoecdysone maskisterone (Ahmad, 1995); *Dysderus koenigii* by CSI penfluron (Prakash et al., 2007); *Agrotis ipsilon* by CSI diflubenzuron (Abdel-Aziz and Awad, 2010), *S. gregaria* by CSI teflubezuron (Teleb, 2011); *E. integriceps* by the juvenoid pyriproxyfen (Zibaee et al., 2012); *Ephestia kuehniella* by IGR pyriproxyfen and CSI hexaflumuron (Rahimi et al., 2013), *Glyphodes pyloalis* by juvenile hormone (Khosravi et al., 2014); etc.

The prevalent promoting effect of Novaluron on THC, in the present study, could be attributed to the enhanced encapsulation of foreign/toxic molecules through process of melanization because melanin deposition during encapsulation is commonly initiated by haemocytes and/or phenoloxidase enzyme circulation in the plasma (Rolff and Siva-Jothy, 2002; Nappi and Christensen, 2005). Also, it may be due to the release of sessile haemocytes and the activation of mitotic division of the haemocytes, because many insect species possess populations of sessile haemocytes (Ratcliffe and George, 1976) which might be activated in response to some insecticides or IGRs. Moreover, the increase in THC could be suggested as an immune response against pathogen or any foreign body, such as the introduced CSIs, in the present study (Chu et al., 1993; Anderson et al., 1995; Ordas et al., 2000).

On the other hand, the predominant inhibitory effect of Cyromazine on THC in *S. littoralis*, in the present investigation, may be correlated with the decrease of some hemocyte types involved in phagocytosis and nodule formation. Reduction of THC may be due to the toxicities of IGRs and their inhibitory effects on the insect endocrine organs and secretion, nodule formation, larval hematopoietic function or the cell proliferation (Sharma et al., 2003; Sabri and Tariq, 2004; Pandey et al., 2007; Zhu et al., 2012; Zibaee et al., 2012). In addition, THC declination may be attributed to the death of pathological cells by degeneration (Sendi and Salehi, 2010).

3. Differential haemocyte populations in *S. littoralis* as affected by Novaluron and Cyromazine.

It is important to point out that the increasing counts of some haemocyte types and decreasing counts of others may be due to the transformation of some types into other ones for achieving the phagocytic function or other tasks in the battle against the biotic targets like bacteria, yeast and apoptic bodies, as well s against and abiotic materials such as particles of Indian ink (Hernandez et al., 1999; De Silva et al., 2000). The particular haemocytes reported to be phagocytic varies among insect taxa, and in some cases discrepancies even exist in the literature among studies on the same species (Tojo et al., 2000). Moreover, DHCs fluctuate not only as a consequence of different instars of the insect but also within a given instar. These changes may be a result of developmental processes (Gelbic et al., 2006). DHCs in haemolymph of last instar larvae of S. littoralis had been changed depending on the hemocyte type, CSI and larval age.

Novaluron caused a slight increase in PRs population during the first half of last larval instar of *S. littoralis*, in the present study, but slightly decreased population during the second half. Cyromazine exerted a predominant inhibitory effect on the population of PRs, regardless the larval age. The decreasing PRs population is, to a great extent, in conformity with those decreasing PRs reported in the same insect by flufenoxuron (Zohry, 2006) and other insects, such as *R. kumarii* by some organophosphorous insecticides (George and Ambrose, 2004) and *A. ipsilon* by CSI diflubenzuron (Abdel-Aziz and Awad, 2010). On the other hand, increasing PRs count was reported in *M. separata* by IGR hydroprene (Wang et al., 1993), *R. kumarii* by the insecticide endosulfan (George and Ambrose, 2004), *S. gregaria* by CSI teflubenzuron (Teleb, 2011), etc. Although PRs are progenitor stem cells which can

differentiate into other types of hemocytes according to light and electron microscopy observations (Yamashita and Iwabuchi, 2001; Lavine and Strand, 2002), their exact function is still unknown (Ribeiro and Brehelin, 2006). As reported by Liu et al. (2013), PRs in *B. mori* can differentiate into PLs and GRs. However, the general reduction of PRs population in larvae of *S. littoralis*, in the present study, may be attributed either to the cytotoxic effects of CSIs on the mitotic division of PRs, conversion to other types of cells or to the inhibitory effects on the activity of haematopoietic organs responsible for PRs production (Zhu et al., 2012; Zibaee et al., 2012).

In the present study on *S. littoralis*, Novaluron slightly enhanced PLs population during the first half of last larval instar but slightly prohibited during the second half. Cyromazine remarkably promoted their population, regardless the larval age. The enhancement of PLs is, to some extent, in agreement with the increasing PLs count in some insect species by various insecticides or IGRs, such as *S. gregaria* nymphs by Lambdacyhalothrin and Deltamethrin (Al-Hariri and Suhail, 2001) and teflubenzuron (Teleb, 2011); *R. kumarii* by endosulfan (George and Ambrose, 2004); *A. ipsilon* by diflubenzuron (Abdel-Aziz and Awad, 2010); *S. litura* by hexaflumuron (Zhu et al., 2012). On the other hand, the slight decrease of PLs population during the second half of larval instar, as a response to Novaluron in the present study, is in accordance with the decreasing count of PLs in the same insect species by LC_{50} of flufenoxuron (Bakr et al., 2007) and other insects, such as *R. kumarii* by some insecticides (George and Ambrose, 2004), *S. gregaria* by some insecticides, spinosad and proclaim (Halawa et al., 2007), etc. The role of PLs in phagocytosis is disputed because some authors believed that they are phagocytes (Tojo et al., 2000; Ling and Yu, 2006) but other authors reported no phagocytic function (Neuwirth, 1973; Beaulaton, 1979). The decreasing PLs population in the current work on S. littoralis can be explained by their transformation into other types of hemocytes (Beaulation and Monpeyssin, 1976; George, 1996) since they are highly polymorphic cells (Gupta and Sutherland, 1966). Also, Novaluron may impaired the haematopoietic organs which responsible for the production of these hemocytes (Tiwari et al., 2002). On the other hand, we cannot provide an appreciable interpretation to the increasing PLs population, as generally found in last instar larvae of all ages by Cyromazine or during the first half of instar by Novaluron, at the present time!!

One of the main functions of GRs is phagocytosis as reported by Wago (1980) in *B. mori*, Raina (1976) in *Pectyinophora gossypiella*, Tojo et al. (2000) in *Galleria mellonella*, Nardi et al. (2001) in M. sexta; Essawy et al. (1985) in *Heliothis armigera*, Pendland and Boucias **(**1996) in *Spodoptera exigua*, Butt and Shields (1996) in *Lymantria dispar* and Costa et al. (2005) in *S. littoralis*. In the current investigation on *S. littoralis*, GRs count decreased throughout the last larval instar, with few exceptions, after treatment with Novaluron. Moreover, Cyromazine exhibited a potent inhibitory effect on these hemocytes in larvae of all ages. This result is evidently correspond to some reported results of decreasing GRs in the same insect species after treatment with the drug metyrapone (Gelbic et al., 2006), LC_{50} of flufenoxuron (Bakr et al., 2007), or some compounds derived from urea waste (Hassan et al., 2013), as well as decreasing GRs count in other insect species, such as *Rhodnius prolixus* nymphs by wounding (Lia-Fook, 1968), *R. kumarii* by endosulfan (George and Ambrose, 2004) and *S. litura* by hexaflumuron (Zhu et al., 2012). In contrast, GRs population was enhanced in *S. gregaria* by Lambdacyhalothrin and Deltamethrin (Al-Hariri and Suhail, 2001) or teflubenzuron (Teleb, 2011) and *A. ipsilon* by Diflubenzuron (Abdel-Aziz and Awad, 2009). However, the general decrease of GRs in *S. littoralis* larvae by CSIs, in the present study, may be interpreted by the death of a lot of them due to their detoxification activity against the toxic molecules (Kurihara et al., 1992; Butt and Shields, 1996; Nardi et al., 2001; Barakat et al., 2002; George and Ambrose, 2004; Costa et al., 2005). Also, it may be due to their differentiation into other types of hemocytes since GRs can differentiate into SPs in another lepidopteran, *B. mori* (Liu et al., 2013).

The available literature contains very few reports of increasing SPs count in insects, as a response to insecticides or IGRs, such as in *A. ipsilon* by Diflubenzuron (Abdel-Aziz and Awad, 2010). In the present study on *S. littoralis*, Novaluron enhanced SPs population throughout the last larval instar, with few exceptions, while Cyromazine exhibited a similar enhancing effect only during the middle duration but prohibited them at two limits of instar. The major promoting action of these CSIs disagrees with the decreasing count reported in some insects by some insecticides and IGRs, such as *S. gregaria* nymphs by Lambdacyhalothrin and Deltamethrin (Al-Hariri and Suhail, 2001) or teflubenzuron (Teleb, 2011), *M. separata* by KK-42 (Wang et al., 1993) and *P. demoleus* by methoprene (Sendi and Salehi, 2010). In Lepidoptera, SPs are quite different from GRs overloaded with phagocytosed material. The functions of SPs are unknown until now (Ribeiro and Brehelin, 2006) but Sass et al. (1994) suggested their responsibility for transporting cuticular components. However, the general increase of SPs population after treatment with the present CSIs may be due to their enhancing effects on the differentiation of SPs or transformation of other hemocytes into SPs in last instar larvae of *S. littoralis*. The exact mode of action is still obscure!!

In the present work, OEs population was enhanced by both CSIs during the second half of last larval instar of S. littoralis. Such enhancement is in agreement with increasing OEs count in the same insect species as a result to treatment with hexaflumuron (Abu El-Magd et al., 1994: Zhu et al., 2012), as well as in other insects, such as *S.*

gregaria as response to laminarin (derived from the brown seaweed *Laminaria digitata*) (Abu El-Magd, 1992) or teflubenzuron (Teleb, 2011). Increasing of OEs population in the present study may be attributed to their role in the detoxification of toxic materials and activating action of CSIs on the hematopoietic organs or cell mitotic division. Similar to unaffected OEs count in *S. littoralis* after treatment with LC_{50} of flufenoxuron (Zohry, 2006), no effect was exhibited by CSIs on these hemocytes in 2-day old larvae of the same species, in the present work. This may be due to the hemocyte resistance to penetration of these compounds and remain unaffected. An exceptional case of decreasing OEs at the beginning of last instar by Novaluron, in the present work, agrees, to some extent, with the reported decreasing OEs in the same insect species by teflubenzuron (Abdel-Al et al., 2011) and in *S. gregaria* by Lambdacyhalothrin and Deltamethrin (Al-Hariri and Suhail, 2001). The decreasing OEs at this time of larval life may be due to degeneration of some cells for releasing precursors of prophenoloxidase (PPO) that likely play a role in melanization of haemolymph and an important immunity protein in insects (Ribeiro et al., 1996). Otherwise, recent studies in different insect species show that additional hemocyte types contain PPO (Liu et al., 2013).

4. Qualitative haemocyte profile in *S. littoralis* as affected by Novaluron and Cyromazine.

As reported by Miselyunene (1976) for Pieris rapae, El-Kattan (1995) for *Plodia interpunctella*, Barakat et al. (2002) for *S. gregaria* and Bakr et al. (2007) for *S. littoralis*, some pathogenic microorganisms, insecticides or IGRs caused some disruptive alterations in the haemocytes basing on changes in the plasma membrane (erosion and extrusion of their cytoplasmic contents), vacuolization and lysis of the cytoplasm and nuclear changes. In the present study on *S. littoralis*, CSIs exhibited various serious cytopathological effects on all types of hemocytes, except Cyromazine which failed to affect OEs. The most common symptoms of morphological disorders and intracellular disturbances of hemocytes appeared as darkly stained cells, destroyed plasma membranes, extruded cytoplasmic contents, cytoplasm lysis and formation of cytoplasmic vacuoles. However, OEs were the least affected hemocytes as previously reported in *D. cingulatus* after treatment with the insecticide Acephate (Qamar and Jamal, 2009) and *S. gregaria* after treatment with teflubenzuron (Teleb, 2011).

The morphological disorders of *S. littoralis* haemocytes, in the present study, may be attributed to the action of CSIs on the 'actin' which localized in the lamellar extensions of the cells. Any naturally originating pesticidal molecule may exert its activity by targeting actins (Anunradha and Annadurai**,** 2008). No appreciable interpretation of the intracellular disturbances in hemocytes by CSIs has been available now!! The question whether the hemocytes are affected directly or via some physiological or endocrinological pathway is yet to be answered in spite of reports that developmental effects caused by IRGs and botanicals were attributed to disruption of endocrine events (Schmutterer, 1990).

In **conclusion**, Novaluron or Cyromazine can be used as a synergistic agent in the microbial control of *S. littoralis* owing to their prohibiting effects on those hemocytes responsible for phagocytosis and subsequently potentiate the pathogen efficiency.

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