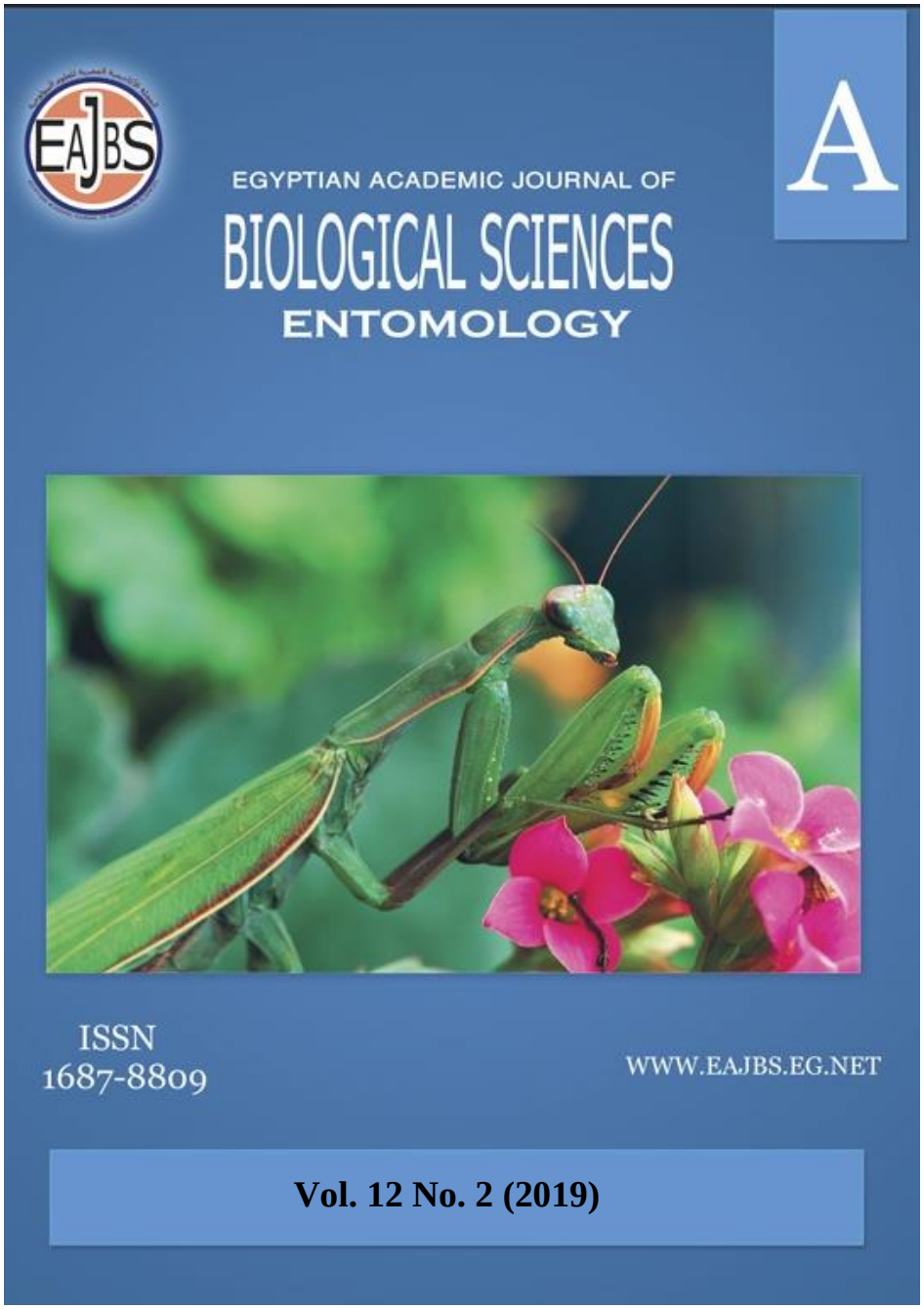

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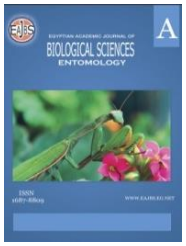

Disruptive Effects of Certain Chitin Synthesis Inhibitors on Some Haemogram Parameters in the Egyptian Cotton Leafworm, *Spodoptera littoralis* (Lepidoptera: Noctuidae)

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ABSTRACT

The Egyptian cotton leafworm, *Spodoptera littoralis*, is a key pest attacking several economically important crops in Egypt and several parts in the world. The objective of the present study was to investigate the effects of novaluron and cyromazine, chitin synthesis inhibitors, on the larval haemogram of this pest. Five main types of the circulating hemocytes, *viz*., plasmatocytes (PLs), granulocytes (GRs), prohemocytes (PRs), oenocytoides (OEs), and spherulocytes (SPs) had been categorized in last instar larvae. The most important differentiating characters of each type were described. After treatment of the freshly moulted penultimate instar larvae with LC_{50} of novaluron or cyromazine (2.71 and 74.44 ppm, respectively), the successfully moulted last (6th) instar larvae were used to assess the haemogram responses. Novaluron remarkably induced the total hemocyte counts (THCs) at two limits of the larval instar. In contrast, cyromazine exhibited a general inhibitory effect on THC during the majority of larval instar. As a response to novaluron treatment, PRs and PLs counts had been slightly induced during the first half of instar but slightly reduced during the second half. Also, GRs population decreased but SPs population increased along most larval period. With respect to the effect of cyromazine, count of PRs and GRs had been remarkably inhibited but PLs was significantly enhanced along the larval life. The same compound exhibited diverse effects on SPs, depending on the larval age. In addition, OEs count was enhanced by both compounds during the second half of larval instar. Both compounds exhibited serious cytopathological effects on all types of hemocytes, except cyromazine against OEs.

INTRODUCTION

In insects, the circulating haemocytes perform primary functions in the body, such as phagocytosis, coagulation to prevent loss of blood, encapsulation of foreign bodies, nodule formation, detoxification of metabolites and biological active materials, as well as storage and distribution of nutritive materials to various tissues (for detail, see: Garcia and Rosales, 2002; Zhou *et al*., 2004; Ling and Yu, 2006; Ribeiro and Brehelin, 2006; Siddiqui and Al-

Khalifa, 2012a; Chavan *et al*., 2017). Haemocytes have been studied mostly in Lepidoptera, Hymenoptera, Coleoptera and Diptera (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; Ghoneim *et al*., 2015 b) as well as Dictyoptera (Chiang *et al.*, 1988), Heteroptera (Sanjayan *et al*., 1996), Hemiptera (George and Ambrose, 2004) and Orthoptera (Barakat *et al*., 2002; Tanani, 2010). Insects have no acquired immune system, like of the higher animals, but a well-developed innate response. Their cellular defense refers to the haemocyte-mediated immune responses, such as phagocytosis, encapsulation and nodulation (Schmidt *et al*., 2001; Lavine and Strand, 2002). Sharma *et al*. (2003) reported that the number and proportion of different haemocytes were beneficial for insects to develop environmental fitness. Therefore, knowledge of insect haemocytes is very important to physiologists, biochemists and toxicologists, because the alteration in the number of cells and structure reflects changes in several physiological and biochemical processes (Qamar and Jamal, 2009; Berger and Jurčová, 2012).

The Egyptian cotton leafworm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) is native to Africa but lives throughout different parts in the world (Shonouda and Osmam, 2000). It is a serious pest of cultivated crops in Africa, Southern Europe, Middle East, Asia Minor and the Mediterranean area (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, soybean and vegetables (El-Khawas and Abd El-Gawad, 2002). In Egypt, *S. littoralis* is a key pest attacking several economically important crops (Raslan, 2002; El-Aswad *et al*., 2003; Korrat *et. al*., 2012). When large numbers of the pest are present complete crop loss is possible (El-Khawas and Abd El-Gawad, 2002).
Over the past four decades, the intensive and repeated uses of conventional insecticides against *S. littoralis* has led to various problems, such as the resistance development in the pest against several synthetic pesticides, dangerous effects on the natural enemies, pollinators and other non-target insects, as well as serious toxicological problems to humans and ecosystem (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, several institutions have devoted great effort for developing safe alternatives of the conventional insecticides (Dahi *et al*., 2009; Hussain, 2012). At present, the use of insect growth regulators (IGRs) has been considered as a potential alternative of conventional insecticides for controlling this pest (Raslan, 2002). IGRs are bio-rational compounds disrupting the normal development of several insects (Henrick *et al*., 1973). The chitin syntheses inhibitors (CSIs) are categorized in IGRs which interfere with chitin biosynthesis and thus prevent the moulting process in a number of insect species (Gijswijt *et al*., 1979; Hammock and Quistad, 1981). These compounds, also, affect the hormonal regulation resulting in different physiological disorders (DeLoach *et al*., 1981). Novaluron is a benzoylphenyl urea with good activity against some insects, such as the Colorado potato beetle (Cutler *et al*., 2005a,b, 2007; Alyokhin *et al*., 2009), *S. littoralis* (Ghoneim *et al*., 2015 a; Hamadah *et al*., 2015, 2016; Tanani *et al*., 2016; Basiouny *et al*., 2016) and *Pectinophora gossypiella* (Ghoneim *et al*., 2017), as well as it has only low mammalian toxicity (Barazani, 2001; Ishaaya *et al*., 2002, 2003). In Egypt, its residues tend to dissipate with a half-life of 2.08 days and the safe use of it on various crops was possible (Malhat *et al*., 2014). Cyromazine was primarily used to control immature houseflies on poultry farms (Awad and Mulla, 1984). It was recommended to use in the pest control programs (Schuster, 1994). The effects of this compound were closer to that of CSIs rather than that of juvenile hormone analogues (Darriet *et al*., 2008). Disturbance of different physiological activities in the dangerous insects, *viz*. feeding, moulting,

reproduction and immune system, may be targeted in the field of pest control (Pandey *et al*., 2012). Among the environmental factors affecting insect hemocytes, and functionally and/or morphologically are insecticides (Zibaee, 2011). Therefore, the objective of the current investigation was to assess the effects of two CSIs, novaluron and cyromazine, on the most important haemogram parameters in *S. littoralis* larvae.

MATERIALS AND METHODS

1. Experimental Insect:

A culture of the Egyptian cotton leafworm *Spodoptera littoralis* (Lepidoptera: Noctuidae) was established under the laboratory controlled conditions (27±2ºC, 65±5% R.H., photoperiod 14 hrs L and 10 hrs D) at Faculty of Science, Al-Azhar University, Cairo, Egypt. This culture was originated by a sample of pupae obtained from the susceptible culture maintained for several generations at Plant Protection Research Institute, Giza, Egypt. Rearing procedure was conducted 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 jars containing a layer of dry sawdust. The developing larvae were provided with fresh castor bean leaves *Ricinus communis*, as food, every day. The pupae were collected and kept in clean jars provided with a layer of moistened saw dust. All jars had been kept in suitable cages provided with branches of fresh *Nerium oleader*, as oviposition sites for emerging adult females. These adults were provided with 10% honey solution as a food source. Moths were allowed to lay eggs on branches and the egg patches were collected every day to be transferred into Petri dishes for the next generation.

2. Tested Compounds and Larval Treatments:

In the present study, the tested chitin synthesis inhibitors (CSIs) were Novaluron (Rimon, Chemtura Corporation, Middlebury, CT) [1-[chloro-4-(1,1,2-trifluoromethoxyethoxy) phenyl] -3- (2,6-difluorobenzoyl) urea] and Cyromazine (Trigard) [N-cyclopropyl-1,3,5-triazine-2,4,6-triamine] which were supplied by Sigma-Aldrich Chemicals (https://www.sigmaaldrich.com). In a preliminary experiment, the freshly moulted penultimate instar (5^{th}) larvae were treated with a range of concentrations of Novaluron (10.0-0.0001 ppm), and Cyromazine (200.0-0.001 ppm). LC_{50} values were determined in 2.71 and 74.44 ppm of these compounds, respectively. The 5^{th} instar larvae were treated with LC_{50} of each compound (2.71 and 74.44 ppm, respectively). The successfully moulted 6^{th} instar larvae were used to assess the effects of these compounds on the most important haemogram parameters.

3. Haematological Characterization:

Collection of Haemolymph:

Haemolymph samples were collected from both treated and control last instar larvae (of the ages: 0-, 2-, 4-, and 6-day old). The haemolymph was obtained by amputation of the prothoracic legs, before coxa, using fine scissors. Gentle pressure was carried out on the thoracic region to draw out sufficient haemolymph drops received by a non-heparinized capillary tube. For three replicates, the haemolymph from two individuals was never mixed.

Total Haemocyte Count:

Furthermore, 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 dispensed 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 usually10.

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 slipcover. The haemocytes were viewed under a 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 the 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:

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

Cytopathological Study:

To record the possible haemocyte deformations, caused by the tested compounds, photomicrographs were obtained by using a light microscope equipped with a camera at a magnification 10 X 40 = 400.

4. Statistical Analysis of Data:

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

RESULTS

1. The Circulating Hemocytes in *S. littoralis* Larvae:

Identification and Description:

On the basis of cell shape, cytoplasmic ratio and cytoplasmic inclusions as well as the shape of nucleus, free circulating hemocytes in last instar larvae of *S. littoralis*, in the current study, had been characterized into **five** main types, *viz*., plasmatocytes (PLs), prohemocytes (PRs), spherulocytes (SPs), granulocytes (GRs) and oenocytoids (OEs). The most important diagnostic characters of each type can be provided as follows.

PRs were seen with variable size (3-7 µm wide and 6-8 µm long). They were nearly ovoid, round or spherical. Few organelles were seen in the deeply stained abundant cytoplasm. The nucleus was large and centrally located occupying most of the cell volume (Fig. 1).

PLs were seen polymorphic and spindle-, oval- or spherical cells. When spherical, their diameters varied between 13-26 µm. When oval, their length varied between 26-34 µm width 15-30 µm. The basophilic (faintly stained) cytoplasm was rich in organelles, such as an amount of rough endoplasmic reticulum, scattered chromatin masses and several tapering projections. The nucleus was large (occupying 40-50% of the cell volume) and appeared as round, elongate or spherical and centric or eccentric in position with a distinct nucleolus. Several PLs had been observed in haemolymph as dense cells

with pale nuclei and punctate chromatin granules. They appeared singles, in pairs or occasionally in small clusters of 4-8 cells (Fig. 2).

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

SPs were found as basophilic or acidophilic cells with variable size (8-20 µm wide and 7-24 µm long). Their shape was round or oval and characterized by numerous cytoplasmic contents and intracytoplasmic spherules occupying almost all cytoplasm. The nucleus was seen small, centric or eccentric in position, mostly deformed by the spherules. The cell surface was homogenous but exhibited cytoplasmic projections corresponding to the spherules (Fig. 4).

OEs were observed as the largest hemocyte kind, spherical (22-35.5 µm in diameter) or oval (18.7-25 µm long, 26.5-35.6 µm wide). When stained with Giemsa stain, cytoplasm was seen homogenous basophilic. It included darkly stained small eccentric nucleus and scarce organelles containing round acidophilic granules (Fig. 5).

2. Effects of the tested compounds on total hemocyte count

Depending on the data distributed in Table (1), Novaluron significantly induced the production of hemocytes at the two limits of last larval instar because the total hemocyte count (THC) remarkably increased with 13.54 and 24.18% in haemolymph of 0- and 6-day old larvae, respectively. On the contrary, production of the hemocytes was reduced during the middle period of instar because THC significantly decreased in 22.18 and 21.54%, in 2- and 4-day old larvae, respectively. After treatment of 5th instar larvae of *S. littoralis* with LC_{50} of cyromazine, THC data in the last instar larvae were listed in Table (1). Depending on these data, cyromazine exhibited an extended inhibitory effect on the hemocyte production during the majority of life, since THC 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 the 6-day old larvae since the hemocyte count increased as clearly shown in increasing THC (14268.47±27.26 in comparison of 11650.00±45.83 cell/mm^3 in control larvae).

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).

3. Effects of the Tested Compounds on Differential Hemocyte Count:

Data assorted in Table (2) obviously revealed that novaluron exhibited diverse effects on the differential hemocyte counts (DHCs), depending on the hemocyte type and the larval age. With regard to PRs, slightly induced population had been determined during the first half of instar (12.97 and 31.92% increments in 0- and 2-day old larvae,

respectively) while slightly reduced population was recorded during the second half (8.36 and 34.54% reductions in 4- and 6-day old larvae, respectively). A similar trend was recorded for PLs, *viz*., increasing count during the first half of the instar (25.82 and 4.58% increments in 0- and 2-day old larvae, respectively) but regressed count during the second half (25.00 and 26.20% reductions in 4- and 6-day old larvae, respectively). According to data of the same table, production of GRs was prohibited along most larval period (18.56, 48.79 and 63.01% reductions in 0-, 2- and 4-day old larvae, respectively) but slightly enhanced at the end of instar (6.98% increment). On the other hand, SPs count had been affected in a reverse trend, i.e., their population was drastically suppressed only at the beginning of instar (26.54% reduction) but slightly or pronouncedly induced along most larval period (8.82, 66.31 and 48.28% increments in 2-, 4- and 6-day old larvae, respectively). In connection with OEs, their population suffered a strong inhibitory effect of novaluron only in the freshly moulted last instar larvae (24.81% reduction) but no effect was detected on 2-day old larvae. Moreover, the haemocyte population was enhanced along the second half of life (33.00 and 24.81% increments in 4- and 6-day old larvae, respectively).

As obviously shown in Table (3), cyromazine exhibited a prominent inhibitory effect on the production of PRs, since their count was dropped in throughout the larval period (25.75, 45.43, 37.50 and 34.54% reductions in 0-, 2-, 4- and 6-day old larvae, respectively). A reverse effect of the tested compound was exhibited on PLs because their count significantly increased (42.75, 19.07, 11.36 and 33.57% increments in 0-, 2-, 4- and 6-day old larvae, respectively). With regard to GRs, cyromazine exhibited a prevalent suppressing effect on their population, since it was unexceptionally reduced (39.50, 58.52, 71.75 and 72.09% reductions in 0-, 2-, 4- and 6-day old larvae, respectively). On the other hand, the tested compound exhibited a contradictory effect on the population of SPs, depending on the age of larvae. In connection with SPs, their count slightly decreased at the two limits of larval instar (26.54 and 17.24% reductions in 0- and 6-day old larvae, respectively) but increased during the middle duration of this instar (8.82 and 27.34% increments in 2- and 4-day old larvae, respectively). The unaffected OEs population in 2-day old larvae was an exceptional case because cyromazine considerably enhanced the population of these hemocytes in larvae of other ages (24.81, 33.00 and 24.81% increments in 0-, 4- and 6-day old larvae, respectively).

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).

4. Cytopathological Effects of the Tested Compounds on *S. littoralis:*

In the present study, it is important to investigate the cytopathological effects of novaluron and cyromazine on PRs. The photomicrographs presented in Plates 1 and 6 obviously demonstrated some morphological aberrations and influenced intracellular constituents. Treatments with each of these compounds resulted in darkly stained PRs with destroyed membranes and protruded cytoplasmic contents.

With regard to PLs, novaluron caused some morphological deformities, such as cell membrane rupture and hemocyte microaggregation. Also, some vacuoles and extruded cytoplasmic constituents had been observed (Fig. 2). In addition, cyromazine treatment led to cell membrane rupture, cytoplasm lysis and cytoplasmic protrusion (Plate 7). In connection with GRs, some cells had destroyed membranes, lysed and vacuolated cytoplasm as a response to the treatments with both compounds (Fig.s 3 and 8). As clearly shown in Plates (4 and 9), SPs had been dangerously affected by the tested compounds, since the plasma membrane of some cells was destroyed and vacuoles were produced in the cytoplasm. Only Novaluron could affect OEs as clearly seen in Fig. (5). Some OEs appeared with degenerated darkly stained nuclei and destroyed cell membranes (Plate 5B) as well as other cells appeared with lysed cytoplasm and granulated nuclei (Fig. 5C).

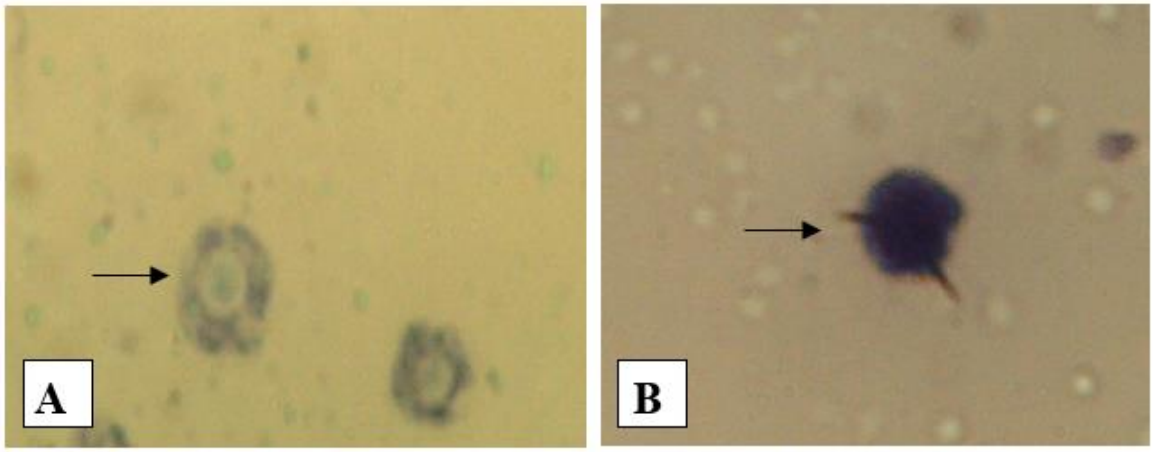

Fig. 1: Photomicrographs of PRs of *S. littoralis* last instar larvae (Geimsa stain, 400x). (A): Normal cell. (B): Hemocyte deformation by LC_{50} of Novaluron, darkly stained with destroyed membrane and extruded cytoplasmic contents.

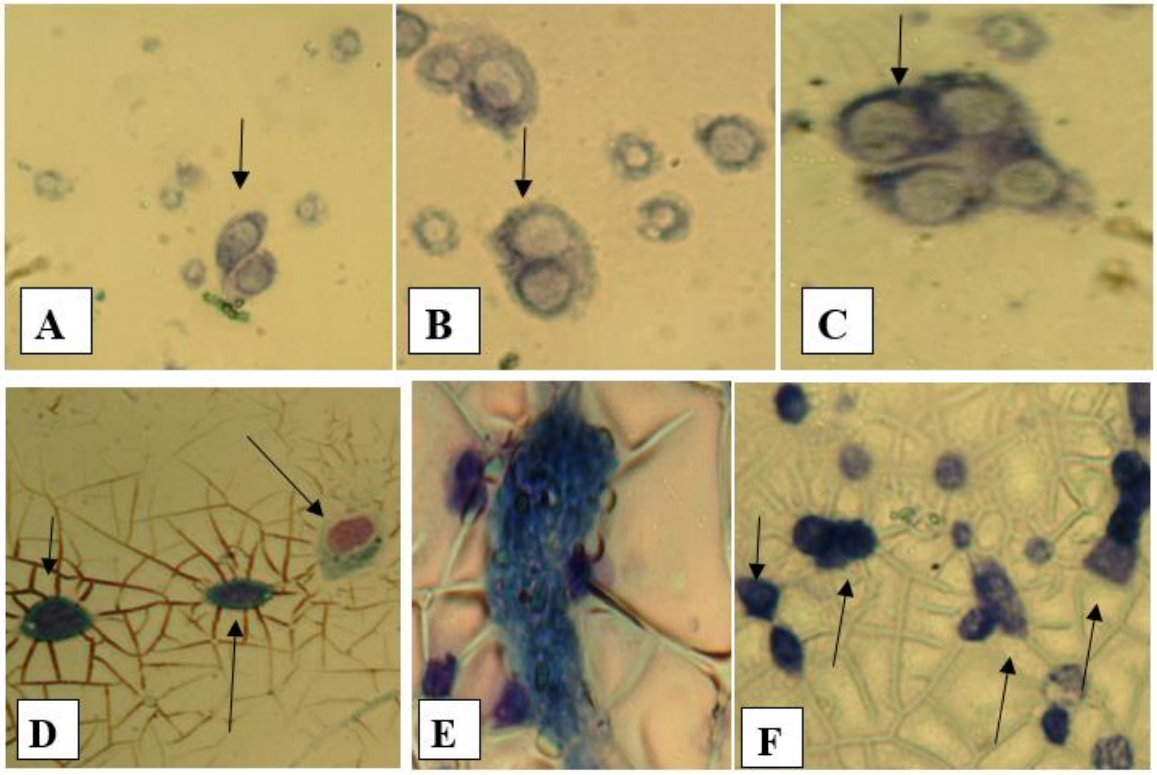

Fig. 2: Photomicrographs of PLs of *S. littoralis* last instar larvae (Geimsa stain, 400x). (A): Normal spindle-shaped PLs. (B): Normal oval-shaped PLs in pairs. (C): Normal oval-shaped PLs in cluster of four cells. Hemocyte deformities by LC_{50} of Novaluron: (D); Lysed PL (at right) and two vacuolated 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.

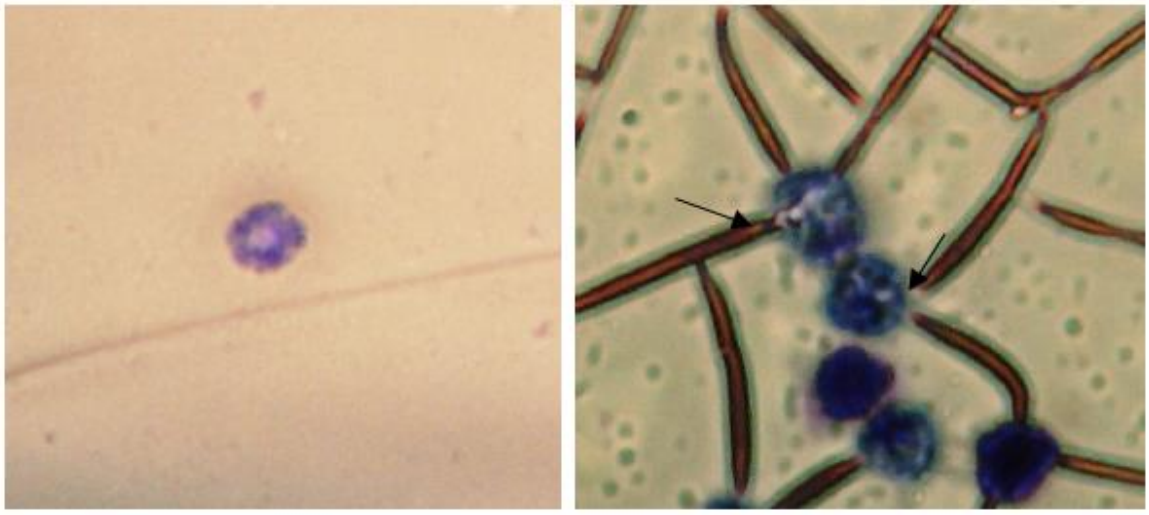

Fig. 3: Photomicrographs of GRs of *S. littoralis* last instar larvae (Geimsa stain, 400x). (A): Normal cell. (B): Hemocyte deformation by LC_{50} of Novaluron, showing destroyed cell membranes and vacuolated cytoplasm.

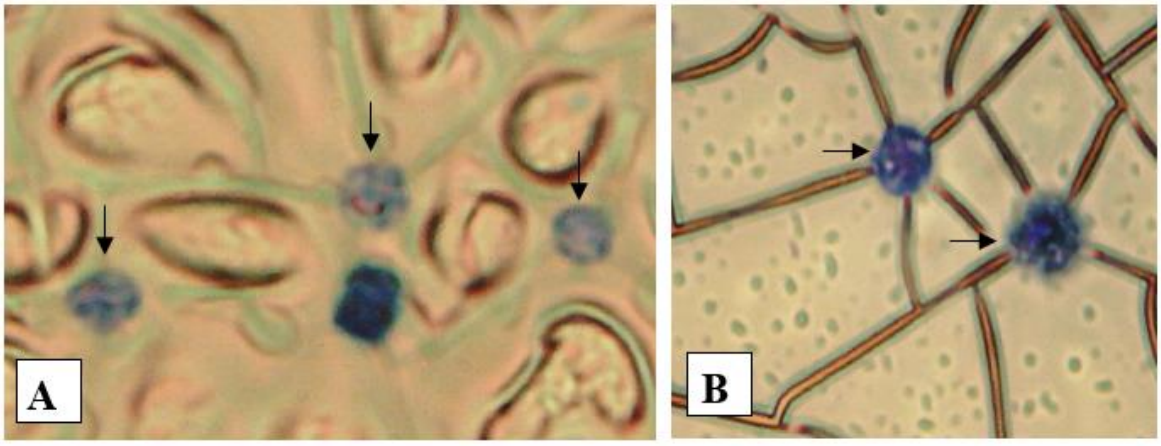

Fig. 4: Photomicrographs of SPs of *S. littoralis* last instar larvae (Geimsa stain, 400x). (A): Normal cell. (B): Hemocyte deformation by LC_{50} of Novaluron, showing destroyed cell membranes and vacuolated cytoplasm.

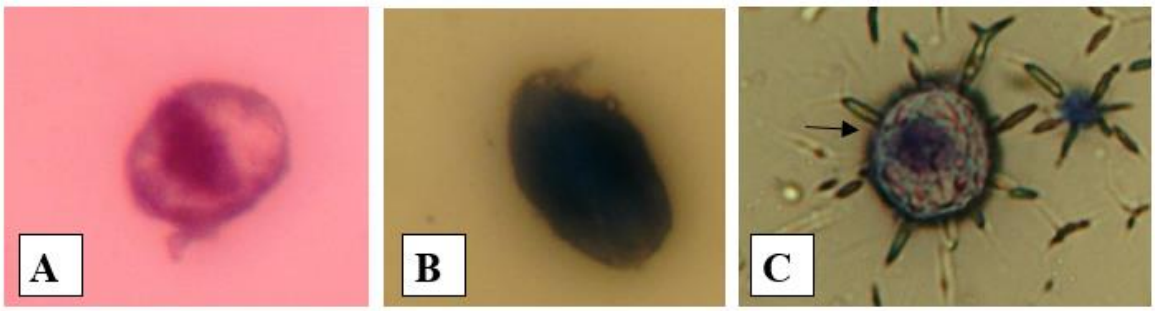

Fig. 5: Photomicrographs of OEs of *S. littoralis* last instar larvae (Geimsa stain, 400x). (A): Normal cell. Hemocyte deformation by LC_{50} of Novaluron: (B): OE with degenerated darkly stained nucleus and cytoplasm as well as destroyed cell membrane. (C): OE with lysed cytoplasm and granulated nucleus.

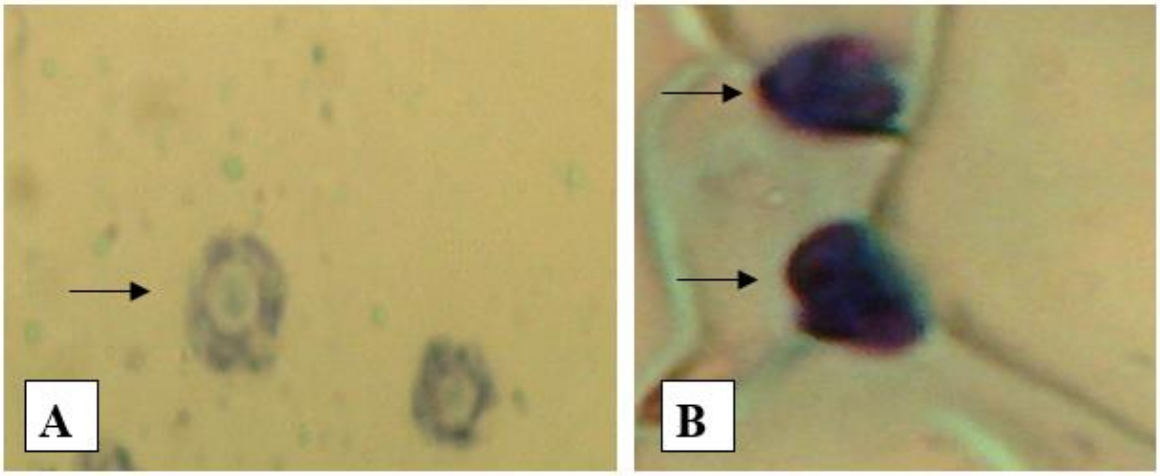

Fig. 6: Photomicrographs of PRs of *S. littoralis* last instar larvae (Geimsa stain, 400x). (A): Normal cell. (B): Hemocyte deformation by LC_{50} of Cyromazine, darkly stained with destroyed membrane and extruded cytoplasmic contents**.**

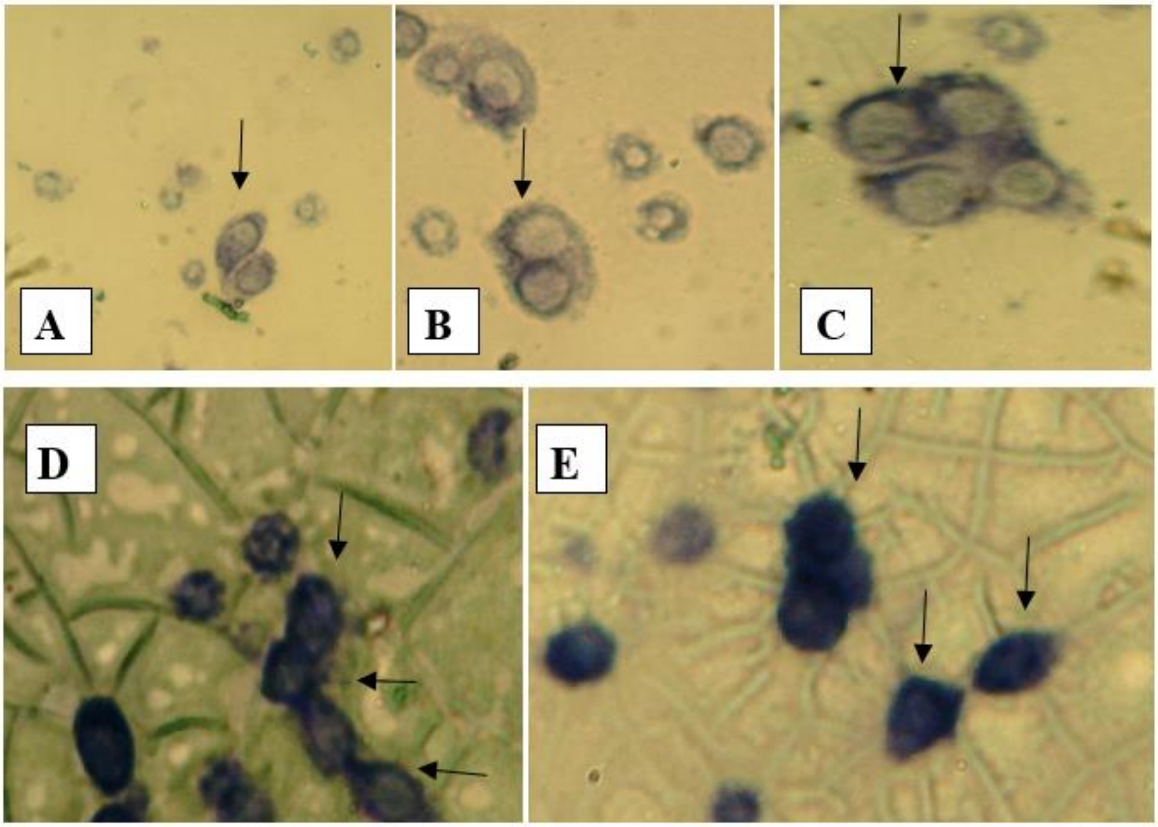

Fig. 7: Photomicrographs of PLs of *S. littoralis* last instar larvae (Geimsa stain, 400x). (A): Normal spindle-shaped PLs. (B): Normal oval-shaped PLs in pairs. (C): Normal oval-shaped PLs in cluster of four cells. Hemocyte deformities by LC_{50} of Cyromazine: (D): PLs with destroyed cell membranes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

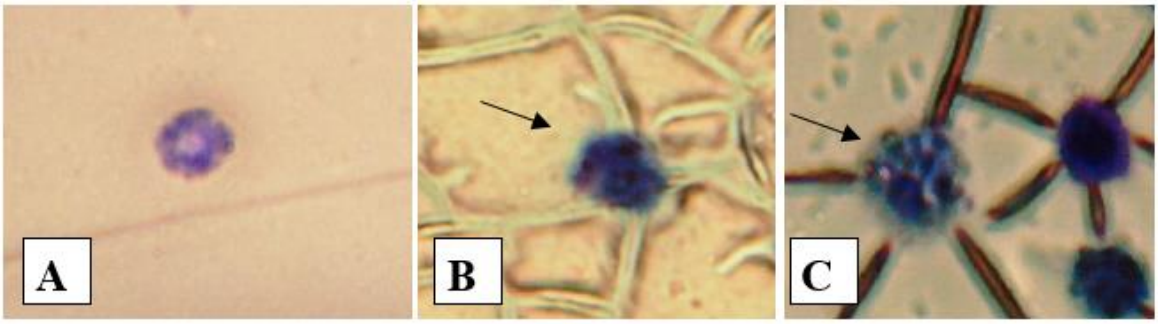

Fig. 8: Photomicrographs of GRs of *S. littoralis* last instar larvae (Geimsa stain, 400x). (A): Normal cell. Hemocyte deformities by LC_{50} of Cyromazine, (B): GR with destroyed cell membrane and lysed cytoplasm, (C): GR with destroyed cell membrane and vacuolated cytoplasm.

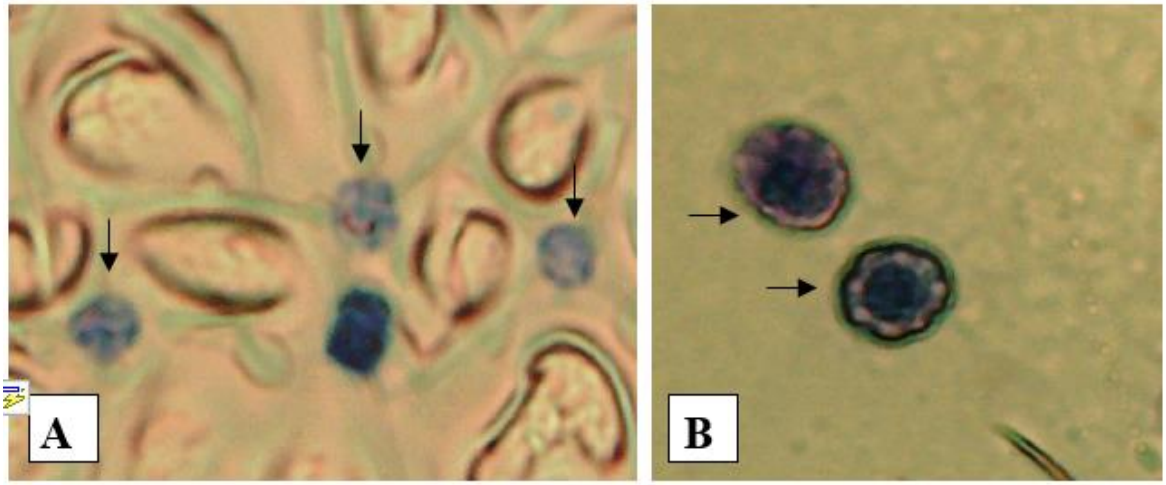

Fig. 9: Photomicrographs of SPs of *S. littoralis* last instar larvae (Geimsa stain, 400x). (A): Normal cell. (B): Hemocyte deformation by LC_{50} of Cyromazine, showing destroyed cell membranes and cytoplasm with continuous vacuole (upper cell) or separated vacuoles (lower cell).

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. Identification of Circulating Hemocyte Types in *S. littoralis*:

In the light of many reported studies (Wigglesworth, 1959; Jones, 1962; Arnold, 1974; Gupta, 1979; Al-Khalifa and Siddiqui, 1985), categories of the circulating haemocyte types in insects range from four to seven or from three to nine. Depending on several studies (Joshi and Lambdin, 1996; Hernandez *et al*., 1999; De Silva *et al*., 2000; Siddiqui and Al-Khalifa, 2012b), the most common types of haemocytes, as described from different species in various orders, are Prohemocytes (PRs), Granulocytes (GRs) and Oenocytoids (OEs).

Seven types of circulating hemocytes have been identified in various insects (Gupta, 1985; Brehélin and Zachary, 1986). Six types of hemocytes were described in *Diatraea*

saccharalis (Falleiros *et al*., 2003), *Papilio demoleus* (Jalali and Salehi, 2008) and *Pectinophora gossypiella*, *viz*., PRs, Plasmatocytes (PLs), Spherulocytes (SPs), OEs, GRs and Adipohemocytes (ADs) (Ghoneim *et al*., 2017). Five classes of were identified in different insect species, such as *Manduca sexta* (Miller and Stanely, 2000), *Poekilocerus bufonius* (Al-Robai *et al*., 2002), *Spodoptera litura* (Sharma *et al.*, 2003), *Ostrinia furnacolis* (Jian *et al*., 2003), *Bombyx mori* (Ling *et al*., 2003a; Tan *et al*., 2013) and *Rhynchophorus ferrugineus* (Hamadah and Tanani, 2017). Four categories were identified in some insect species (Masconi *et al*., 1989; Peter and Ananthakrishnan, 1995; Gelbic *et al*., 2006). Three main types were identified in some insects, such as *Schistocerca gregaria* (Tanani, 2010). Only two main types could be identified in *Drosophila* spp. (Lavine and Strand, 2002), *Melanoplus sanguinipes* (Meranpuri *et al*., 1991) and *P. demoleus* (Sendi and Salehi, 2010).

In the present study, morphological characteristics of the circulating hemocytes revealed five main types in the haemolymph of last instar larvae of *S. littoralis*, *viz*., PRs, PLs, GRs, SPs and OEs. This result disagreed with several reported results in which less number of haemocytes had been identified, such as only four types, without GRs (Osman *et al*., 1984), cytocytes (Abdel-Rahman *et al*., 2000) or OEs (Gelbic *et al*., 2006). Also, four types had been distinguished in the larval haemolymph of the same insect by some researchers (Harpaz and Zelcer, 1969; Gelbič *et al*., 2006; Asiri, 2017). On the other hand, our result consistently agreed with the reported five types of circulating hemocytes in last instar larvae of the same insect by many authors (Zohry, 2006; Hassan *et al*., 2013; Shaurub *et al*., 2014; Abou-Taleb *et al*., 2015).

However, these diverse results might be attributed to the insect species itself or its developmental stage and some technical difficulties for identification as well as the characters adopted by other researchers (George and Ambrose, 2004; Ribeiro and Brehelin, 2006). In addition, hemocyte morphology and thus the type identification have been affected by some factors influencing the haemolymph physical properties or biochemical composition (Carrel *et al*., 1990), the physiological condition of the insect (Chapman, 1998) and the insect developmental stages (For a recent review, see Ghoneim, 2019). Therefore, classification of the circulating hemocytes in an insect has been recommended to be revised several times (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 reported by Romosen and Stofolano (1998), total hemocyte count (THC) has been varied from insect to another insect and depends upon the developmental stage and physiological state of the insect itself as well as upon the technique followed. On the other hand, in his comprehensive review, Ghoneim (2019) discussed different factors intervening in the THC in insects, such as the brain endocrine complex following some initial stimulus as well as the ecdysteroids, which may regulate the population of haemocytes. In addition, synthetic insecticides and insect growth regulators (IGRs) intervene in the intermediary metabolism and immune capability of insects reflecting on some changes in the hemocyte population, differentiation and phagocytosis (for some detail, see Hernandez *et al*., 1999; De Silva *et al*., 2000; Qamar and Jamal, 2009).

In the current study on *S. littoralis*, Novaluron remarkably induced the hemocyte population at two limits of the larval instar, because THC pronouncedly increased but decreased during the middle period. On the other hand, Cyromazine exhibited an inhibitory effect throughout the majority of larval instar, with few exceptions. The induced THC by Novaluron was in agreement with some of reported results for the same insect species by different chitin synthetic indicators (CSIs), such as diflubenzuron (Osman *et al*., 1984), flufenoxuron or 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, a similar THC induction was reported for other insect species, such as *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. Also, injection of *B. mori* larvae with 20-ecdysone led to significantly enhanced hemocyte density at approximately 12-18 h post-injection (Ling *et al*., 2003b). THC increased in *Coccinella septempunctata* after treatment with the bacteria-derived spinosad (Suhail *et al*., 2007) and *Eurygaster integriceps* after treatment with methoxyfenozide (Zibaee *et al*., 2012). Recently, treatment of *Rh. ferrugineus* last instar larvae with pyriproxyfen resulted in significantly increased THC in the haemolymph (Hamadah and Tanani, 2017). Also, both Novaluron and Diofenolan exhibited strong inducing effects on THC in larvae of *P. gossypiella* (Ghoneim *et al*., 2017)

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

The general inducing effect of Novaluron on THC, in the present study, may be due to the induced encapsulation of foreign/toxic molecules through the melanization process, since melanin deposition during encapsulation has been commonly initiated by haemocytes and/or phenoloxidase circulation in plasma (Rolff and Siva-jothy, 2002; Nappi and Christensen, 2005). Also, it may be attributed to the release of sessile haemocytes and/or activation of mitosis of haemocytes, because many insects possess populations of sessile haemocytes which might be activated in response to some insecticides or IGRs. Moreover, the increased THC in the present investigation on *S. littoralis* might be considered as an immune response against a pathogen or any foreign body, such as the introduced CSIs (Chu *et al*., 1993; Anderson *et al*., 1995; Ordas *et al*., 2000).

On the other hand, the prevalent prohibitory effect of Cyromazine on THC in *S. littoralis*, in the current investigation, might be correlated with the decrease of some hemocyte types involved in the phagocytosis and/or the nodule formation. Reduction of THC might be due to the toxicities of IGRs and their suppressing effects on the insect endocrines 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 might be due to the death of pathological cells *via* the degeneration (Sendi and Salehi, 2010).

3. Differential Haemocyte Populations in *S. littoralis*:

In the current study, the differential haemocyte populations or differential haemocyte (DHCs) in haemolymph of last instar larvae of *S. littoralis* had been changed depending on the hemocyte type, the tested CSI and the larval age, as should be discussed herein.

PRs Population: Novaluron caused an insignificant increase in the PRs population during the first half of the last larval instar of *S. littoralis*, but a slight decrease during the second half. On the other hand, Cyromazine exhibited a prevalent inhibitory effect on the PRs population, regardless the larval age. The decreasing PRs count was, to some extent, in conformity with those reported results of decreasing PRs in the same insect after larval treatment with flufenoxuron (Zohry, 2006) as well as in other insects, such as *R. kumarii* by some insecticides (George and Ambrose, 2004) and *A. ipsilon* by diflubenzuron (Abdel-Aziz and Awad, 2010). On the other hand, increasing PRs population was reported in *M. separata* after treatment with hydroprene (Wang *et al*., 1993), *R. kumarii* after treatment with endosulfan (George and Ambrose, 2004), *S. gregaria* by CSI teflubenzuron (Teleb, 2011), *Rh. ferrugineus* after treatment of with sublethal concentrations of pyriproxyfen (Hamadah and Tanani, 2017), etc. Although PRs are progenitor stem cells which can be transformed into other hemocyte types depending on the light and electron microscopy observations (Yamashita and Iwabuchi, 2001; Lavine and Strand, 2002), the exact function of this type of haemocytes is still unknown (Ribeiro and Brehelin, 2006). As reported by Liu *et al*. (2013), PRs in *B. mori* can transform into PLs and GRs. However, the general declination of PRs population in larvae of *S. littoralis*, in the present investigation, might be attributed either to the cytotoxic effects of the tested CSIs on the mitotic division of PRs, conversion to other cell types or to their inhibitory effects on the activity of haematopoietic organs responsible for PRs production (Zhu *et al*., 2012; Zibaee *et al*., 2012).

PLs Population: Novaluron slightly induced the PLs population during first half of the last larval instar of *S. littoralis*, in the present work, but slightly reduced during the second half. On the other hand, Cyromazine remarkably enhanced the PLs population, regardless the age of larvae. The PLs enhancement was, to a great extent, in agreement with the increasing PLs count in a number of insect species after treatment with some insecticides or IGRs, such as *S. gregaria* nymphs after treatment with Lambdacyhalothrin or Deltamethrin (Al-Hariri and Suhail, 2001) or teflubenzuron (Teleb, 2011); *R. kumarii* after treatment with endosulfan (George and Ambrose, 2004); *A. ipsilon* after treatment with diflubenzuron (Abdel-Aziz and Awad, 2010); *S. litura* after treatment with hexaflumuron (Zhu *et al*., 2012). On the other hand, the slight decrease in PLs count during the second half of larval instar, as a response to Novaluron in the present study, was in accordance with the decreasing PLs count in the same insect after treatment with LC_{50} of flufenoxuron (Bakr *et al*., 2007) and other insects, such as *R. kumarii* by some insecticides (George and Ambrose, 2004), *S. gregaria* after treatment with some insecticides, spinosad and proclaim (Halawa *et al*., 2007), etc. Some authors (Tojo *et al*., 2000; Ling and Yu, 2006) reported that the role of PLs in phagocytosis has been disputed because they may perform as phagocytes while other authors (Neuwirth, 1973; Beaulaton, 1979) reported no phagocytic function. The decreasing PLs population in the current study on *S. littoralis* can be explained by their transformation into other hemocyte types (George, 1996) because they are highly polymorphic cells (Gupta and Sutherland, 1966). Also, Novaluron might halted the haematopoietic organs which responsible for the production of this hemocyte type (Tiwari *et al*., 2002). On the other hand, no appreciable interpretation could be provided to the increasing PLs population, as found in 6^{th} instar larvae of the present insect *S. littoralis* at all ages by Cyromazine or during the first half of instar by Novaluron, at the present time!!

GRs Population: In the current study, treatment of *S. littoralis* larvae with novaluron led to decline of GRs population throughout the last larval instar, with few exceptions. Moreover, GRs population in larvae of all ages cyromazine was considerably inhibited. These results were evidently in agreement with some reported results of decreasing GRs in

the same insect after treatment with metyrapone (Gelbic *et al*., 2006), flufenoxuron (Bakr *et al*., 2007), or some urea waste-derived compounds (Hassan *et al*., 2013), as well as declined GRs population in other insects, such as *R. kumarii* by endosulfan (George and Ambrose, 2004) and *S. litura* by hexaflumuron (Zhu *et al*., 2012). In contrast, GRs population was induced in *A. ipsilon* by Diflubenzuron (Abdel-Aziz and Awad, 2010) and *S. gregaria* by teflubenzuron (Teleb, 2011) or Lambdacyhalothrin and Deltamethrin (Al-Hariri and Suhail, 2001).

Although phagocytosis has been reported as one of the main functions of GRs in haemolymph of different insect species, such as *B. mori* (Wago, 1980), *Galleria mellonella* (Tojo *et al*., 2000), *M. sexta* (Nardi *et al*., 2001), *Heliothis armigera* (Essawy *et al*., 1985), *Spodoptera exigua* (Pendland and Boucias, 1996), *Lymantria dispar* (Butt and Shields, 1996) and *S. littoralis* (Costa *et al*., 2005**)**, the general decrease of GRs in *S. littoralis* larvae by the tested CSIs, in the current study, might be interpreted by the death of a lot of them owing to their detoxification activity against the toxic molecules (Nardi *et al*., 2001; Barakat *et al*., 2002; George and Ambrose, 2004; Costa *et al*., 2005). Also, it might 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).

SPs Population: Very few studies reported that SPs population increase in insects, as a response to some insecticides or IGRs, such as *A. ipsilon* by Diflubenzuron (Abdel-Aziz and Awad, **2010**). In the current study on *S. littoralis*, novaluron induced the SPs population throughout the last larval instar, with few exceptions, while cyromazine exhibited a similar effect only during the middle period and inhibited their population at two limits of the larval instar. The major inducing action of these CSIs disagreed, however, with the decreasing population as reported in some insects by some insecticides and IGRs, such as *S. gregaria* nymphs by teflubenzuron (Teleb, 2011) or Lambdacyhalothrin and Deltamethrin (Al-Hariri and Suhail, 2001), *M. separata* by KK-42 (Wang *et al*., 1993) and *P. demoleus* by methoprene (Sendi and Salehi, 2010). The functions of SPs are exactly 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 larval treatment with the tested CSIs in the present investigation might be due to their inducing effects on the SPs differentiation or the transformation of other hemocytes into SPs in the last instar of *S. littoralis*. The exact mode of action is still obscure!!

OEs Population: As recorded in the present study on *S. littoralis*, larval treatment with Novaluron or Cyromazine enhanced the OEs population during the second half of the last instar. A similar increase of OEs count in the same insect was reported after treatment with hexaflumuron (Abu El-Magd *et al*., 1994: Zhu *et al*., 2012), as well as in other insects, such as *S. gregaria* after treatment with teflubenzuron (Teleb, 2011). Induced OEs population in the present investigation might be due to their role in the detoxification of toxic materials and activating action of the tested CSIs on the hematopoietic organs or cell mitosis. In agreement with the unaffected OEs count in *S. littoralis* after treatment with flufenoxuron (Zohry, 2006), no effect was recorded for CSIs on these hemocytes within the 2-day old larvae of the same species, in the present study. This result might be understood in the light of hemocyte resistance to penetration of these compounds and thus remain unaffected.

An exceptional case of declined OEs count at the beginning of last instar by novaluron, in the present study, agreed, to some extent, with the reported decreasing OEs population in the same insect by teflubenzuron (Abdel-Al *et al*., 2011) and in *S. gregaria* by Lambdacyhalothrin or Deltamethrin (Al-Hariri and Suhail, 2001). The declined OEs population at this time of larval instar might be due to degeneration of some cells for

releasing precursors of prophenoloxidase (PPO) that likely plays a role in melanization of haemolymph and an crucial immunity protein in insects (Ribeiro *et al*., 1996). Otherwise, recent studies in different insects show that additional hemocyte types contain PPO (Liu *et al*., 2013).

In general, the increasing populations of some haemocyte types and decreasing counts of others might be due to the transformation of some types into other types in order to achieve the phagocytic function or other functions in the battle against biotic targets, like bacteria, yeast and apoptotic bodies, as well as against abiotic materials, such as particles of Indian ink (Hernandez *et al*., 1999; De Silva *et al*., 2000). Furthermore, DHCs fluctuate not only as a consequence of different larval instars of the insect but also within a given instar. These changes may be a result of developmental processes (Gelbic *et al*., 2006).

4. Qualitative Profile of Haemocytes in *S. littoralis*:

Some pathogenic microorganisms, insecticides and IGRs have been reported to cause some disruptions in insect haemocytes basing on alteration in the plasma membrane (erosion and extrusion of their cytoplasmic contents), lysis and/or vacuolization of the cytoplasm and some nuclear changes, as recorded in different insects, such as *Plodia interpunctella* (El-Kattan, 1995), *S. gregaria* (Barakat *et al*., 2002) and *S. littoralis* (Bakr *et al*., 2007), *Rh. ferrugineus* (Hamadah and Tanani, 2017) and *P. gossypiella* (Ghoneim *et al*., 2017). In the current study on *S. littoralis*, both Novaluron and Cyromazine exhibited serious cytopathological effects on all hemocyte types, except OEs which had not been affected by Cyromazine. The major symptoms of cytological disorders appeared as darkly stained cells, destroyed plasma membranes, protruded cytoplasmic contents, cytoplasm lysis and appearance of cytoplasmic vacuoles. However, OEs were the least affected hemocyte type as reported in *D. cingulatus* after treatment with Acephate (Qamar and Jamal, 2009) and *S. gregaria* after treatment with teflubenzuron (Teleb, 2011).

The cytological disorders of *S. littoralis* haemocytes, in the present study, might be due to dangerous action of the tested CSIs on the 'actin' which localized in the lamellar extensions of the cells, sine naturally originating pesticide molecule may exhibit its activity by targeting actins (Anunradha and Annadurai**,** 2008). No conceivable interpretation of the intracellular disruptions in hemocytes of *S. littoralis* by the tested CSIs, in the present study, has been provided right now!! Although Schmutterer (1990) reported that the developmental effects caused by IGRs and/or botanicals can be attributed to the disruption of some endocrine events, a question that the hemocytes are affected directly or *via* some physiological or endocrinological pathways remains to be answered.

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

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