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Qualitative and quantitative changes in the Haemogram of desert locust Schistocerca gregaria (Orthoptera: Acrididae) by extracts of Nigella sativa (Ranunculaceae).

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ABSTRACT

The present work was carried out aiming to assess the *Nigella sativa* extracts on some hematological parameters of nymphs and adults of the dangerous pest *Schistocerca gregaria*. Only three haemocyte types were recognized in haemolymph of nymphs and adults, *viz*., plasmatocytes (PLs), granulocytes (GRs) and coagulocytes (CGs). In the haemolymph, total haemocyte count increased or decreased depending on the developmental stage, nymphal age and extract. All *N. sativa* extracts exhibited prevalent inhibitory effects on PLs in nymphs and adults. In contrast, GRs was generally enhanced in nymphs and adults by both methanol and petroleum ether extracts while promoted by n-butanol extract in nymphs and inhibited adults. Also, enhanced or inhibited CGs depended on the developmental stage, nymphal age, extract and concentration level. The majority of *N. sativa* extracts caused several morphological and intracellular disorders in some hemocytes. The cytopathological symptoms could be described as: cell lysis, destroyed plasma membranes, extruded cytoplasmic contents and vacuolated cytoplasm and nuclei.

Keywords:- Coagulocytes ; Cytopathology ; Granulocytes ; Haemocyte count; Methanol; n-butanol; Petroleum ether; Plasmatocytes;

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1. INTRODUCTION

The desert locust *Schistocerca gregaria* (Forskal)(Orthoptera: Acrididae) has been ranked together with other migratory locusts among the most important crop pests in Africa. Damage caused by the desert locust is a consequence of its polyphagous behaviour, high density of the population, and the nature to aggregate and swarm. Each individual gregarious locust is able to consume roughly its own weight (about 2 grams) in foliage daily [1, 2, 3]. Most recent large-scale outbreaks of it occurred in 1986-1989 and in 2003-2005, mostly on the African continent [4]. Current locust control operations are mainly based on organophosphorus pesticides as a result of the banning of organochlorines [5]. The widespread use of such synthetic pesticides has considerable drawbacks, such as the development of insect resistance to insecticides, increased costs, handling hazards, concerns about insecticide residues, and great threats to both human and environmental health [6]. Therefore, many institutions have intensified their efforts in the search for integrated locust control measures. Much attention has been devoted to use plant extracts or plant constituents that have insecticidal effects [7, 8, 9] because they are generally pest-specific, relatively harmless to non-target organisms and they are biodegradable and consequently harmless to the environment [10, 11]. Because of the multiple sites of action through which the plant materials can act, the probability of developing a resistant population is very low [12].

Nigella plants are widely distributed in countries which border the Mediterranean Sea, central Europe and western Asia [13]. There are many species classified in the genus *Nigella* (Ranunculaceae) [14, 15]. Among the most important medicinal crops in Egypt is *Nigella sativa* which is commonly called as known as black seed or black cumin [16] and "Habbat al-barakah" (the seed of blessing) in Arabic. Seeds of *N. sativa* and their oil have a long history of folklore usage in various systems of medicines. Sharma *et al*. [17] reviewed the medicinal, pharmacological, traditional value and folk remedies of this herb. In pest control, Deshpande *et al*. [18] reported that oleic and linoleic acid as insecticidal components from *N. sativa* which were found to be toxic to *Callosobruchus chinensis* and similar results were obtained [19, 20]. *N. sativa* extracts exhibited toxic effects on *Spodoptera littoralis* [21] and *S. gregaria* [22]. They disrupted the growth, development and metamorphosis of the latter insect [22]. Also, Ahmad *et al*. [23] studied the insecticidal activity of extracts from this herb against the larvae of *Trogoderma granarium* under laboratory conditions. Recently, Khan *et al.* [24] reported the disturbing effects of the acetone seed extract of *N. sativa* on biology and invasion of the stored product pest *Tribolium castaneum*.

In the field of entomology, hemocytes are an attractive research arena for many scientists [25]. In most insects, there are several types of circulating hemocytes and these hemocytes have been the focus of research on cell development and differentiation [26, 27]. 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 [26, 28]. Although the type of immunocytes and their exact role in insects are debatable [29], the primary functions of the insect haemocytes are: coagulation, phagocytosis, encapsulation, detoxification and storage and distribution of nutritive materials (for reviews, see: 26, 27, 30, 31, 32, 33, 34, 35, 36). Insect hemocytes can produce many immune proteins like antibacterial peptides and PPO [37, 38].

The insect pests may be controlled by disturbing their physiological activities, i.e. feeding, molting, reproductive and immune systems [39]. Among the environmental factors affecting insect haemocytes, morphologically and functionally, are insecticides [40]. To a great extent, similar effects of plant products had been recorded for some insects such as *Periplaneta americana* [41], *Dysdercus koenigii* [42], *Cyrtacanthacris tatarica* [43] and *Spodoptera litura* [44]. Plant extracts, at the sub-lethal levels, might be enough to interfere with the function of specific receptors of many insect-species hemocytes, or cause ultrastructural alteration which interfere with normal hemocyte function [45]. For these reasons, the present work was carried out aiming to investigate the influences of *N. sativa* extracts on the haemocyte profile of the locust *S. gregaria*.

2. MATERIALS AND METHODS

2.1. Experimental insect

The desert locust *Schistocerca gregaria* Forskal was used as an experimental insect in the present study. The insects were reared and handled under the crowded conditions of Hunter-Jones [46]. Depending on the improvements of Ghoneim *et al*. [47] Insects were reared in wooden formed cages provided with electric bulbs (150 watt) adjusted to a photoperiod of 12L:12D and to maintain an ambient temperature of 32±2°C. Fresh clean leaves of the Egyptian clover *Trifolium alexandrinum*, in winter, and the leaves of leguminous plant *Sesbania aegyptiaca,* in summer, were used for feeding insects in the stock culture. On the other hand, *T. alexandrinum* leaves only were offered as food for insects of the experimental work.

2.2. Plant extracts

Samples of *Nigella sativa* seeds were purchased from an Egyptian market. The samples were air-dried, powdered and kept in tightly closed amber coloured glass containers for protecting from light, at low temperature. Dried and pulverized powder of *N. sativa* (2 kg) was exhaustively separately extracted with methanol (1.7 Lx3). The combined alcohol extracts were concentrated to 400 ml, diluted with 400 ml of water and the next successively extracted with petroleum ether (5x400 ml) was concentrated to dryness under reduced pressure giving (11 and 90 g), and n-butanol (5x400 ml) extracts were concentrated to dryness under reduced pressure giving (75 and 55 g).

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2.3. Hematological parameters

The early penultimate (4^{th}) instar nymphs were allowed to feed for only 24h on fresh *T. alexandrinum* leaves treated with each of two concentration level (15.0 and 7.0 %) of *N. sativa* methanolic, petroleum ether or n-butanolic extract. For the determination of the total and differential haemocyte counts, the haemolymph was collected from last instar nymphs (of early-, mid- and late-age) and newly emerged adults. The haemolymph was obtained by non heparinized capillary tube after amputation the coxa of the hind leg with fine scissors and gentle pressure on the thorax and abdomen. Three replicates were used and the haemolymph from two individuals was never mixed.

2.3.1. Total haemocyte count

The haemolymph was collected into thoma-white blood cell diluting pipette to the mark (0.5). Diluting solution (NaCl- 4.65 gm, KCl – 0.15 gm, $CaCl_2$- 0.11 gm, Crystalviolet- 0.05 gm and acetic acid – 1.25 ml / liter distilled water) was taken up to the mark (l l) on the pipette (dilution is 20 times). The first 3 drops were discharged to avoid errors. The mixture was dispended to the chamber of counting slide. After 3 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 [48] 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.

2.3.2. Differential haemocyte count:

Stained preparations, according to Arnold and Hinks [49], haemolymph 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 Barakat *et al*. [50]. The percentages of haemocyte types were calculated by the formula:

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

2.4. Statistical analysis of Data:

Data obtained were analyzed by the Student's *t*-distribution, and refined by Bessel correction [51] for the test significance of difference between means.

3. RESULTS

3.1. Identification of circulating hemocytes in nymphs and adults of *S. gregaria*

Only three haemocyte types were recognized in the nymphs and adults of *S. gregaria*, in the present study: plasmatocytes (PLs), granulocytes (GRs) and coagulocytes (CGs) (see Plate 1). The normal PLs can be described as spindle-, oval- or spherical-shaped cells containing basophilic cytoplasm (faintly stained). The large rounded nucleus is centric or ecentric and occupied 40-50 % of the cell volume, as well as contained scattered chromatin masses. Cytoplasm enclosed a moderate amount of rough endoplasmic reticulum (see Plate 1 A, B, C). The normal GRs appeared as spherical- or oval-shaped cells with basophilic cytoplasm (deeply stained) which contained large number of acidophilic granules. Granulocyte had an eccentric nucleus occupying 58.3-66.6 % of the cell volume. Some GRs contained fillopodia (see Plate 1 D, E, F). CGs appeared as spherical- or oval-shaped cells with pale hyaline cytoplasm containing scattered granules (see Plate 1 G).

3.2. Total haemocyte count (THC) of *S. gregaria* as affected by *N. sativa* extracts

After treatment of the penultimate instar nymphs with different extracts of *N. sativa*, data of THC were distributed in Table (1). In haemolymph of the early-aged nymphs, methanol extract exhibited a remarkable stimulatory effect on THC (87.6 and 127.5 % increments, at the highest and lowest concentration levels, respectively) while the n-butanol extract exhibited a slight inhibitory effect on such THC.

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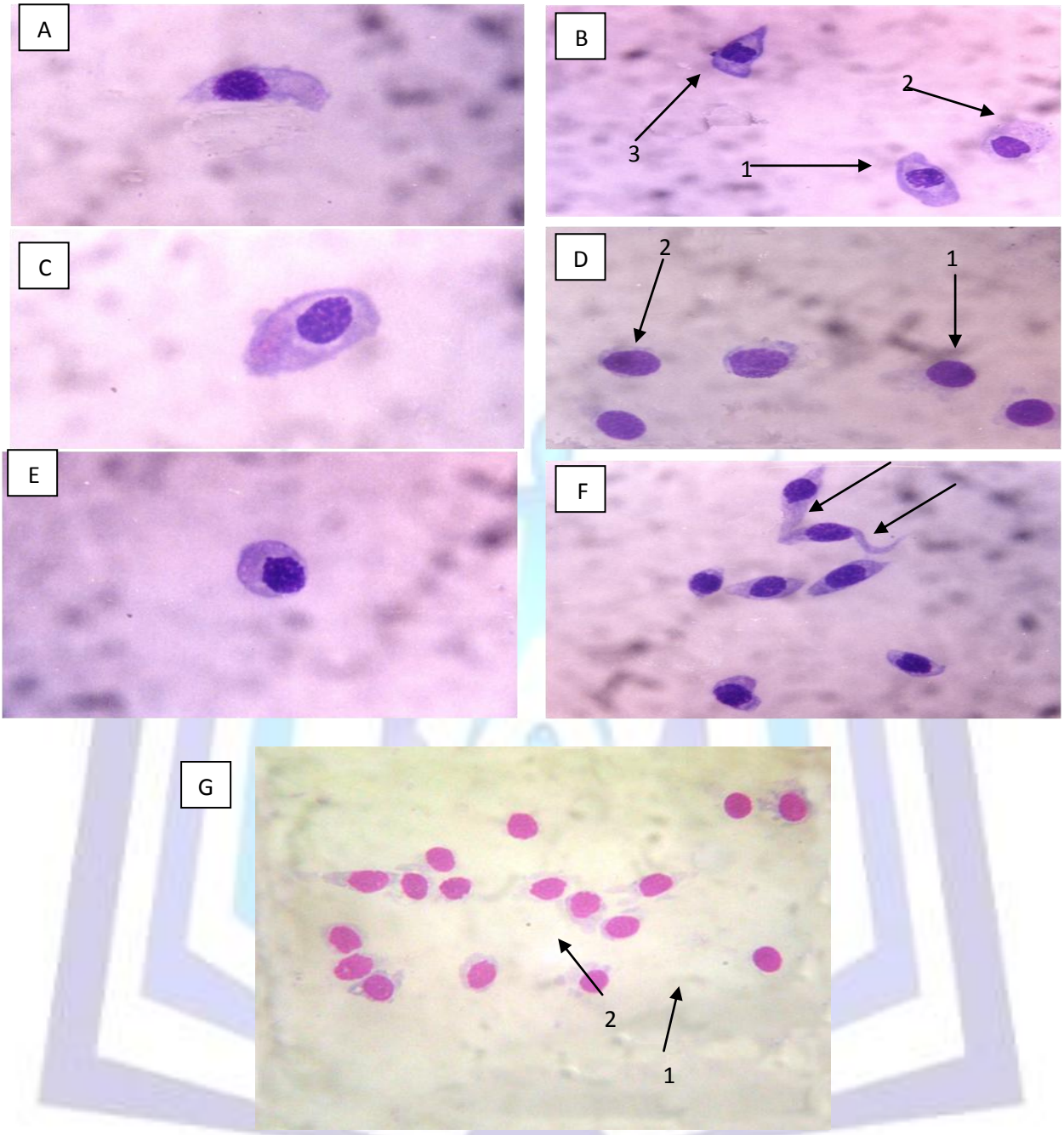

Plate 1: Photomicrographs of normal circulating hemocytes of *S. gregaria*. (A): Spindle-shaped PLs with eccentric nucleus. (B) 1: oval- shaped PLs with centric nucleus, 2: round-shaped PLs with eccentric nucleus, 3: spindle-shaped PLs with centric nucleus. (C): Oval-shaped PLs with eccentric nucleus. (D) 1: round-shaped GRs, 2: oval- shaped GRs. (E): Round-shaped GRs with clear eccentric nucleus and without granules. (F): GRs with fillopodia. (G) 1: round-shaped CGs, 2: oval- shaped CGs.

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Table 1: Altered THC (cell/mm^3) in nymphs and adults of *S. gregaria* by different extracts of *N. sativa.*

Extract	Conc.		Nymphal age			Newly emerged adults
			Early-aged	Mid-aged	Late-aged	
Methanol	15.0	mean ± SD	4783.3 ± 152.8 d	7283.3 ± 208.1 c	3216.7 ± 104.1 d	2200.0 ± 150.0 d
		Change %	+87.6	+16.2	-49.5	-31.3
	7.5	mean ± SD	5800.0 ± 200.0 d	4533.3 ± 125.8 d	3033.3 ± 202.1 d	1383.3 ± 125.8 d
		Change %	+127.5	-27.7	-52.4	-56.8
Petroleum ether	15.0	mean ± SD	1850.0 ± 132.3 c	5983.3 ± 104.1 b	4666.7 ± 160.7 d	3166.7 ± 125.8 a
		Change %	-27.5	-4.5	-26.7	-1.0
	7.5	mean ± SD	5616.7 ± 125.8 d	6866.7 ± 104.1 b	8733.3 ± 104.1 d	2933.3 ± 125.8 a
		Change %	+120.3	+9.6	+37.2	-8.3
n-butanol	15.0	mean ± SD	2333.3 ± 152.8 a	17416.7 ± 175.6 d	5800.0 ± 180.3 b	916.7 ± 175.6 d
		Change %	-8.5	+177.9	-8.9	-71.4
	7.5	mean ± SD	2400.0 ± 150.0 a	6466.7 ± 125.8 a	5816.7 ± 175.6 b	3633.3 ± 152.8 b
		Change %	-5.9	+3.2	-8.6	+13.5
Control		mean ± SD	2550.0 ± 180.3	6266.7 ± 125.8	6366.7 ± 125.8	3200.0 ± 132.3

Conc.: Concentration levels (%), mean ± SD followed with the letter (a): not significantly different (P>0.05), (b): significantly different (P<0.05), (c): highly significantly different (P<0.01), (d): very highly significantly different (P<0.001).

In addition, the effect of petroleum ether extract depended on the concentration level since THC significantly decreased at the highest concentration level (1850.0 ±132.3, compared to 2550.0 ±180.3 cell/mm^3 of control nymphs) but significantly increased at the lowest one (5616.7 ±125.8, compared to 2550.0 ±180.3 cell/mm^3 of control nymphs).

In haemolymph of mid-aged nymphs, only n-butanol extract promoted the hemocyte production since increasing THC was estimated (177.9 and 3.2 % increments, at highest and lowest concentration levels, respectively) while treatment with other extracts resulted in increasing or decreasing THC, depending on the concentration level. However, all extracts exhibited inhibitory effects on the hemocyte production in the late-aged nymphs, with few exceptions.

In respect of the newly emerged adults, hemocyte production was remarkably reduced by the action of methanol extract (31.3 and 56.8 % reductions, at highest and lowest concentration levels, respectively), but were slightly decreased by petroleum ether extract (1.0 and 8.3 % of reductions, at the same concentration levels, respectively). In response to the n-butanol extract, THC was significantly declined at the highest concentration level (916.7±175.6, in comparison with 3200.0±132.3 cell/mm^3 of control adults) but significantly raised at the lowest concentration level (3633.3±152.8, in comparison with 3200.0±132.3 cell/mm^3 of control adults).

3.3 Differential haemocyte count (DHC) of *S. gregaria* as affected by *N. sativa* extracts

To shed some light on DHC in nymphs and adults as affected by *N. sativa* extracts, data assorted in Table (2) clearly show a prevalent prohibiting action of methanol extract on the production of PLs in haemolymph of nymphs and adults. In contrast, GRs production was generally enhanced in nymphs and adults by the same extract, with an exception of

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decreased count in the mid-aged nymphs at the lowest concentration level (20.3% reduction). With regard to CGs, methanol extract evidently promoted their production in nymphs and adults except the late-aged nymphs (38.1 and 37.5% reductions at the highest and lowest concentration levels, respectively).
Considering the petroleum ether extract, data arranged in Table (3) obviously reveal a predominant inhibitory effect on the production of PLs. On the contrary, it enhanced the GRs production since their counts unexceptionally increased in nymphs and adults. Depending on data of the same table, petroleum ether extract promoted the CGs production in the mid-aged nymphs and newly emerged adults, regardless the concentration level (61.4 and 50.5% increments in nymphs and 51.2 and 45.0% increments in adults, at the highest and lowest concentration level, respectively). On the other hand, production of these hemocytes was tremendously inhibited by the same extract in early- and late-aged nymphs, regardless the concentration level.

Table 2: Altered DHC (%) in nymphs and adults of *S. gregaria* by methanol extract of *N. sativa*.

Developmental stage		Conc.		PLs	GRs	CGs
Nymphal age	Early-aged	15.0	mean ± SD	12.3 ± 1.5 d	35.0 ± 1.0 d	52.7 ± 2.5 c
			change %	-67.4	+50.2	+32.7
		7.5	mean ± SD	13.0 ± 1.7 d	28.3 ± 2.5 b	57.0 ± 3.0 c
			change %	-65.5	+21.5	+43.6
		Control	mean ± SD	37.7 ± 1.2	23.3 ± 1.2	39.7 ± 2.3
	Mid-aged	15.0	mean ± SD	13.7 ± 1.2 d	28.7 ± 1.5 c	57.7 ± 2.5 d
			change %	-49.3	-20.3	+55.9
		7.5	mean ± SD	6.3 ± 0.6 d	48.7 ± 1.2 d	45.7 ± 1.5 c
			change %	-76.7	+35.3	+23.5
		Control	mean ± SD	27.0 ± 2.0	36.0 ± 1.0	37.0 ± 1.0
	Late-aged	15.0	mean ± SD	4.0 ± 2.1 a	54.7 ± 2.1 d	41.3 ± 3.1 d
			change %	-24.5	+95.4	-38.1
		7.5	mean ± SD	4.7 ± 2.9 a	53.7 ± 1.5 d	41.7 ± 1.5 d
			change %	-11.3	+91.8	-37.5
		Control	mean ± SD	5.3 ± 1.5	28.0 ± 1.0	66.7 ± 2.3
Newly emerged adults		15.0	mean ± SD	3.3 ± 1.5 d	62.7 ± 2.5 c	34.0 ± 1.7 c
			change %	-85.7	+22.9	+30.8
		7.5	mean ± SD	11.7 ± 1.5 d	56.0 ± 1.7 b	32.3 ± 2.5 b
			change %	-49.1	+9.8	+24.2
		Control	mean ± SD	23.0 ± 1.7	51.0 ± 2.0	26.0 ± 1.0

Conc., a, b, c, d: see footnote of Table (1). PLs: plasmatocytes, GRs: granulocytes, CGs: coagulocytes.

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Table 3: Altered DHC (%) in nymphs and adults of *S. gregaria* by petroleum ether extract of *N. sativa*.

Developmental stage		Conc.		PLs	GRs	CGs
Nymphal age	Early-aged	15.0	mean ± SD	16.7 ± 1.5 d	49.3 ± 2.5 d	34.0 ± 1.0 b
			change %	-55.7	+111.6	-14.4
		7.5	mean ± SD	5.7 ± 1.5 d	61.3 ± 1.5 d	34.3 ± 2.1 b
			change %	-84.9	+163.1	-13.6
		Control	mean ± SD	37.7 ± 1.2	23.3 ± 1.2	39.7 ± 2.3
	Mid-aged	15.0	mean ± SD	12.3 ± 1.5 d	41.0 ± 1.0 c	59.7 ± 2.5 d
			change %	-54.4	+13.9	+61.4
		7.5	mean ± SD	5.0 ± 1.0 d	40.0 ± 1.0 c	55.7 ± 1.5 d
			change %	-81.5	+11.1	+50.5
		Control	mean ± SD	27.0 ± 2.0	36.0 ± 1.0	37.0 ± 1.0
	Late-aged	15.0	mean ± SD	2.0 ± 1.7 a	58.0 ± 1.0 d	40.0 ± 1.0 d
			change %	-62.3	+107.1	-40.0
		7.5	mean ± SD	2.7 ± 1.2 a	53.7 ± 1.5 d	43.7 ± 2.5 d
			change %	-49.1	+91.8	-34.5
		Control	mean ± SD	5.3 ± 1.5	28.0 ± 1.0	66.7 ± 2.3
Newly emerged adults		15.0	mean ± SD	9.3 ± 1.5 d	51.3 ± 0.6 a	39.3 ± 1.5 d
			change %	-59.6	+0.6	+51.2
		7.5	mean ± SD	10.3 ± 2.5 c	52.0 ± 1.7 a	37.7 ± 1.2 d
			change %	-55.2	+2.0	+45.0
		Control	mean ± SD	23.0 ± 1.7	51.0 ± 2.0	26.0 ± 1.0

Conc., a, b, c, d: see footnote of Table (1). PLs, GRs, CGs: see footnote of Table (2).

Data distributed in Table (4) exiguously show a prevalent inhibitory effect of n-butanol extract on the production of PLs, regardless the concentration level or the developmental stage. As easily seen in the same table, GRs production was generally stimulated by n-butanol extract in nymphs. The most remarkably increasing count was determined in the early-

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aged nymphs (144.6% increment, at the highest concentration level) but the least increasing count was estimated in the mid-aged nymphs (4.7% increment, at the lowest concentration level). In contrast, GRs count in adults was drastically regressed (17.1 and 22.2% reductions, at the highest and lowest concentration levels, respectively). The n-butanol extract pronouncedly enhanced the CGs production in adults (103.8 and 51.2% increments, at the highest and lowest concentration levels, respectively) as well as in early- and mid-aged nymphs while it prohibited CGs production in the late-aged nymphs (59.1 and 46.5% reductions, at the highest and lowest concentration levels, respectively).

Table 4: Altered DHC (%) in nymphs and adults of *S. gregaria* by n-butanol extract of *N. sativa*.

Developmental stage		Conc.		PLs	GRs	CGs
Nymphal age	Early-aged	15.0	mean ± SD	16.3 ± 0.6 d	57.0 ± 1.7 d	56.0 ± 1.7 c
			change %	-56.8	+144.6	+41.1
		7.5	mean ± SD	8.7 ± 2.1 d	35.3 ± 1.5 d	56.0 ± 1.0 d
			change %	-76.9	+51.5	+41.1
		Control	mean ± SD	37.7 ± 1.2	23.3 ± 1.2	39.7 ± 2.3
	Mid-aged	15.0	mean ± SD	7.0 ± 1.0 d	41.3 ± 1.5 c	51.7 ± 2.5 d
			change %	-74.1	+14.7	+39.7
		7.5	mean ± SD	12.7 ± 0.6 d	37.7 ± 0.6 a	49.7 ± 0.6 d
			change %	-53	+4.7	+34.3
		Control	mean ± SD	27.0 ± 2.0	36.0 ± 1.0	37.0 ± 1.0
	Late-aged	15.0	mean ± SD	4.3 ± 1.5 a	68.4 ± 2.9 d	27.3 ± 1.5 d
			change %	-18.9	+144.3	-59.1
		7.5	mean ± SD	4.7 ± 1.5 a	59.7 ± 2.3 d	35.7 ± 1.2 d
			change %	-11.3	+113.2	-46.5
		Control	mean ± SD	5.3 ± 1.5	28.0 ± 1.0	66.7 ± 2.3
Newly emerged adults		15.0	mean ± SD	4.7 ± 2.1 d	42.3 ± 1.5 c	53.0 ± 1.0 d
			change %	-79.6	-17.1	+103.8
		7.5	mean ± SD	21.0 ± 1.0 a	39.7 ± 0.6 d	39.3 ± 1.2 d
			change %	-8.7	-22.2	+51.2
		Control	mean ± SD	23.0 ± 1.7	51.0 ± 2.0	26.0 ± 1.0

Conc., a, c, d: see footnote of Table (1). PLs, GRs, CGs: see footnote of Table (2).

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3.4 Qualitative haemocytes profile of *S. gregaria* after nymphal treatments with plant extracts

After treatment of the penultimate instar nymphs with extracts of *N. sativa*, some morphological disorders were observed in haemocytes of the last instar nymphs and newly emerged adults. As clearly seen in Plate (2 E), no morphological disorders were caused in PLs while some of GRs were lysed by the action of petroleum ether extract. Also, some GRs appeared as small darkened cells after treatments with methanol or petroleum ether extract (see Plate 2 B). With regard to CGs, photomicrographs of Plate (2E&F) clearly show some lysed cells but others appeared with ruptured cell membrane and extruded cytoplasmic contents, irrespective of the plant extracts.

As obviously shown in Plate (2 A), numerous vacuoles had been formed in the nuclei some PLs by n-butanol extract. Concerning GRs, the nymphal treatments with petroleum ether extract resulted in the formation of little or many vacuoles in cytoplasm (see Plat 2 C&D). Also, little vacuoles appeared in cytoplasm of CGs as response to the disruptive action, regardless the extract (see Plate 2 G).

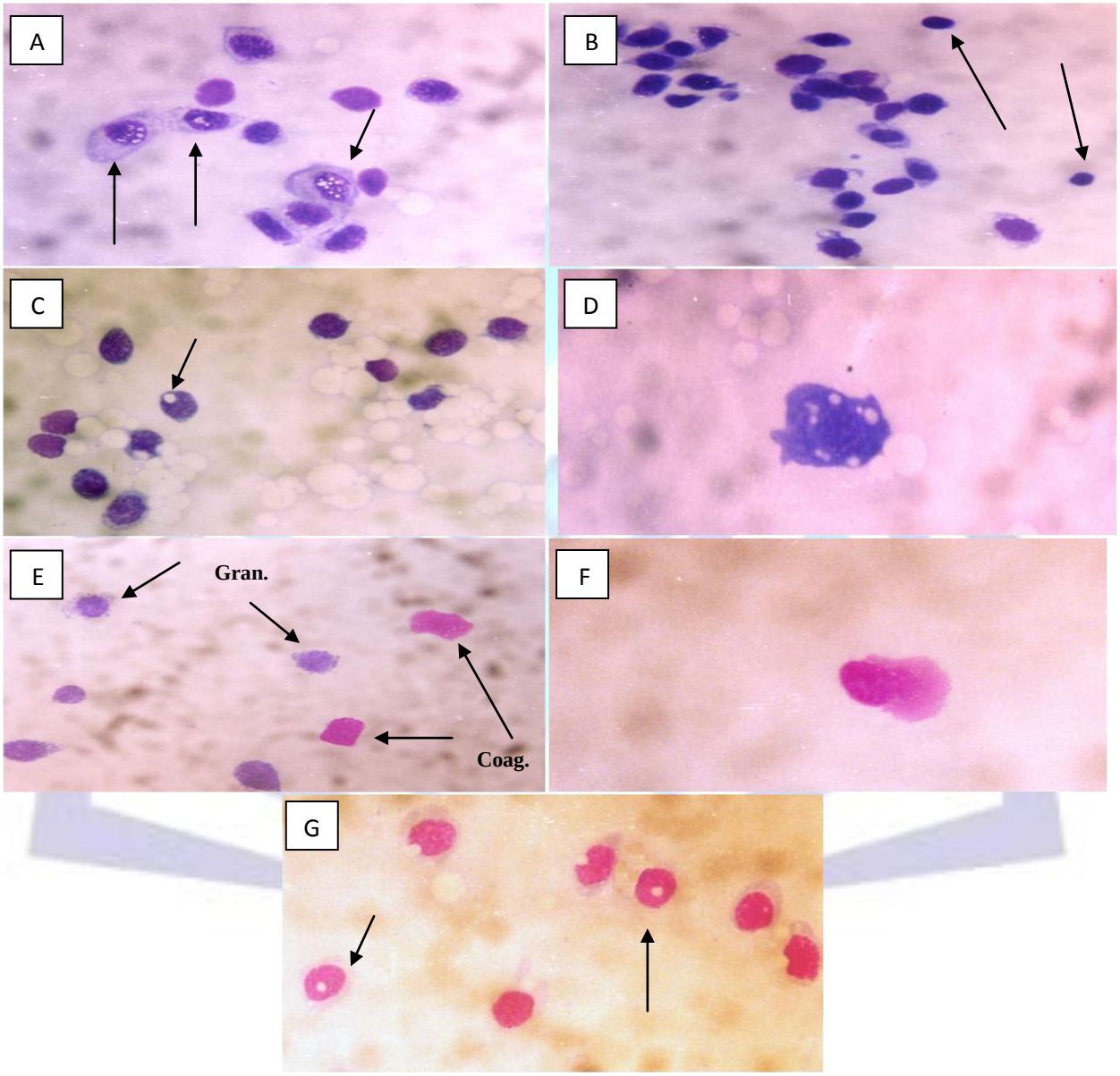

Plate 2: Photomicrographs of deformed haemocytes in *S. gregaria* as a response to extracts of *N. sativa*. (A): PLs with vacuoles in the nucleus after treatment with n-butanol extract. (B): small darkened GRs after treatment with methanol and petroleum ether extracts. (C): GRs contained vacuole after treatment with petroleum ether extract. (D): Lysed GRs with vacuoles after treatment with petroleum ether extract. (E): lysed GRs and CGs, regardless the extract. (F): Destroyed cell membrane and extruded cytoplasmic contents in CGs, regardless the extract. (G): CGs with vacuolated cytoplasm, regardless the extract.

4. DISCUSSION

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 [52]. Haemocytes have been studied mostly in Lepidoptera, Hymenoptera, Coleoptera and Diptera [26, 35, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63] as well as Dictyoptera [64], Heteroptera [30] and Hemiptera [65]. Little work has been reported in the available literature for orthopterans, notably the acridids [50, 66].

4.1. Identification of circulating hemocyte types in *S. gregaria*

Classified categories of haemocyte types range from four to seven [67] or between three and nine [48, 68, 69, 70, 71]. There is confusion between various haemocyte types such as prohemocytes (PRs) and plasmatocytes (PLs) as well as granulocytes (GRs) and adipohaemocytes (ADs) [72, 73]. As reported in the literature, seven types of hemocytes have been frequently described in various insects [55, 74]. Six types of hemocytes were identified in *Diatraea saccharalis* [75] and *Papilio demoleus* [76]. Five distinct classes of haemocytes were identified in different insect species, such as *Manduca sexta* [57], *Poekilocerus bufonius* [77], *S. litura* [78], *Ostrinia furnacolis* [79], *S. littoralis* [62, 80]. Four types of haemocytes were identified in some other insect species [43, 54, 61, 81, 82, 83, 84]. Two haemocyte types only could be identified in *Drosophila* spp. [26]) and *Melanoplus sanguinipes* [56, 85]. However, Sendi and Salehi [86] identified only two major hemocyte types in *P. demoleus* basing on their role in immunity, i.e., PLs and GRs.

In the present study, only three main hemocyte types could be identified in the haemolymph of last instar nymphs and adults of *S. gregaria*: PLs, GRs and coagulocytes (CGs). Thus, the present result disagrees with a lot of previously mentioned records for various insect species and six hemocyte types in haemolymph of the same locust [87] but consistently agrees with Tanani [66] who characterized PLs, GRs and CGs in the same locust. However, the differences might be attributed to the differences in insect species or even the developmental stage of the same species, several technical difficulties for identification and the characters adopted by other workers [35, 65]. The hemocyte classification, types and morphology are often influenced by some factors affecting the haemolymph physical properties or biochemical composition [88], physiological condition of the insect [89] and developmental stages of the same species. Therefore, the classification has been revised several times for the same species [35, 36, 52, 67, 90, 91, 92].

4.2. Total haemocyte population in *S. gregaria* as affected by *N. sativa* extracts

Several factors could modulate immune responses of insects such as insecticides, hormones, environmental temperature, cations, etc. [93]. 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 [52]. Therefore, the present study on the desert locust *S. gregaria* aimed to investigate the effects of *N. sativa* extracts on the total haemocyte count (THC) and differential haemocyte counts (DHCs). In the haemolymph of early-aged last instar nymphs, THC remarkably increased by methanol extract but slightly decreased by n-butanol extract of *N. sativa* seeds. However, effect of petroleum ether extract depended on the concentration level. In mid-aged nymphs, only n-butanol extract promoted the hemocyte production, regardless the concentration level, while effects of other extracts depended on the concentration level. In late-aged nymphs, all extracts exerted prohibiting action on THC with few exceptions. In respect of the newly emerged adults, THC was reduced by the action of both methanol and petroleum ether extracts. At the highest concentration level of n-butanol extract, THC was pronouncedly declined but significantly raised at the lowest one.

The increasing THC in nymphs of some ages is in agreement with some of reported results for other insects, such as *P. americana* as response to several foreign particles [94]; *S. littoralis* by the chitin biosynthesis inhibitors diflubenzuaron [54], flufenxuron and chlorfluazuron [95] and some compounds derived from urea waste and rice straw [80]; *S. litura* by ecdysone [96]; *Gryllus bimaculatus* [83], *Acanthaspis pedestris* [97] and *Rhynocoris kumarii* by some insecticides [65]. Also, enhancement of THC was reported for *S. littoralis* by azadirachtin (Azt.) and its preparation Margosan-0 [59], *Agrotis ipsilon* by acetone extract of *Melia azedarach* [60], *Parasarcophaga surcoufi* by Azt. [58], and *Coccinella septempunctata* by Azt. and spinosad [98].

On the other hand, decreasing THC in adults and nymphs of some ages of *S. gregaria* as response to *N. sativa*, in the present study, correspond to similar results reported for several insects by various insecticides, IGRs and plant extracts, such as *P. americana* by Azt. [41]; *C. tatarica* by Azt. [43]; *Helicoverpa armigra* by plant oils of *Artemisia annua*, *Ageratum conyzoides* and *Azadirachta indica* [99]; *Rh. kumarii* by the insecticide endosulfan [65]; *S. littoralis* by flufenoxuron [95]; *S. gregaria* by some conventional insecticides, spinosad and proclaim [87]; *C. septempunctata* by abamectin [98]; *S. litura* by Azt. [78] and essential oils of *Acorus calamus* [44]; *Papilio demoleus* by IGR methoprene [86] and certain plant extracts [39]; *Eurygaster integriceps* by *A. annua* extracts [100]; *Dysdercus cingulatus* by some Azt. preparations [101]; *S. littoralis* by certain concentration levels of some compounds derived from urea and rice straw [80]; etc.

The increase in THCs could be attributed to the enhanced encapsulation of foreign/toxic molecules through process of melanization; melanin deposition during encapsulation is commonly initiated by haemocytes and/or phenoloxidase enzyme circulation in the plasma [102, 103]. However, the increasing THCs in nymphs or adults of *S. gregaria*, in the present study, as responses to the effects *N. sativa* seed extracts, could be attributed to the defensive action of haemocyte detoxification of these extracts [104]. Also, the increase of THCs 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 [105] which might be activated in response to some insecticides or plant extracts. Moreover, the increase in THC could be considered as an immune response against pathogen or any foreign body such as the

introduced plant extracts [106, 107, 108]. It may be important to mention that the brain endocrine complex is involved in haemocyte accumulation following some initial stimulus [109]. Jones [72] suggested that ecdysteroids can regulate the number of haemocytes. Because Azt. could be a responsible factor for the modification of haemolymph ecdysteroid titers [1101, 111], some extracts of *N. sativa*, in the present study on *S. gregaria*, may act as an antiecdysteroid materials promoting the increasing THC.

As previously mentioned, some of the plant extracts used in the present study prohibited the haemocyte production as observed in decreasing THC, depending on the nymphal age of *S. gregaria*. Such decreased THC may be correlated with the decrease of some hemocyte types involved in phagocytosis and nodule formation. As suggested by many authors [78, 112, 113, 114, 115], reduction of THC may be due to the toxicities of botanicals, their inhibitory effects on the endocrine glands and their secretion in insects, of nodule formation and inhibition of larval hematopoietic function or the cell proliferation. In addition, THC declination may be attributed to the death of pathological cells by degeneration [86].

4.3. Differential haemocyte populations in *S. gregaria* as affected by *N. sativa* extracts

It is important to point out that the increasing counts of some haemocyte types and decreasing counts of other types 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 and abiotic materials such as particles of Indian ink [116, 117] or chemical compounds of plant extracts. 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 [118].

In the present study on *S. gregaria*, *N. sativa* seed extracts prohibited DHC of some identified hemocyte types and enhanced other types. With regard to PLs, all extracts exhibited prevalent inhibitory effects on their production, or count in particular, in haemolymph of nymphs and adults, regardless the concentration level. This result agrees, to some extent, with decreasing PLs count in some insects by some insecticides, IGRs or botanicals, such as *Rh. kumarii* by some insecticides [65], *S. littoralis* by LC_{25} or LC_{50} of Azt. [59] or LC_{50} of the chitin synthesis inhibitor flufenoxuron [95], *S. gregaria* by some conventional insecticides, spinosad and proclaim [87], *C. tatarica* [43] and *P. surcoufi* by Azt. [58]. etc. On the contrary, decreasing PLs population in the present study disagree with the reported increasing PLs in *S. littoralis* as a response to some urea compounds derived from urea waste or rice straw [80]. The role of PLs in phagocytosis is disputed because some authors believed that they are phagocytes [34, 118] but other authors reported no phagocytic function [119]. However, decrease of PLs count in the present study on *S. gregaria* might be due to the transformation of these haemocytes into other haemocyte types [104, 120]. In other words, the decreasing PLs counts could be attributed to the fact that the PLs are highly polymorphic and might be converted into other types of haemocytes [121]. On the other hand, *N. sativa* extracts may prohibit the hematopoietic organs which are responsible for the production of PLs [122].

In contrast to the effects on PLs, both methanol and petroleum ether extracts of *N. sativa* seeds generally enhanced the GRs count in nymphs and adults of *S. gregaria*, in the current work. Also, a similar promoting effect was exhibited by n-butanol extract on these hemocytes only in nymphs but their count was exceptionally regressed in adults. These results are, to a great extent, in accordance with the increasing GRs count in *S. littoralis* as a response to the Azt. preparation Margosan-0 [59] and *Rh. kumarii* to some insecticides [65]. Otherwise, the exceptional case of decreasing GRs count in adults of *S. gregaria* by n-butanol extract of *N. sativa*, in the present study, come in agreement with those reduced count as reported for *Rhodnius prolixus* nymphs after wounding [123], *P. surcoufi* after treatment with Azt. [58] and *S. littoralis* larvae after treatment with LC_{50} of flufenoxuron [95] or some compounds derived from urea waste and rice straw [80].

One of the main functions of granulocytes is phagocytosis as reported by Wago [124] in *Bombyx mori*, Raina [125] in *Pectyinophora gossypiella*, Tojo *et al*. [118] in *Galleria mellonella*, Nardi *et al*. [126] in *Manduca sexta*, Essawy *et al*. [127] in *Heliothis armigera*, Pendland and Boucias [128] in *Spodoptera exigua*, Butt and Shields [129] in *Lymantria dispar*, Costa *et al*. [130] in *S. littoralis*. However, the increasing counts of GRs in the current work on *S. gregaria* may be explained by the transformation of some haemocytes into GRs [121] which reveals their role in the detoxification of the toxic compounds in the present plant extracts [65, 131, 132]. Otherwise, the decreasing number (or ratio), in the exceptional case, may be due to their role in the phagocytic activity [50].

One of the scarcely mentioned haemocyte types in the literature is CGs which could be characterized in the nymphs and adults of *S. gregaria* in the present study. CGs type was considerably enhanced by both methanol and n-butanol extracts of *N. sativa* in adults and the majority of nymphs. On the other hand, petroleum ether extract promoted the hemocyte type in adults but tremendously prohibited it in the majority of nymphs. Thus, the predominant increasing CGs count, in the present study, may be attributed to their role in phagocytosis [133].

4.5. Qualitative haemocyte profile in *S. gregaria* as affected by *N. sativa*

As affected by some pathogenic microorganisms, insect growth regulators, chitin synthesis inhibitors or plant extracts, the changed characterization of haemocytes was based on: changes in the plasma membrane (erosion and extrusion of their cytoplasmic contents), vacuolization and degeneration of the cytoplasm, nuclear changes (pycnosis, karyorrhexis, granulosis and division of nuclei) as reported in the larvae of *Pieris rapae* [134], *Plodia interpunctella* [135], *S. gregaria* [50] and *S. littoralis* [95]. In spite of the use of plants for insect control as a source of non-toxic compounds, haemocoelic injection of Azt. into last instar nymphs of *C. tatarica* resulted in bulging of cytoplasm, membrane rupture and extrusion of cytoplasmic contents of PLs while GRs contained vacuoles in cytoplasm and nucleus and no deformities were observed in PRs and SPs [43]. Some phytochemicals like plumbagin and Azt. caused cytopathological disturbances in hemocytes which somewhat similar to be produced by hormones or hormone analogues [78, 136]. In connection with the morphological disorders of hemocytes in *S. gregaria* by seed extracts of *N. sativa*, in the present study, all extracts failed to affect PLs but some of GRs were lysed or appeared as small darkly stained cells after treatment with petroleum ether

extract. Some of CGs were degenerated and other ones appeared with destroyed membranes and extruded cytoplasmic contents, regardless the extract. These morphological disorders of some haemocytes may be attributed to the action of the present plant extracts on the 'actin' which localized in the lamellar extensions of the cells as interpreted for *Drosophila melanogaster*, *S. litura* and *Plutella xylostella* by Anuradha and Annadurai [63] who concluded that Azt. or any naturally originating pesticidal molecule may exert its activity by targeting actins.

From the intracellular point of view, numerous vacuoles had been caused in the nuclei of some PLs in *S. gregaria* by n-butanol extract of *N. sativa* and similar vacuoles appeared in cytoplasm of some GRs by petroleum ether extract. Also, vacuolated cytoplasm was observed in some CGs, regardless the extract. Similar vacuoles could not be observed in the cytoplasm of PLs after treatment of larvae of *S. littoralis* with Azt. or its preparation Margosan-0 [137]. Also, bulging of some PLs and lysis of other ones were caused by Azt. in last instar larvae of *P. surcoufi* [58]. Not the formation of cytoplasmic vacuoles, but the cytoplasmic contents of GRs were observed in last instar larvae of *P. surcoufi* after treatment with Azt. [58]. These effects may be attributed to certain chemical constituents since several constituents had been identified, such as conjugated linoleic acid, thymoquinone, nigellone (dithymoquinone), melanthin, nigilline, damascenine, tannins, flavonoids, saponins, alkaloids, 20% proteins and 37% lipids, dithymoquinone carvacol and anethole 4-terpinole [17,138, 139, 140, 141]. 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 botanicals, such as Azt., were attributed to disruption of endocrine events [7,8].

In conclusion, the exact mode of action of *N. sativa* extracts, in the present study, on the haemocyte constituents of *S. gregaria* is unfortunately available now. Further cytological and ultrastructural investigations should be needed to explicate such damaging intracellular effects of the present plant extracts in haemocytes. However, *N. sativa* seed extracts can be used as a synergistic agent for pathogens owing to the prohibition of those hemocytes responsible for phagocytosis and subsequently potentiate the pathogen efficiency for control the desert locust *S. gregaria*.

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