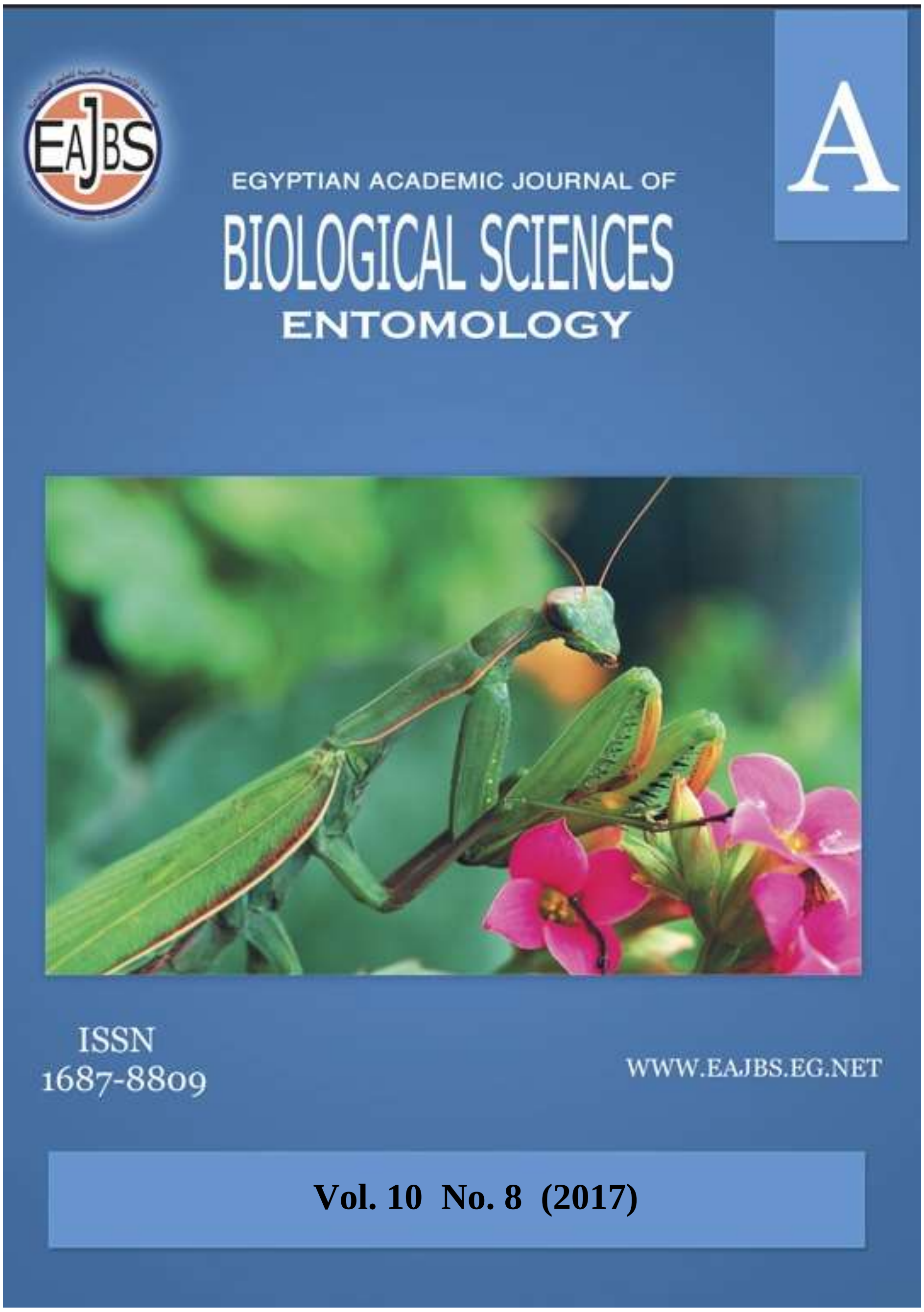

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Disruptive Impacts of Selected Insecticides on Larval Haemogram parameters of the Red Palm Weevil, *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae)

Hamadah, Kh. Sh.* & Tanani, M.A.
Faculty of Science, Al-Azhar University, Madenit Nasr, Cairo, Egypt
*Corresponding author: E.mail: khalid_hamadah@Azhar.edu.eg

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ABSTRACT

The present study was conducted to investigate the effect of three insecticides, pyriproxyfen (admeral), neemazal (azadirachtin) and spinetoram (Radiant), against the red palm weevil *Rhynchophorus ferrugineus.* Two sublethal concentrations of each compound had been estimated in a preliminary test: LC_{50}: 1067.5 ppm, LC_{75}: 2317.5 ppm (pyriproxyfen), LC_{50}: 14600 ppm, LC_{75}: 27100 ppm (neemazal), LC_{50}: 18.37 ppm, LC_{75}: 88.60 ppm (spinetoram). These concentrations were tested against the immune cells (Total hemocyte count, Differential hemocyte count and hemocyte deformations). Depending on the present study, five types of hemocytes were observed as prohemocyte, granulocyte, plasmatocyte, oenocyte and spherulocyte. Total hemocyte count was significantly increased irrespective to the insecticide. All insecticide induced the prohemocyte while the other types were declined. However, the tested insecticide exhibited pathological symptoms on insect haemocytes morphology in the cell membrane, cytoplasm and nucleus.

INTRODUCTION

The red palm weevil or the sago palm weevil (RPW), *R. ferrugineus* (Oliver), (Coleoptera: Curculionidae) was first recorded in Emirates at 1986 and spread to the Gulf States and spanned the red sea into North Africa as the latest record since 1992 in Egypt. A significant damage was caused to wide genera of palms, and this makes it desirable control (EPPO 2008). In Egypt, control programs of RPW mostly rely on the use of various conventional insecticides. Other than, the attained benefits from uses of insecticides but excessive uses cause many problems to human, beneficial insects, residual toxicity and pollution. So there is a great need to use a safe alternative insecticide with the new mode of action as microbial insecticides, plant extracts and insect growth regulators. Spinosad (microbial insecticide) is abiotic insecticide derived from soil dwelling bacteria, *Saccharopolyspora spinosa* (Mertz and Yao) that exerts its toxic action by contact or ingestion. It targeting a nicotinic acetylcholine receptor as well as ɤ-aminobutyric acid (GABA) gated chloride channels causes insect paralysis (Salgado *et al.* 1997; Watson 2001; Sparks 2004; Sarfraz *et al.* 2005). It possesses the low risk to mammals and predatory insects, parasitoids and honeybees, degrades by sunlight, and has a novel mode of action (Bret *et al.* 1997; Salgado 1997). Pyriproxyfen (juvenile hormone analogue, JHA) disrupt the function of the endocrine system by preventing the larvae from reaching to the adult stage. It has relatively low mammalian toxicity and was used for controlling public health pests, white flies, *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) and *Trialeurodes vaporariorum* (Westwood), the oblique banded leaf roller, *Choristoneura rosaceana* (Harris) and 1st instar larvae of *Spodoptera exigua* (Ishaaya & Horowitz 1992; Miyamoto *et al.* 1993; Ellsworth *et al*. 1997; Ishaaya *et al.* 1994; Sial & Brunner 2010; Moadeli *et al.* 2014). The insecticidal activity of plant extracts has considered as attractive alternatives to synthetic chemical insecticides for little threats to the environment and human health (Koul & Walia 2009). Neem seed and leaf extracts, as well as, azadirachtin and Neemazl (20% azadirachtin content), are active as antifeedant, repellents, disrupt growth, inhibit moulting and oogenesis (Butterworth & Morgan 1968; Zanno *et al.* 1975; Steets 1976; Rembold & Sieber 1981; Koul 1984; Garcia & Rembold 1984; Dorn *et al.* 1986; Richer *et al.*1997; Ghoneim *et al.* 2000). Insect haemocytes are comparable to the leucocytes of the vertebrates (Jones 1962, 1975; Ahmed & Khan 1988; Han & Gupta 1989; Gupta 1979, 1991; Bardoloi & Hazarika 1992). These are mainly responsible for cellular and humoral immune responses including phagocytosis, encapsulation, nodule formation as well as antimicrobial peptides (Hoffmann 2003; Kanost *et al*. 2004). Therefore the purpose of this study was to compare the efficacy of three compounds represents three insecticidal categories spinetoram (microbial insecticides), pyriproxyfen (juvenile hormone analogue) and neemazal (plant extracts) on haemograme (Total haemocyte count (THC), Differential haemocyte count (DHC) and Histopathology of haemocytes) of the dangerous pest of the date palms to determine the affected immunity extent.

MATERIALS AND METHODS

Insect Culture:

The red palm weevil *R. ferrugineus* colony was established from field – collected pupae. The weevil was reread on sugarcane stem at 29^{o}C ± 2 and 60-70 % RH, the light intensity of about 30 foot-candles is provided with fluorescent tubes **(**Rahalkar *et al*. 1972; Rananvare *et al*. 1975).

Insecticides:

The following chemicals, spinetoram 5% (radiant), pyriproxyfen 10% (admeral) and neemazal (azadirachtin 20%) were kindly supplied from the Laboratory of Insecticides, Agricultural Research Centre, Doqqi, Giza, Egypt.

Haematological Studies:

LC_{50} & LC_{75} were calculated for each insecticide from our previous study (Hamadah & Tanani 2013), and prepared by dissolving in distilled water**.** These concentrations are LC_{50}: 1067.5 ppm and LC_{75}: 2317.5 ppm for (pyriproxyfen), LC_{50}: 14600 ppm and LC_{75}: 27100 ppm for (neemazal), and LC_{50}: 18.37 ppm and LC_{75}: 88.60 ppm for (spinetoram). The 0-day old 5th (newly moulted last instar of *Rh. ferrugineus*) were fed on fresh internode piece of sugarcane stem after dipping in different concentration levels of each insecticide for 24h.

For the determination of total, differential haemocyte counts and haemocytes morphology, the haemolymph was collected after 24h treatment of the 0-day old 5th instar larvae. The haemolymph was obtained by non-heparinized capillary tube after amputation of one or two prothoracic legs, before coxa of the larva using fine scissors and gentle pressure on the thorax and abdomen. Three replicates were used and the haemolymph from two individuals was never mixed.

Total haemocyte count (THC): The haemolymph was collected into Thomas – white blood cell diluting pipette to the mark (0.5). Diluting solution (NaCl – 4.65 g, KCl – 0.15 g, $CaCl_2$ – 0.11 g, Crystal violet – 0.05 g and acetic acid – 1.25 ml / l distilled water) was taken up to the mark (1) 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 3 min, 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.

Differential haemocyte count (DHC) and pathological symptoms: Stained haemolymph preparations were carried out, according to Arnold & Hinks (1979). The haemolymph was smeared on clean glass slides, allowed to dry for 1 – min, and fixed for 2 – min with drops of absolute methyl alcohol. Fixed cells were stained with Giemsa῾s solution (diluted 1 : 20 in distilled water) for 20 min, 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 oil immersion objective with Olympus microscope at a magnification 100 X 40 = 4000 and 100 cells per slide were examined for both DHC and pathological symptoms. The cell shape, cytoplasmic ratio, cytoplasmic inclusions and shape of the nucleus were used for classification of haemocytes using the classification scheme of some authors (Al-Khalifa & Siddiqui 1999; Gadelhak 2005; Manachini *et al*. 2011). The percentages of haemocyte types were calculated by the formula:

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

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 the difference between means.

RESULTS

Identification of Hemocytes:

In untreated haemocyte-types were identified for their size, morphology and dye-staining properties**.** Five types of haemocytes were identified as follow: 1) Prohaemocyte (pro.): strongly basophilic, round and small haemocytes. 2) Granulocyte (gra.): Oval or round and their cytoplasm is mildly basophilic. 3) Plasmatocyte (pla.): Oval or spindle and vacuolated cytoplasm with high nucleus/cytoplasm ratio. 4) Oenocyte (oen.): Round (regular in shape) with round eccentric nucleus and nucleus more basophilic than cytoplasm. 5) Spherlocyte (sph.) (also known as cystocyte): irregular in shape contain basophilic inclusion (spherules) (Fig. 1). The most abundant type of haemocyte was pro. (69.7%) followed by gra. (32.0%), pla. (10.0%), oen. (6.3%) and sph. (3.7%) (Fig. 2). All insecticide significantly increased THC except the lowest concentration of spinetoram (LC_{50}) by

2133.3±125.8 vs 2350.0±100.0 cell/mm^3 of control. However, the highest THC was recorded for pyriproxyfen (LC_{50}) by 8266.7±132.3 cell/mm^3 (Fig. 2). To some extent, the THC was drastically induced with the increase in population mainly of pro. (Fig. 2&3). With respect to the DHC, pro. was significantly induced and the highest value was recorded for neemazal (LC_{50}) 86.3±1.2 followed by (LC_{75}) for spinetoram 84.3±1.5 vs 69.7±1.2 % of control. While the highest concentration of neemazal (LC_{75}) was significantly prohibited it to 56.7±0.6%. On the other hand, the other haemocyte types were drastically declined when compared with that of control insects except the highest concentration of both pyriproxyfen and neemazal promoted the Oen. type to 8.3 ± 0.6 and 14.3±1.2 respectively, vs 6.3±0.6% of control insects (Fig.3).

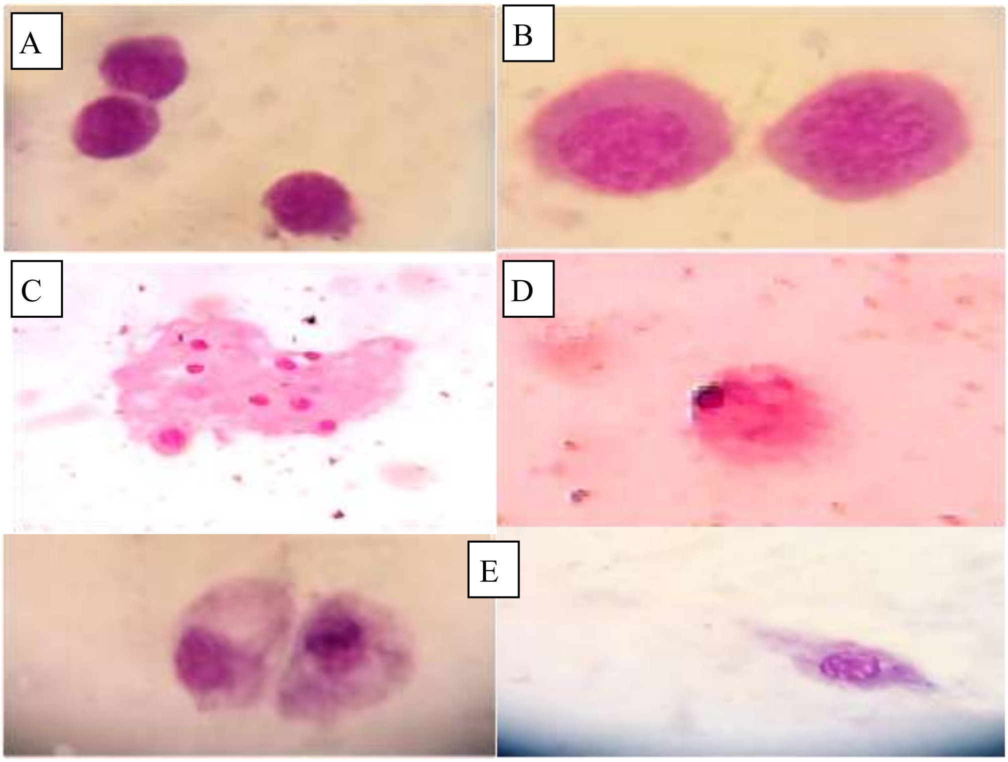

Fig. 1. Normal hemocytes of the red palm weevil *Rh. ferrugineus*. Where: A) Prohemocyte:. B) Granulocyte. C) Spherulocyte. D) Oenocyte. E) Plasmatocyte.

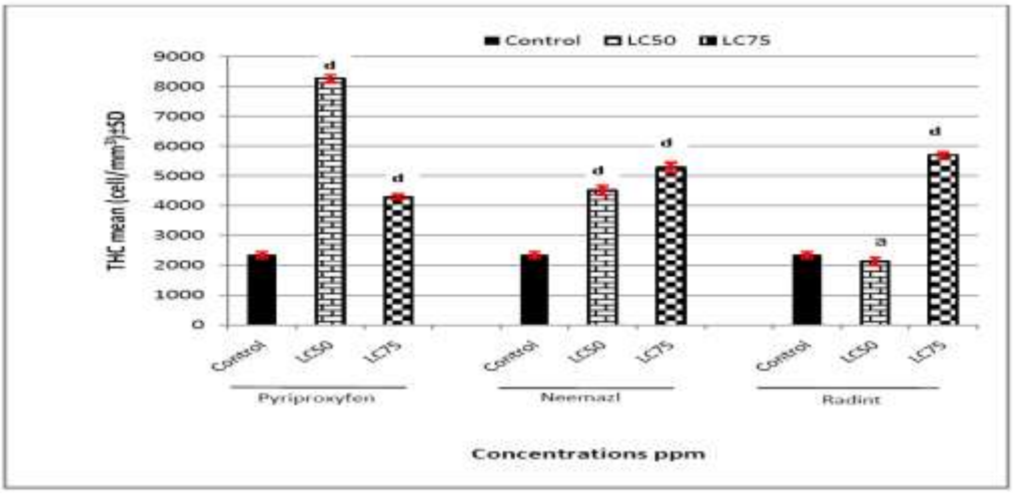

Fig. 2. Total hemocyte count (mean±SD) of *Rh. ferrogenius* after 24 h treatment of larvae (5th instar) during feeding treatment by Pyriproxyfen, neemazal and spinetoram. Where: a: nonsignificant (P>0.05), d: extremely significant (P<0.001).

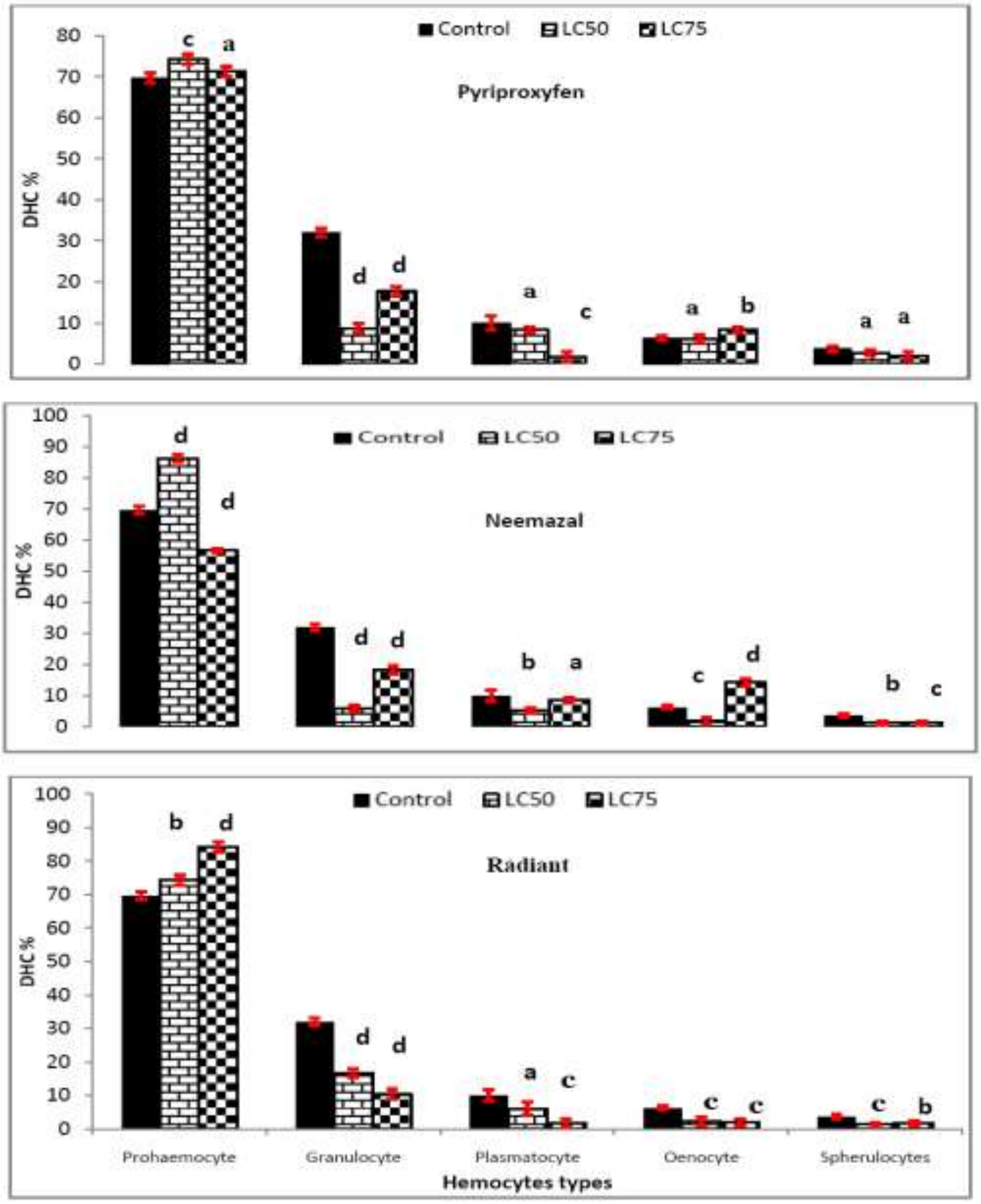

Fig. 3. Differential hemocyte count (mean±SD) of *Rh. ferrugineus* after 24 h treatment of larvae (5^{th}) during feeding by pyriproxyfen, neemazal and spinetoram. Where: a: nonsignificant (P>0.05), b: significant (P<0.05), c: very significant (P<0.01), d: extremely significant (P<0.001).

Effect on Hemocytes Morphology:

Generally, the tested insecticides in this study seem to affect the cell membrane, cytoplasm and nucleus of the haemocyte. The most sensitive cells were found to be the pro. followed by gra., pla. and oen. but no effect was observed for sph.. pyriroxyfen was the compound that induces more deformations to haemocytes followed by neemazal or spinetoram. However, the unique effect was recorded as the deeply stained cytoplasm of gran. by pyriproxyfen LC_{50} and pla. vacuole by spinetoram LC_{75} (Fig.4). For more details, pyriproxyfen LC_{50} activate pro. vacuole, gran. vacuole, deeply stained cytoplasm of gran., deeply stained pro. aggregation and distortion of the cell membrane of oen. with pycnotic nucleus (Fig.4, A, E, D, C and G). pyriproxyfen LC_{75} promote lysed pro., deeply stained pro. aggregation, distortion of the cell membrane of pla. and distortion of the cell membrane of oen. with pycnotic nucleus. (Fig.4, B, C, F, G). neemazal LC_{50} stimulates distortion of cell membrane of pla. and deeply stained pro. (Fig.4, F, H). Neemazal LC_{75} caused distortion of the cell membrane of pla., distortion of the cell membrane of oen. and deeply stained pro. (Fig.4, F,G, H). spinetoram LC_{75} caused deeply stained pro. (Fig.4, H). spinetoram LC_{50} induced aggregated deeply stain pro., distortion of cell membrane of oen. with pycnotic nucleus, deeply stain pro. and pla. vacuole (Fig.4, C, G, H, I).

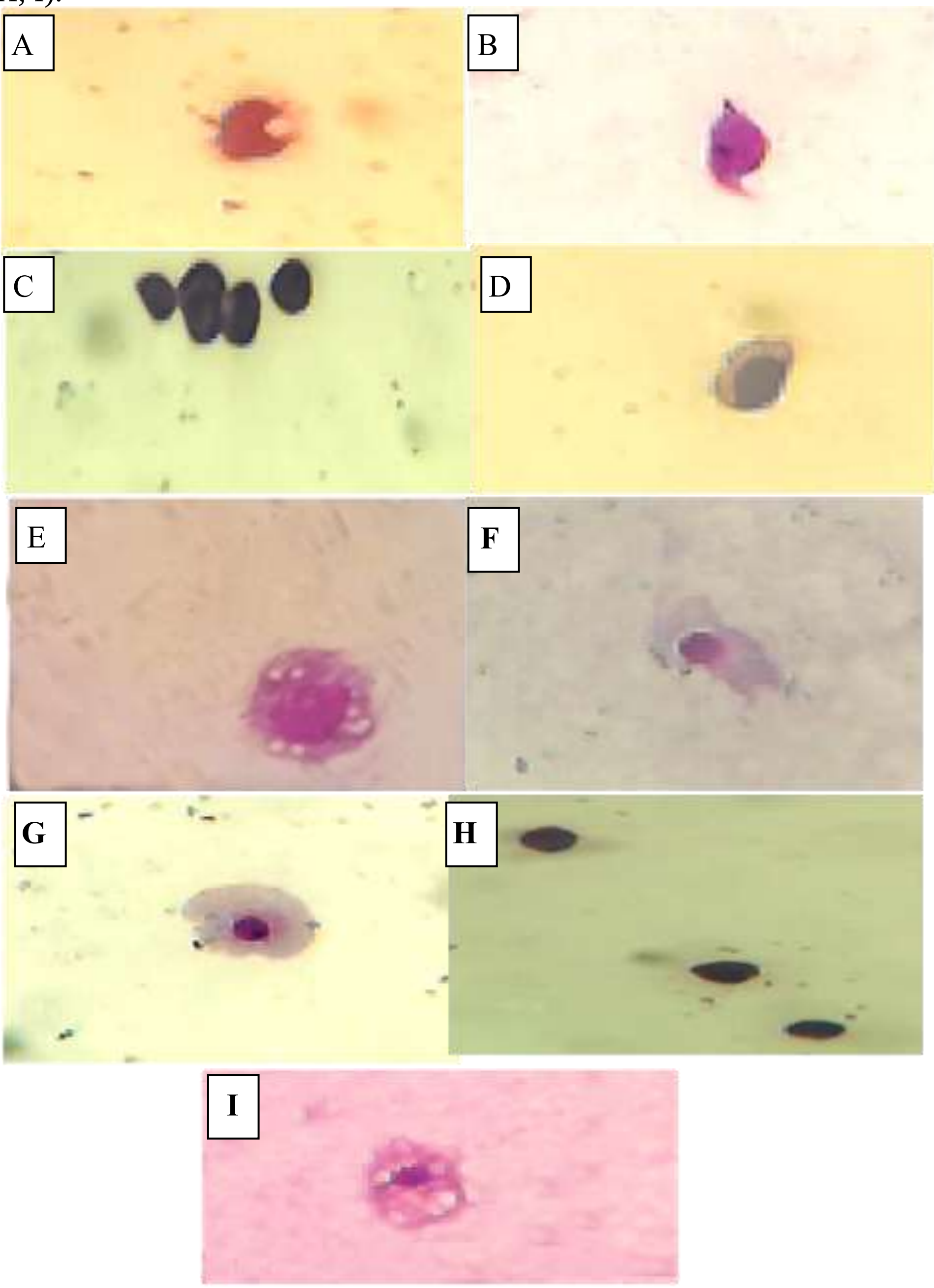

Fig. 4. Malformed hemocytes of *Rh. ferrugineus* after 24 h treatment of larvae (5^{th}) during feeding by pyriproxyfen, neemazal and spinetoram. Where: A: Prohemocyte vacuole, B: Lysed prohemocyte, C: Deeply stained prohemocyte aggregation, D: Deeply stained cytoplasm of granulocyte, E: Granulocyte vacuole, F: Distortion of cell membrane of Plasmatocyte, G:Distortion of cell membrane of oenocyte with pycnotic nucleus, H: deeply stained prohemocyte, I: Plasmatocyte vacuole.

DISCUSSION

Several factors can change the Insect's immune responses such as insecticides, hormones, environmental temperature, etc. (Mandato 1998). However, the haemocytes cellular immunity, may also be included in metabolism and detoxification of xenobiotics (Kurihara *et al*. 1992; Rahimi *et al.* 2013). So the current study was conducted to find effects of three compounds represent three insecticidal categories spinetoram (microbial insecticides), pyriproxyfen (JHA) and neemazal (plant extracts) on insect cellular immunity by evaluating numbers of haemocytes (THC & DHC) and Haemocyte morphology. We identified five types of haemocytes: pro., gra., pla. oen. and sph. in the last instar larvae (5^{th}) of RPW. The identification depends on the RPW hemocyte literature: the same five types were recorded by Gadelhak (2005) and Manachini *et al.*, 2011 while six types (pro., pla., gra., oen., sph., and adipohemocytes) were recorded by Al-Khalifa & Siddiqui (1999). In this study, pro. was the most abundant type of haemocytes. Other studies on the same insect, RPW, showed that other types of haemocytes were the most abundant as pla. by 50% (Manachi *et al.* 2011), pla. by ≥70% (Al-Khalifa & Siddiqui 1999), or gra. by 27.58% (Gadelhak 2005). On the other hand, the differences in determination of haemocytes types can be controversial but may be attributed to the differences in species or stage of the same species, some factors, physiological conditions, technical difficulties for identification and the characters adopted by other workers (Carrel *et al.* 1990; Chapman 1998; George & Ambrose 2004; Ribeiro & Brehelin 2006). With respect to THC, all tested insecticides significantly increased it after 24h of treatment of last instar larvae 5^{th} RPW. For DHC, all types significantly decreased in % except the Pro. type that significantly increased in comparison with that of the control insects with few exceptions. The increased of THC was associated with the increase of Pro., in general. Several studies exhibited the effect of IGRs and other insecticides on THC and DHC. Ghasemi *et al.* (2014) showed changes in the total and differential counts (increase and decrease) of the Mediterranean flour moth, *Ephestia kuehniella* hemocytes when treated with pyriproxyfen and methoxyfenozide. On the other hand, Zibaee *et al*. (2012) reported that pyriproxyfen reduced total haemocyte, plasmatocyte and granulocyte numbers in adults of *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae). Significant reduction in the THC of the treated insects by methoprene in *Papilio demoleus* (Sendi & Salehi 2010). Dimilin decreased THC in *Agrotis ipsilon* Fabricius and significantly increased plasmatocytes, granulocytes, and spherule cells as well as a decrease of prohemocytes (Nahla & Awad 2010). Hexaflumuron increased THC and DHC of *Spodoptera litturalis* (Zhu *et al*. 2012). Juvenoid injection into the last nymphal instar of cockroach caused a 50% reduction of haemocytes in the adult (Gupta 1985) Insecticides dimethoate and chlorpyriphos increased THC of the *Platynotus belli* larvae that inversely proportional to the concentration (Chavan *et al*. 2017). Novaluron and diofenolan compounds promote the *Pectinophora gossypiella* larvae (6 and 48 h post-treatment) to produce increasing total haemocyte population. The DHC of each haemocyte type was enhanced or inhibited as a response to the effect of the tested compounds (Ghoneim *et al.* 2017). Also, enhancement of THC was reported for *Spodoptera littoralis* by azadirachtin and its preparation margosan-0 (Ayaad *et al.* 2001), *Agrotis ipsilon* by acetone extract of *Melia azedarach* (El–Sheikh 2002), *Parasarcophaga surcoufi* by azadirachtin (Rizk *et al.* 2001), and *Coccinella septempunctata* by azadirachtin and spinosad (Suhail *et al.* 2007). Azadirachtin 5% caused a slight increase of THC in the early-aged nymphs of the desert locust, *Schistocerca gregaria* but considerable decreasing THC in late-aged ones (Hamadah 2009). THC increased 1 min after treatment of *Coccinella septempuntata* L. with azadirachtin and spinosad, but decreased after application of abamectin. DHC, azadirachtin increased percentage of pla, gra. and sph., decreased pro. and oen. Spinosad increased the percentage of pla., oen. and sph., and decreased percentage of pro. and gra.. Abamectin treatments increased the percentage of gra. and sph., and decreased percentage of pro. and pla. (Suhail *et al.* 2007). In general, the total number of hemocytes is known to change in association with both of detoxification and immune defenses (Kurihara *et al*. 1992), so it is not surprising that these compounds in our study affect haemocyte abundance and variation. The promoted THC in this study is believed dependent to ecdysone titre (Prasada Rao *et al*. 1984). However, Akai & Sato (1971) reported that the increase in ecdysteroids level in *Bombyx mori* L. haemolymph caused a strong release of hemocytes from the haematopoietic organs. However, the increase might also be correlated to the degree of the defensive action of haemocytes that involved in detoxification and to decreased blood volume (Feir 1979; George 1996; George & Ambrose 2004). All types of haemocytes were significantly decreased except the pro. type that may be attributed to cytotoxic effects, inhibition of larval hematopoietic function or the cell proliferation (Zhu *et al*. 2012; Zibaee *et al*. 2012). Some pathological symptoms, in the cell membrane, cytoplasm and nucleus, were recorded and the most sensitive cells were pro., gra., pla. and oen. while no effect on sph. irrespective of the tested insecticide. These symptoms similar to the effect by some the insecticides (Azam & Ilyas 1986; Younes *et al*. 1999; Haq *et al*. 2005), IGRs (Sendi & Salehi 2010; Bakr *et al.* 2007; Ghoneim *et al*. 2015, 2017), entomopathogenic microorganisms and its exotoxins (Venkova 1972; Barakat *et al.* 2002) and phytochemicals (Saxena & Tikku 1990; Sharma *et al*. 2003, Hamadah 2009; Ghoneim *et al.* 2015). The reason for the sensitivity of these cells either could be phagocytic cells attracted to any foreign substance and become suffer from it (Sendi & Salehi 2010) or may be attributed to the action on the 'actin' which localized in the lamellar extensions of the cells (Anunradha & Annadurai 2008).

Conclusion, the present study showed that pyriproxyfen, neemazal and spinetoram affect the RPW immunity and this gives them the opportunity in their use in integrated pest management with sublethal concentrations. However, the affected immunity will reflect on insect life because haemocytes play various roles as the defense against parasites and pathogen, wound repair and moulting.

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