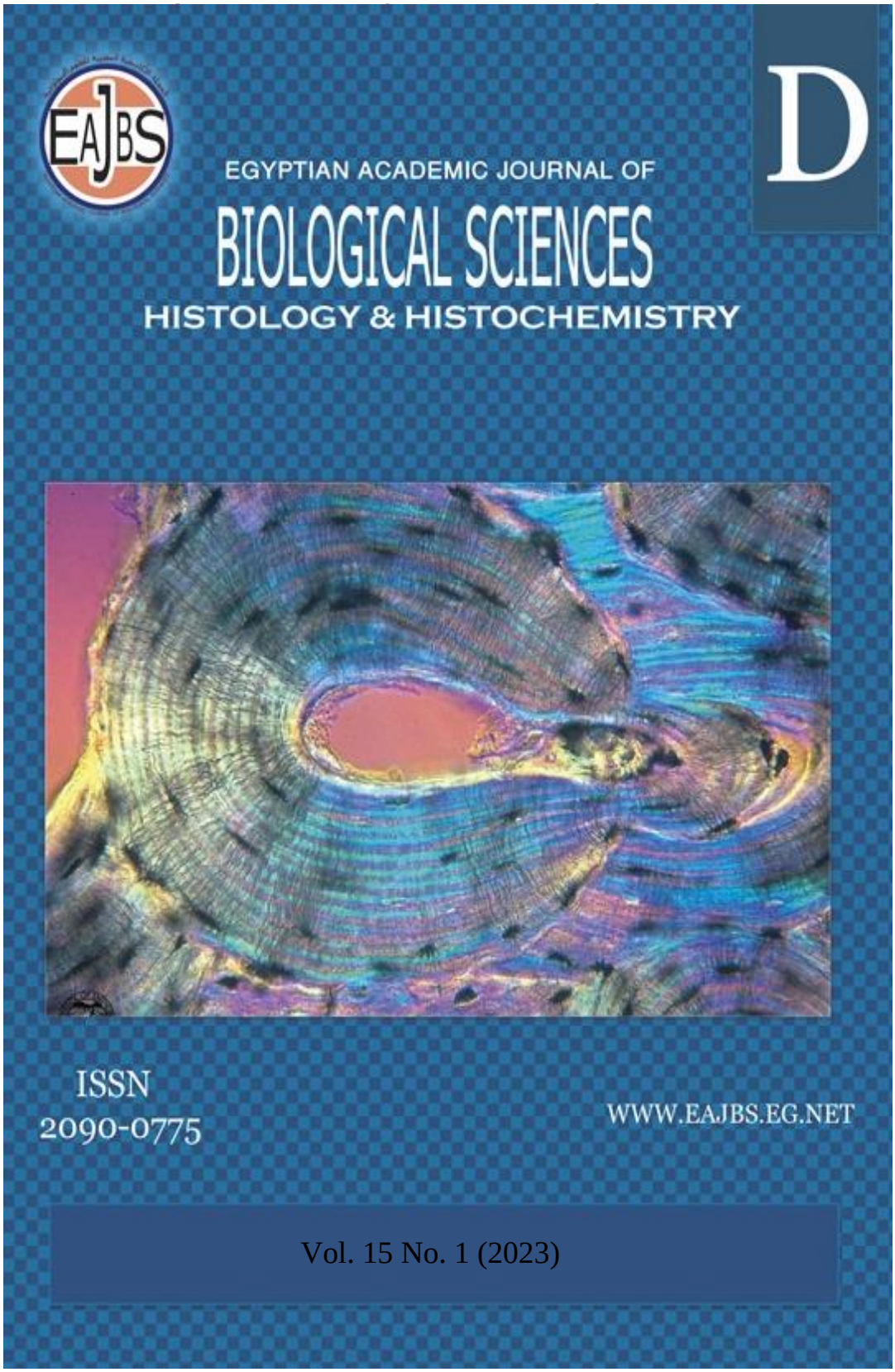

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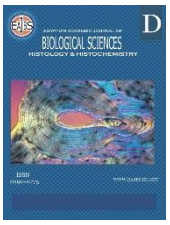

Toxicological and Histopathological Studies of Nano Chitosan and Spinosad on Red Palm Weevil, *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae) under Laboratory

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Rhynchophorus ferrugineus, Toxicology, Histopathology, nano chitosan and Spinosad.

ABSTRACT

The red palm weevil (RPW) *Rhynchophorus ferrugineus* is a main pest of date palm in a number of the Arabian Gulf States which includes Saudi Arabia. This work aimed to evaluate the efficacy of nano chitosan and Spinosad against mortality, some biological aspects, some enzymes & histological, different haemocyte counts and total haemocyte counts of 9th instar larvae of *R. ferrugineus*.

Nano chitosan and Spinosad concentrations of (0.5, 0.1 and 0.05 %) had been tested against these mentioned insects' 9th instar larvae at different exposure times ranging from 1 to 7 days. The mortality percentage increased with the increase in exposure times at all tested concentrations. The results revealed that LC_{50} (0.11) for nano chitosan and (0.30) for Spinosad.

Nano chitosan affects some biological aspects of *R. ferrugineus* as it led to an increase in the longevity of larvae, pupa and adults (male and female). The female didn't put any eggs so, the sterility was 100% compared with Spinosad and control.

Nano chitosan decreased changes in enzyme activity of ALP, PO, GPT and GOT recorded (-13.31, -20.88, -10.02 and -35.0%), respectively compared with control. Spinosad decreased changes in enzyme activity of ALP was recorded -35.62% compared with control. While it increased changes in enzyme activity of PO, GPT and GOT were recorded (40.79, 45.05 and 21.43%), respectively compared with the control. As well as, nano chitosan and Spinosad decreased the total protein and total carbohydrates recorded (13.3, 11.67 and 9.03, 10.66 mg/g.b.w.) respectively, in comparison to the control (20.4 and 13.13 mg/g.b.w.), respectively. Results showed that treating 9th instar larvae of *R. ferrugineus* with nano chitosan and Spinosad significantly decreased different haemocyte counts and total haemocyte counts when compared to the control. Also, appeared some abnormalities in haemolymph cell morphology.

The histological changes in the midgut of the 9th instar larvae of *R. ferrugineus* showed great abnormalities and destruction compared with the control.

INTRODUCTION

The red palm weevil (RPW) *Rhynchophorus ferruginous* (Coleoptera: Curculionidae) additionally referred to as the Asian palm weevil is an invasive key pest of palms (Aceraceae) in various agroecosystems the world over.

The insect is a main pest of date palm in a number of the Arabian Gulf States which includes Saudi Arabia, the United Arab Emirates, the Sultanate of Oman, and Egypt (El-Lakwah *et. al.*, 2011 and Abdelsalam *et. al.*, 2020). The agroclimatic conditions well-known in this area and the particular morphology of the crop, coupled with extensive current date palm farming, have presented the pest with a really perfect ecological habitat (El-Deeb *et. al.*, 2019and Habood *et. al.*, 2022). The RPW larvae bore deep into palm crowns, trunks, and offshoots, commonly hidden from visible inspection till the palms are almost dead. Several weevil generations can also develop within a single tree. Infested trees suffer from decreased productivity. Heavy infestations often result in collapsed trees and thus, an overall lack of crops (Yones, *et. al.*, 2014). Young palm trees (and within side the case of date palms, their offshoots) are intensively traded and transported among and inside countries, consequently, the pest is unfolding worldwide (Giblin-Davis, 2001). As a result of the outstanding harm by this pest on palm trees, we carried out this look to minimize the pest's effect within side the area.

Spinosad is a broad-spectrum insecticide that gives powerful manipulation of insect pests withinside the orders Lepidoptera, Diptera, and Thysanoptera, and a few species of Coleoptera and Orthoptera (Thompson *et al.*, 2000 and Abdelsalam *et. al.*, 2020). Spinosad is a combination of spinosyns A and D; the previous is the major metabolite (Bret *et al*., 1997). Spinosad is poisonous to insects by ingestion and contact (Wanner *et al*., 2000) and has a completely unique mode of movement in the insect nervous system at the nicotinic acetylcholine receptor and GABA receptor sites (Salgado 1997, 1998). Spinosad has low mammalian toxicity (Thompson *et al.*, 2000), and it degrades quickly while uncovered to sunlight (UV light) (Liu *et al*., 1999).

Chitosan extracted from natural chitin that's the structural component within side the exoskeleton of crustaceans via way of means of N-deacetylation, it's far containing D-glucosamine and N-acetyl-D-glucosamine molecules in a linear polymer shape related to the aid of using β-1,4-glycosidic bond, it's been utilized in agriculture as a plant growth promoter (Zayed *et al*., 2017 and Sabbour and Nayera 2019). Chitosan nanoparticles (ChNP) have the traits of chitosan and the properties of nanoparticles including surface and interface effects, small size and quantum size effects (Divya and Jisha 2018). Nanoparticle synthesis is presently intensively researched because of its wide variety of potential applications. As an alternative to chemical synthetic insecticides, the use of nanoparticles as an antimicrobial agent has become more common as technological advances have made their manufacturing extra economical (Eman 2018 and Habood *et. al.*, 2022). Therefore, those compounds are considered useful insecticides within side the manager of insect pests.

The aim of the present study is to spotlight the efficiency of Spinosad and nano-chitosan on some toxicological, biological, biochemical & histological effects, besides haemolymph studies against *Rhynchophorus ferrugineus* under laboratory conditions.

MATERIALS AND METHODS

Mass Rearing *of R. ferrugineus*.

Larvae and pupae of *R. ferrugineus* were collected from infested palm trees. Insects were incubated in transparent plastic boxes (120×60×30 cm) under laboratory conditions, (Ahmed *et al* .2015). Sugarcane steam split longitudinally in 10 cm. Pieces were used as a food source and oviposition substrate. Emerged adults were sexually differentiated and kept in separate containers for bioassay with sugarcane pieces as food.

Tested Materials:

1- Preparation of Chitosan Nanoparticles:

Chitosan nanoparticles were purchased from Nano Gate Company, Cairo, Egypt, and Prepared according to the method of Hasanin *et al*. (2018)

Transmission Electron Microscopy:

The particle size and shape were observed by TEM at the National Research Center - Egypt, operating at 200 kv.

The morphology of chitosan nanoparticles was investigated using TEM (Fig 1). Chitosan nanoparticle was found to be influenced by a Degree of Deacetylation of 90%, spherical shape, appearance of White and average size less than 50 nm.

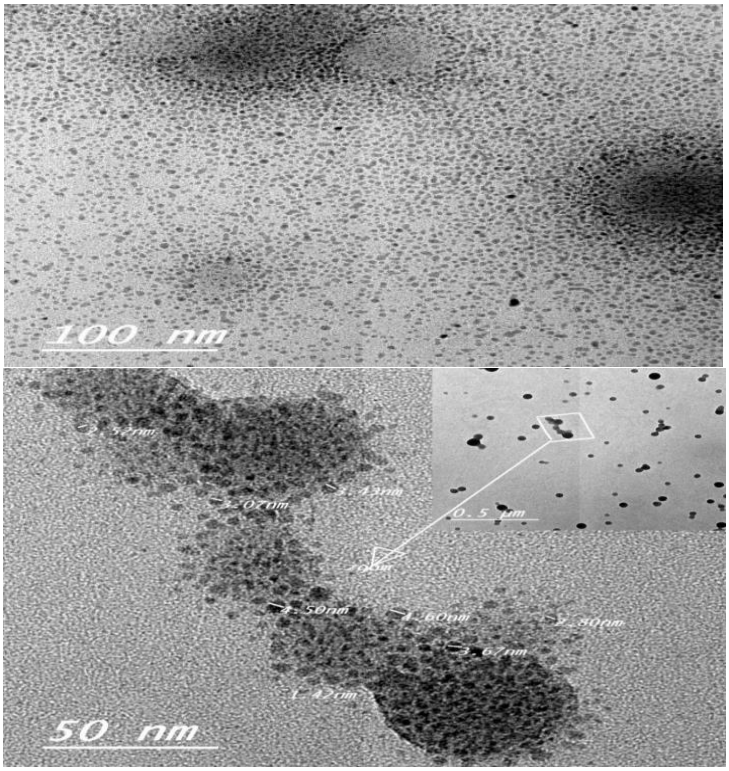

Fig. 1: TEM image of prepared nano chitosan.

2- Spinosad (Tracer 24% SC):

Obtained from Central Agricultural of Pesticides Laboratory (CAPL), Agricultural Research Centre (ARC), Dokki, Giza, Egypt.

Bioassay Experiment:

The ninth larval instar of *R. ferrugineus* was exposed to tested materials using the dipping food technique (Salman *et al*., 2020).

Fresh sugarcane stalks were cut into pieces. These fresh-cut pieces were immersed in prepared concentration solutions for 30 minutes.

Three concentrations were prepared (0.5, 0.1, and 0.05%) for nano chitosan and Spinosad. Three glass jars as replicates were used. Subsequently, ten larvae were introduced into each glass jar and covered with muslin. Ten replicates as control larvae were kept under the same conditions without any treatments. Mortality was evaluated after seven days post-treatment. All tests were carried out at 26 ± 2 º c and 65 ±5 % RH. The number of dead larvae in each jar was assessed and the percentage of mortality was calculated. The experiment was repeated four times The corresponding concentrations using the probit software program (LPD line) to obtain the toxicity regression line and LC_{50} value.

Biological Studies:

For larval and pupal stages, larval and pupal duration were recorded. Adult experimented by placing two pairs of mixed sex. *R. ferrugineus* adults (2-3 days) with treated or untreated sugarcane stem. The longevity of males and females

was calculated and *R. ferrugineus* were left to lay eggs, the number of deposited eggs on treated or untreated was counted. Also, hatchability and sterility percentages were calculated. For each tested concentration, three glass jars as replicates were used and the test was repeated three times.

Biochemical Studies:

9^{th} instar larvae were collected after 24hr treatment with LC_{50} of nano chitosan and Spinosad. They were homogenized in a saline solution. Homogenates were centrifuged at 8000 r.p.m for 15 min at 2ºc. The deposits were discarded and the supernatants were stored at 0ºc till used. For biochemical investigation, Alkaline phosphatase (ALP) activity was evaluated according to klein, Read and Babson (1960) and enzyme activity was recorded using a spectrophotometer at 550 nm. Phenol oxidase (Po) activity was determined using the method of Oppenoort and Welling (1976), and the reaction was measured every 1 min for an hour at 420 nm. The activity of the enzyme was measured as the absorbance change rate per min. Glutamic oxaloacetic transaminase (GOT) and Glutamic pyruvic transaminase (GPT) activity was measured according to Harlod (1975), and enzyme activity was recorded at 546 nm using a spectrophotometer.

The protein content estimation was conducted according to Qari and Shehawy (2020). Total carbohydrates were estimated in the acid extract of the sample by the phenol-sulfuric acid reaction of Dubios *et al*. (1956). Total carbohydrates were extracted and prepared for assay according to Crompton and Birt (1967). Blanks were prepared by substituting distilled water for the sugar solution. The absorbance of the characteristic Yellow- orange color is measured at 440 nm against blank. Total carbohydrate is expressed as µg glucose/ gm fresh weight.

Haemolymph Studies:

9^{th} instar larvae of *R. ferrugineus* were fed on a piece of sugarcane stem after dipping on LC_{50} concentration of nano chitosan and Spinosad for 24 h. For determination of total, differential hemocyte counts and hemocytes morphology, haemolymph was collected after 24 h treatment of 9^{th} instar larvae. The haemolymph was obtained after amputation of one or two prothoracic legs, before coxa of the larva using gentle pressure on the thorax and abdomen. Three replicates were used and haemolymph from two individuals was never mixed.

1- Total Haemocyte Count (THC):

Hacmolymph was collected into Thomas – white blood cell diluting pipette to the mark (0-5). Diluting solution (Nacl – 4.65g, kcl- 0.15g, $cacl_2$ - 0.11g, crystal violet - 0.05g and acetic acid – 1.25ml/1 distilled water) was taken up to 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 the counting slide. After 3 min, a total number of cells recognized in 64 squares of four corners were counted. The number of haemocytes per cubic millimeter was calculated according to formula of Jones (1962).

2- Differential Haemocyte Counts:

Differential haecmocyte count (DHC) was carried out, according to Arnold and Hinks (1979). Haemolymph was smeared on a clean glass slide, allowed to dry for 1- min and fixed for 2- min with drops of methyl alcohol. Fixed cells were stained with Giemsa solution for 20 min, washed several times with water and dipped in distilled water. stained smears were air-dried and mounted in DPX with a slipcover. Haemocytes were viewed under oil immersion (100X) using aleica Balen III stereomicroscope (Leica Ny). Haemocytes morphology was classified by some authors (AL- khalifa and siddiqui 1999; Gadelhak 2005; Manachini *et al*., 2011).

Histological Studies for Examination of The Midgut of Larvae:

All larvae were decapitated and their last abdominal segments were removed and fixed in alcoholic Bouni's fixative for 24h.

Samples were dehydrated at serial concentrations of ethanol alcohol (70, 80, 90 and 100%). Later they were cleared in xylene for 30 min. Samples were embedded in hot paraffin wax by using standard plastic cups. The wax block was so solidified by putting it in cold water.

Blocks were then stuck to the holder of the rotary microtome and cut at five-micron thickness.

There the slides were stained with hematoxylin and Eosin for 30 min. Then rinsed in alcohol series (70,80,90 and 100%) and passed in two changes of xylol for 10-15min/each. Finally, the slide was mounted in Canada balsam and covered with cover glass and dried at 40°C for a day and sections were examined and photographed by a light microscope according to the method of Drury and wallington;1980; Abduallah,2009.

Statistical Analysis:

The dosage mortality response was determined by Probit analysis (Finney, 1971) using a computer program of Noack and Reichmuth (1978). Data are presented as the mean ± standard error (SE) and were analyzed using Student's t-test between treatments and control.

RESULTS AND DISCUSSION

1-Toxicity Studies:

Data of the treated larvae of *R. ferrugineus* with nano chitosan and Spinosad was presented in Table (1). The obtained data indicated that the mortality percentage increased by increasing the applied concentration and prolongation of the exposure period. Nano chitosan was the most effective with both 0.5 and 0.1 % after seven days. The efficacies of nano chitosan on mortality of *R. ferruginens* larvae at 0.5% concentration were 70.0,83.31,95.33 and 100%, respectively, whereas the % mortality rates ranged from 63.76 to 93.33% by treated with a concentration of 0.1%. After treatment at a lower concentration of 0.05% mortality percentages reached 56.67 to 86.36% after 7 days post-treatment. Spinosad gave lower death% where it was only 60.26 to 93.38% and 51.57 to 88.24% with Spinosad at both 0.5 and 0.1%, respectively. Meanwhile, a concentration of 0.05% of Spinosad gave lower deaths % of 48.01 to 78.42% after seven days post-treatment.

Regarding Table (2) the lethal concentration of nano chitosan caused 50% and 90 %; LC_{50} and LC_{90} mortality in *R.ferrugineus* larvae were 0.11 and 1.55% with a slope of 1.12 after seven days post-treatment. Also, results showed that after seven days post – treatments with Spinosad, the LC_{50} and LC_{90} values were 0.30 and 3.68% with a slope of 1.18.

Table 1: Percent larvicidal activity of nano chitosan and Spinosad against *R. ferrugineus* larvae.

Treatments	Concentrations (%)	% Mortality (Days) ±SE			
		1	3	5	7
Nano	0.5	70.00±4.46	83.31±4.53	95.33±2.45	100.00±0.0
chitosan	0.1	63.76±3.45	76.67±2.75	80.00±2.92	93.33±2.71
	0.05	56.67±3.04	66.65±2.90	73.33±4.75	86.36±2.39
Spinosad	0.5	60.20±3.81	72.64±3.25	85.55±2.20	93.38±1.82
	0.1	51.57±2.66	65.80±3.11	77.04±3.92	88.24±3.18
	0.05	48.01±2.28	59.10±2.82	64.22±2.28	78.42±1.46
Control	0.0	0.0	0.0	0.0	0.0

Table (2): Lethal concentrations of nano chitosan and Spinosad against *R. ferrugineus* larvae.

Treatments	Lethal concentrations (%) and their 95% confidence limits			Slope ±SE	R
	LC_{50}	LC_{90}	LC_{95}		
Nano chitosan	0.11 (0.05-0.22)	1.55 (0.75-3.21)	3.27 (1.19-9.0)	1.12± 0.045	0.998
Spinosad	0.30 (0.20-0.45)	3.68 (1.51-8.95)	7.43 (2.37-23.31)	1.18 0.01	0.995

2- Biological Studies:

2.1- Duration of Larval Stage:

Data compiled in Table (3) showed that nano chitosan had a strong effect on the duration of larvae and slowly developed to exhibit the longest duration of 103.77 days. Especially with a 0.5% concentration.

Meanwhile, nano chitosan showed a gradual shortage in larval periods to give means of 90.44 and 77.63 days after treatment with 0.1 and 0.05 concentrations, respectively compared with (45.65 days) at control

2.2- Duration of Pupal Stage:

Also, as tabulated in Table (3), the pupal period ranged from a minimum of 24.0 days to a maximum of 35.0 days by treated with concentrations of 0.05, 0.1 and 0.5% of nano chitosan. On the other hand, pupal periods ranged from 14.3 to 20.6 days after treatment with 0.05,0.1 and 0.5% concentrations of Spinosad compared with (9.33 days) of control

2.3- longevity of Adults:

Data presented in Table (3), revealed that nano chitosan proved to be statistically highly significant on the longevity of males and females.

The longest means of longevities (96.7 days for males and 90.24 days for females) were obtained with adults who emerged from larvae treated with nano chitosan. Meanwhile, Spinosad caused the shortest means of longevities (45.5 days for males and 40.6 days for females) compared with (88.97 days for males and 83.50 days for females) for control

2.4- Female Fecundity of Eggs:

From the obtained results given in Table (3), the insect doesn't lay any eggs with all concentrations of nano chitosan. Also, all concentrations of nano chitosan gave the highest sterility percentage of 100% at (0.5, 0.1 and 0.05%)

Results indicated a significant reduction in the number of eggs and hatchability % of eggs laid per female for Spinosad.

The deposited eggs were 46.66, 95.75 and 105.75 eggs/female at (0.5, 0.1 and 0.05 %) of Spinosad, respectively. Also, a higher concentration of Spinosad (0.5%) gave the highest sterility percentage (98.15%), while, sterility percentage decreased by decreasing Spinosad concentrations compared with (260.33 eggs/ female) and sterility (86.01%) for control.

Table (3): Effect of nano chitosan and Spinosad on some biological aspects of *R. ferrugineus* under laboratory conditions.

Treatments	Conc. %	Mean duration (Days) ± S E		Longevity (Days) ± S E		No. of Eggs/ Female± S E	No. Hatched eggs	% Hatchability	% Sterility
		larva	pupa	Male	Female				
Nona chitosan	0.5	103.77±1.07	35.00±0.28	96.7±0.66	90.24±0.50	0.0	0.0	0.0	100.0
	0.1	90.44±0.34	30.00±0.25	91.44±0.81	87.33±0.53	0.0	0.0	0.0	100.0
	0.05	77.63±0.76	24.03±0.56	89.96±0.33	84.55±0.76	0.0	0.0	0.0	100.0
Spinosad	0.5	48.25±0.18	20.6±1.04	45.5±5.61	40.6±2.8	46.66±9.61	16.0±0.75	34.3±0.40	98.15
	0.1	35.77±0.10	17.7±4.40	38.8±10.5	33.5±6.42	95.75±3.63	48.0±0.91	50.13±0.51	83.22
	0.05	27.66±0.11	14.3±5.51	25.3±9.50	20.6±9.20	105.75±7.30	52.0±0.48	49.9±0.52	79.71
control		45.65±2.30	19.33±0.30	88.97±0.34	83.50±0.76	260.33±0.84	224.0±0.61	86.01±0.40	-

3. Enzyme Activity:

3.1- Alkaline Phosphatase (ALP):

As shown in Table (4) nano chitosan and Spinosad had a significant effect on Alp activity in haemolymph. In general, the tested compounds showed a reduction in the activity of ALP of the treated 9th instar larvae of *R. ferrugineus* than the control. The enzyme activity was measured at LC_{50} of nano chitosan (change -13.31%) followed by LC_{50} of Spinosad (change -35.62%) whereas ALP exhibited variable activity (40.5±8.12 u/l) by nano chitosan and (45.5±6.0 u/l) at Spinosad compared with the control (49.3±4.49 u/l).

3.2- Phenol Oxidase Activity (PO):

As shown in Table (4), nano chitosan treatment at LC_{50} had a significant decreasing effect on phenol oxidase activity compared with that of control, with a change rate (-20.88%). Whereas Spinosad treatment at LC_{50} had a significantly increasing effect on (PO) activity So, the change rate was (40.19%) compared with the untreated control.

3.3- Glutamic Pyruvic Transaminase (GPT):

Data illustrated in Table (4) revealed that GPT activity after treatment with nano chitosan decreased than the untreated ones.

The mean enzyme activity was (70.60±7.40 u/l) with a change of (-10.02%) for nano chitosan. On the contrary, Spinosad increased the enzyme activity (123.0±12.11 u/l) with a change (45.05%) compared with (85.0±7.66 u/l) in the control.

3.4- Glutamic Oxaloacetic Transaminase Activity (GOT):

As shown in Table (4), nano chitosan decreased the enzyme activity (75.86±6.74 u/l) with a change (-35.00%). Meanwhile, Spinosad increased GOT activity (145.68±5.64 u/l) with a change (of 21.43%) compared with (128.11±12.68 u/l).

3.5- Total Protein and Total Carbohydrate:

Results illustrated in Table (4), showed that nano chitosan and Spinosad had significantly decreased total protein and carbohydrate compared with the control. The activity ratio of total protein was (13.3±1.5 and 11.67±1.6 mg /g.b.wt) for nano chitosan and Spinosad, respectively compared with (20.40±1.3 mg/g.b.wt) of control. Whereas, in the case of total carbohydrate the activity ratio (9.03±0.50 and 10.66±0.70 mg/g.b.wt) for nano chitosan and Spinosad, respectively compared with the control (13.13±0.49 mg/g.b .wt).

Table (4): Alkaline phosphatase (ALP), phenol oxidase (PO), Got and GPT activity in haemolymph of the *R. ferrugineus* larvae after 48 hr. treatment with LC_{50} of nano chitosan and Spinosad.

Treatments	Conc. (%)	ALP (u/l) Mean± SE	% change	PO (u/l) Mean ±SE	% change	GPT (u/l) Mean ±SE	% change	GOT (u/l) Mean ±SE	% change	Total Protein (mg/g.b. wt) Mean±SE	Total Carboy drate (mg/g.b. wt) Mean ± SE
Nano Chitosan	LC_{50} (0.11)	40.5 ±8.12	-13.31	23.22 ±2.44	-20.88	70.60 ±7.40	-10.02	75.86 ±6.74	-35.0	13.3 ±1.5	9.03 ±0.50
Spinosad	LC_{50} (0.30)	45.5 ±6.01	-35.62	44.63 ±7.55	40.19	123.0 ±12.11	45.05	145.68 ±5.46	21.43	11.67 ±1.6	10.66 ±0.70
Control		49.3 ±4.49	-	30.33 ±10.76	-	85.0 ±7.66	-	128.11 ± 12.68	-	20.40 ±1.3	13.13 ±0.49

4- Haematological Studies:

Six types of haemocytes were identified as follows:

1- prohaemocyte (pro) 2- plasmatocyte (pla), 3- Granulocyte (gra), 4- oenocyte (oen) amd 5- spherlocyte (sph) 6- Coagulocytes (coag) (Fig. 2). The differential hacmocyte counts (DHC) of the 9th instar larvae of *R. ferrugineus* treated with nano chitosan and Spinosad are shown in Table (5). In the case of Spinosad the highest value was recorded (17.5±1.5) of granulocytes followed by plasmatocytes (15.5±1.0) and the lowest value (4.0±1.5) for spherulocytes. Prohemocytes and

oenocytoids comprised (9.0±2.0 and 8.0±1.6), respectively. on the other hand, nano chitosan decreased (DHC), that were (14.6±1.4 and 13.5±1.2) for granulocytes and oenocytoids, respectively.

Finally, spherulocytes recorded the lowest value compared with (20.6±1.2 and 19.0±3.2) for granulocytes and plasmatocytes and the lowest value (7.7±1.4) for spherulocytes. Prohemocytes and oenocytoids comprised (13.6±0.9 and 10.9±1.4), respectively.

Nano chitosan and Spinosad reduced total haemocytic count in comparison to the control. Total haemocytic count recorded (2133.3±125.8 and 2350.0± 100.0 cells/mm^3) for nano chitosan and Spinosad, respectively compared with the highest value (3165.3±270.0 cells/mm^3) for control.

DHCs for Treated *R. Ferrugineus* by Nano Chitosan and Spinosad:

Figures (3&4**)** show the effects of nano chitosan and Spinosad on haemocytes of 9th instar larvae of *R. ferrugineus*. Treatments with LC_{50} of tested compounds caused abnormalities in surface morphology in some types of blood cells. Haemocytes changes become fragile with irregularity and elongation of cell walls besides, twisting at the ends of spindle cells. Also, the nucleus moved towards the cell wall, losing its central position and cellular vacuolization was observed. The cytoplasm of all cells was bad and faint. Also, the plasma membrane of all the cells becomes fragile leading to a gradual loss of cytoplasm and ultimately only a few interconnected cytoplasmic strands are left.

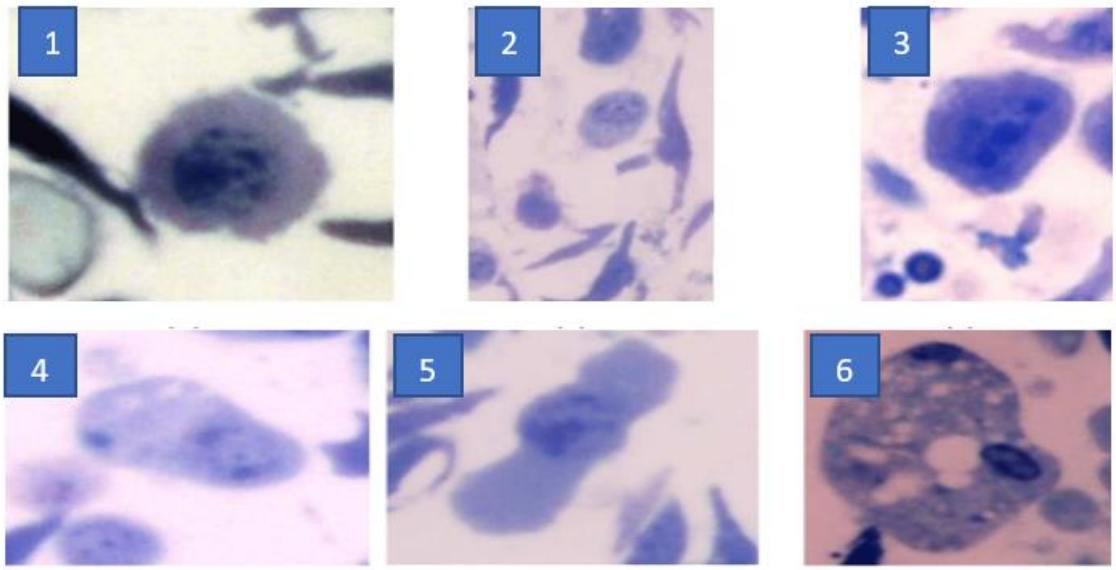

Fig 2: Sections of the six types of haemocytes from haemolymph of *R. ferrugineus*. (1): Prohemocyte (2): Plasmatocyte (3): Coagulocyte (4): Granulocyte (5): Oenocytoid (6): Spherlocyte.

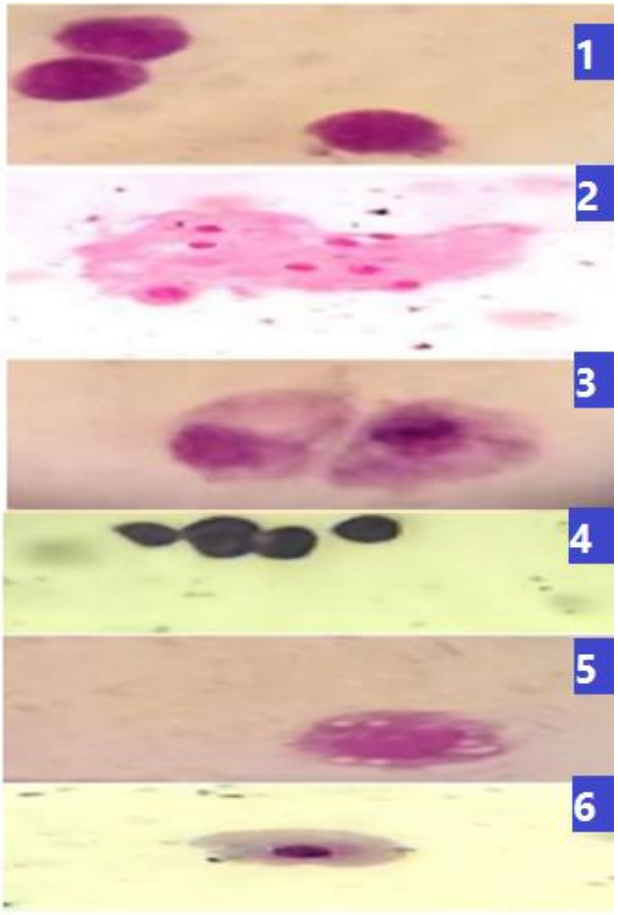

Fig.3: Malformed hemocytes of *R. ferrugineus* after 24 h treatment of larvae (9th) during feeding by nano chitosan.
(1): Prohemocyte (2): Plasmatocyte (3): Coagulocyte (4): Granulocyte (5): Oenocytoid (6): Spherlocyte.

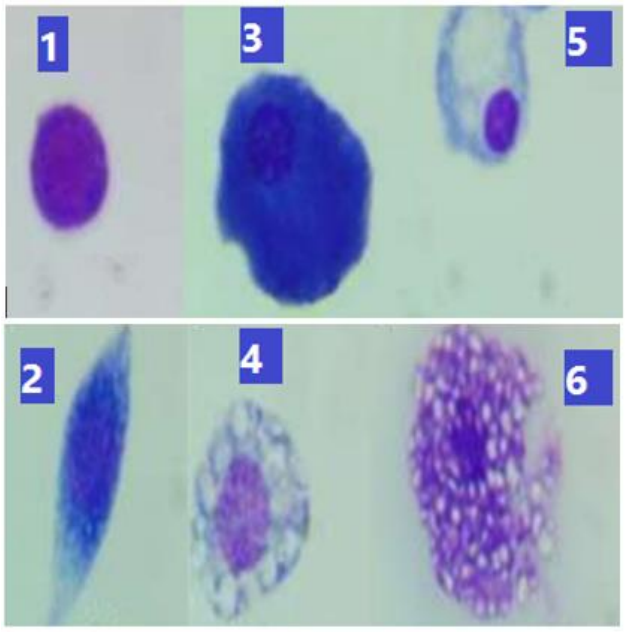

Fig.4: Malformed hemocytes of *R. ferrugineus* after 24 h treatment of larvae (9th) during feeding by Spinosad
(1): Prohemocyte (2): Plasmatocyte (3): Coagulocyte (4): Granulocyte (5): Oenocytoid (6): Spherlocyte.

Table (5): Effect of nano chitosan and Spinosad on different haemocyte counts and total haemocyte counts of 9th instar larvae of *R. ferrugineus*.

Treatments	Pro.	Plas.	Gra.	Coag.	Oen.	Sph.	Total haemocyte Counts (cells/mm^3) Mean ±SE
	Mean± SE						
Nano chitosan	8.7± 1.9	13.5 ±1.2	14.6 ±1.4	8.0 ±0.7	6.6 ±0.7	2.0 ±0.3	2133.3±125.8
Spinosad	9.0± 2.0	15.5 ±1.0	17.5 ±1.5	10.0 ±1.5	8.0± 1.6	4.0 ±1.5	23500.0± 100.0
Control	13.6 ±0.9	19.0 ±3.2	20.6 ±1.2	12.0 ±1.9	13.40 ±1.4	6.39 ±1.4	31615.3 ±277.0

5- Histopathological Studies:

5.1- Mid-gut:

As shown in a cross-section (Fig 5) normal midgut of 9th instar larvae of *R. ferrugineus* contains a longitudinal muscle layer wardly followed by an interior circular muscle layer. Also, appeared epithelium of the midgut is surrounded by the basement membrane. Besides, granular cytoplasm and spherical nuclei. The epithelial shows striated (brush border) severe effects were found in the midgut of the larvae treated with LC_{50} of nano chitosan (Fig 6), great destruction in the epithelium, and large vacuoles were present and muscular layer was broken in some cases.

Regenerative cells could not appear at the base of epithelial cells and become thicker than control. Meanwhile, treated with LC_{50} of Spinosad showed also severe effects. The muscular layer become thicker and regenerative cells were not pronounced. Also, epithelial cells were destroyed and large vacuoles were found between the epithelium and muscular layer compared to those in the control (Fig 7).

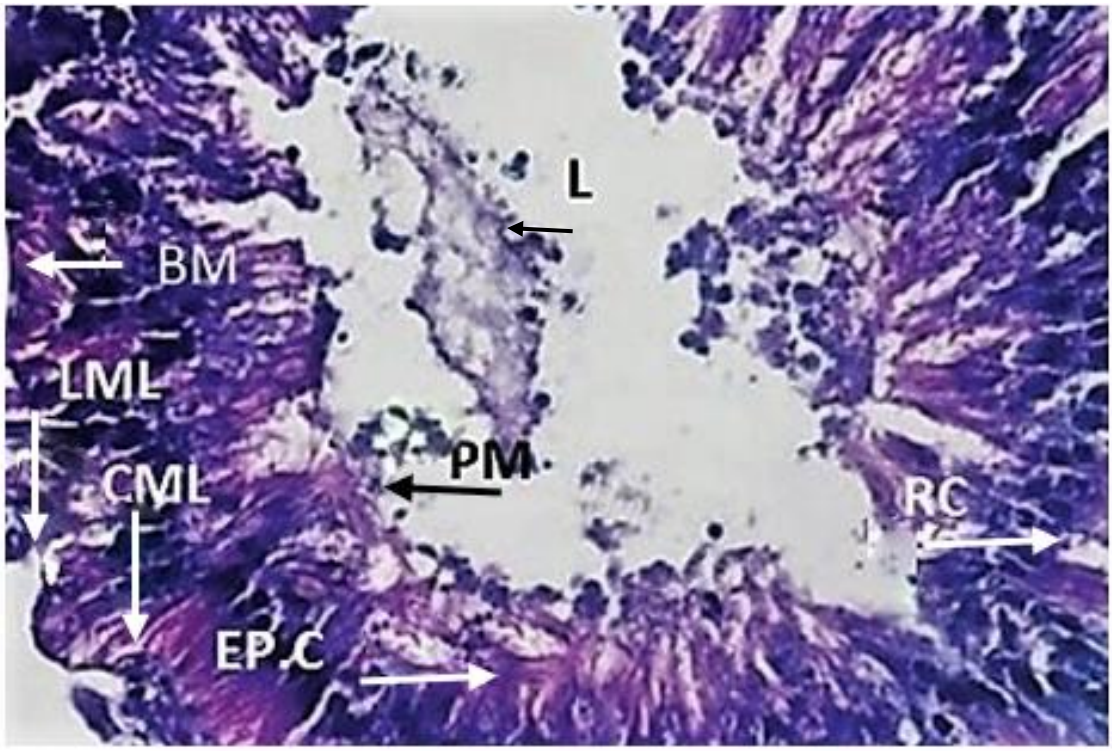

Fig. 5: Cross-sections of the midgut of *R. ferrugineus* larva in control showed:
LML: Longitudinal muscle layer, BM: Basement membrane, CML: Circular muscle layer, Ep. C: Epithelial cell, PM: Peritrophic membrane, RC: Regenerative cell, L: Lumen

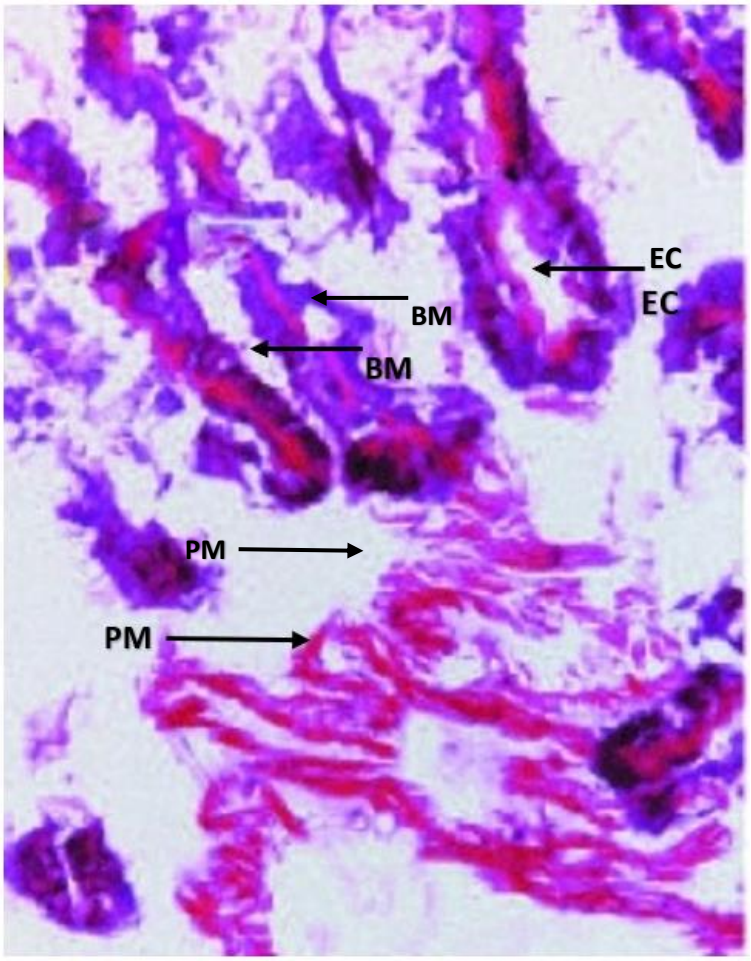

Fig. 6: Cross-sections of the midgut of *R. ferrugineus* larva treated with nano chitosan. PM: Peritrophic membrane, EC: Epithelial cell, BM: Basement membrane

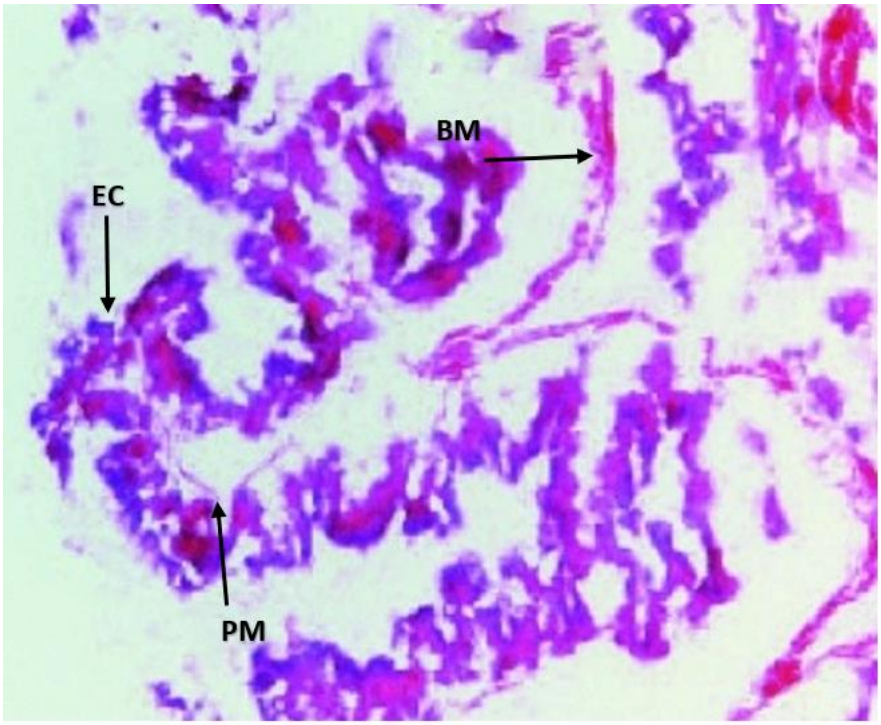

Fig. 7: Cross-sections of the midgut of *R. ferrugineus* larva treated with Spinosad. PM: Peritrophic membrane, EC: Epithelial cell, BM: Basement membrane.

Our results are largely consistent with the previous study El-Ezaby (1997) who revealed that injection of a concentrated insecticide: Spinosad caused a 98% percent mortality rate. Also, injection of a mixture of jujuba oil with insecticide (acetamiprid) was most effective against red palm weevil that attacks date palm cultivars, as it resulted in 100% (Abd El-Fattah *et al*;2019). Many authors found that nano chitosan has insecticidal activity and pest mortality increased with increasing the concentration of nano chitosan (Sabbour 2016) on *Schitocerca gregaria* and Sabbour (2019) on *Saissetia oleae* adults. Similar results were obtained by Sabbour and Solieman (2016) on *Tuta absoluta* and Alakhdar (2020) on *Tetranchus urticae* and *T.cinnabarinus*, they stated that LC_{50} of larvae was more than those of adults.

Elena and Ruiu (2018), found that feeding on sub-lethal concentrations of Spinosad resulted in diverse effects in males and females of *R. ferrugineus*. The value of LC_{50} varied according to the active ingredient in commercial Spinosad and larval instar. It was 18.7 ppm for Radient (Hamadah and Tanani 2013) mixed with the food of the last instar and 123.49 for Tracer 24% (Belal *et al* 2012) for the third instar.Sahab *et al*. (2015) noticed that the number of eggs deposited / female of *Callosobruchus maculatus* significantly decreased after treatment of nano chitosan under laboratory and semi-field conditions.

Besides, treatment of *Cassida vittata* under field conditions with chitosan and nano chitosan found a decline in the number of eggs and percentage of egg hatching (Sabbour and Abdel-Hakim, 2018).

Similar results were recorded on *Schitocerca gregaria* treated with nano chitosan, which found a significant decrease in the number of eggs, the nymph longevity and the percentage of adult females and males. (Alfy *et al*.2020).

Abd El-Naby (2019) found a significant decrease in the total protein of *S.littoralis* as a result of silica and silica nanoparticles treatment.Also, Namasivayam *et al*. (2018) reported a decrease in total protein and total carbohydrate treated with nano chitosan.Also, they declared that nanoparticles are easily ingested by insects and spread to the insect body, more through insect gut lumen into hemolymph and reducing insect's digestive capabilities. Spinetoram inhibited the activities of both ALP and ACP in haemolymph and fat body of *Rh.ferugineus* larvae. (Abdel-Mageed *et al*.2018).

Similarly, both Got and GPT activities had been reduced in the 4th instar larvae of *Tribolium granarium* after rearing on a diet treated with nano chitosan (Younes *et al*;2017).Farag and El-Sabki (2021) tested insecticide methomyl, Spinosad and chloropyrifos on the 9th larval instar of the red palm weevil (RPW), they found that all treatments of insecticides exhibited antioxidant enzymes (catalase and glutathione peroxidase), especially in Spinosad than both methomyl and chlorpyrifos insecticides.

Alzahrani (2019) found a reduction in glutathione content and acetylcholine esterase activity resulting from treatment with four imidacloprid concentrations (10, 15, 20 and 30 ppm).

In addition, AbdbelSalam *et al*. (2020) tested the lethal concentrations for 50% (44.3ppm) of the treated female of *Rhynchophorus ferruginous* on the activity of catalase (CAT), glutathiones transferase (GST) and superoxide dismutase (SOD). The result showed a significant decrease in CAT،GST and SOD activity.

This study demonstrates the possibility of using Spinosad as an insecticide against the females of *R. ferrugineus*.Habood and Badr (2022) found that nano chitosan affects the biology of red palm weevil by decreasing pupation percentage, emergence

percentage, pupal duration, number of eggs /females, hatchability percentage and longevity of females and males as compared to the control.

Also, LC_{50} of nano chitosan disturbed total protein and total carbohydrates. In addition, the activity of enzymes (protease, invertase and amylase) of larvae and adults was disordered. So, we concluded that nano chitosan can be used as a control tool for the red palm weevil.

Gadelhak (2005) found six types of hemocytes namely prohemocytes, granulocytes, plasmatocytes, coagulocytes, enocytoids and spherule cells identified in the hemolymph of *Rhynchophorus ferrugineus.*Ghusemi *et al* (2014) showed changes in total and differential counts (increase and decrease) of the Mediterranean flour moth, *Ephestia kuehniella* hemocytes when treated with pyriproxyfen and methoxy fenozide.

Hamadah (2009) revealed that THC increased 1min after treatment of *Coccinella septempunctata* L. with azadirachtin and Spinosad but decreased after application of abamectin.Also, Spinosad decreased the percentage of pro, pla, sph and gra on the haemogram of *Coccinella septempunctata* (Suhail *et al*.2007).Hamadah and Tanani (2017) tested sublethal concentrations of different insecticides, admiral, azadirachtin and spinetoram on (total hemoayte count, differential hemocyte count and hemocyte deformations). Results showed a significantly decreased in total hemocyte count and differential hemocyte count. All insecticides induced the prohemocyte while the other types declined. In addition, tested insecticide exhibited pathological symptoms on insect hemocytes morphology in cell membrane cytoplasm and nucleus.

Farag *et al*. (2021). Showed that, Spinosad insecticide caused damage in the midgut of *R. ferrugineus* larvae including vacuolar degeneration, proliferation and necrosis of epithelial lining with destruction and separation of basement membrane and with desquamation in its lumen.Abd El-Fattah *et al*. (2021). tested nano chloropyrophous on the histopathological midgut of *R. ferrugineus* larvae and adults. Results showed much damage including vacillation of cytoplasm, analysis and destroyed nuclei of epithelial cells. The larva was more sensitive to total damage in comparison with adults.Osman *et al*. (2016), observed abnormalities in the midgut and developing oocytes of the female of Khapra beetle, *Trogoderma granarium* treated with Carwy oil and pyriproxyfen, these effects comprise destruction of epithelial cells, microvilli and peritrophic membrane were curled and ruptured than those of control. Meanwhile, the germanium and follicular epithelium of developing oocytes of the ovaries showed faint nuclei. This may be due to the lesser content of DNA compared to those of control.

Conclusion

From the aforementioned data, it was found that Spinosad and nano chitosan disturbed the physiology of *R. ferrugineus* larvae and leading to altering their biology and causing their death. Also, the tested material exhibited pathological symptoms of insect haemocytes morphology in the cell membrane. So, we concluded that nano chitosan and Spinosad can be used as a control tool for the Red palm weevil.

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ARABIC SUMMARY

دراسات سمية وهستوباثولوجية لنانو شيتوزان وسبينوساد على سوسة النخيل الحمراء تحت الظروف المعملية.

أميرة محمد الشيوى - سارة عيد الديب - كرم خميس الجيزاوى
قسم وقاية النبات - كلية الزراعة - جامعة بنها - مصر

سوسة النخيل الحمراء *Rhynchophorus ferrugineus* تعتبر أفة رئيسية على التمور في عدد من دول الخليج العربي التي تشمل المملكة العربية السعودية. يهدف هذا العمل إلى تقييم فعالية النانو شيتوزان والسبينوساد على نسب الموت، بعض الجوانب البيولوجية، بعض الإنزيمات، القطاعات الهستولوجية ، وتعداد خلايا الدم المختلفة ، وتعداد خلايا الدم الكلية ليرقات العمر التاسع لسوسة النخيل الحمراء.

استخدمت تركيزات من النانو شيتوزان والسبينوساد وهي (0.5، 0.1، 0.05٪) على يرقات الحشرة في العمر التاسع في أوقات تعرض مختلفة تراوحت من 1 إلى 7 أيام. زادت نسب الموت مع زيادة أوقات التعرض في جميع التركيزات المختبرة. أظهرت النتائج أن التركيز المميت لنص التعداد (LC_{50}) للنانو شيتوزان (0.11) وللسبينوساد (0.30).

تأثير النانو شيتوزان على بعض الجوانب البيولوجية لحشرة النخيل الحمراء حيث أدى إلى زيادة طول عمر اليرقات والعذ ارى والحشرات الكاملة (ذكور وإناث). الأنثى لم تضع أي بيض لذلك كان العقم 100٪ بالمقارنة مع سبينوساد والكنترول.

حدث انخفاض فى نشاط الإنزيمات الحشريه ALP وPO وGPT وGOT المعاملة بالنانو الشيتوزان واعطت (-13.31, -20.88, -10.02- 35.0 %) على التوالي بالمقارنة مع مجموعة الكنترول. بينما قل نشاط إنزيم ALP فى الحشرات المعاملة بسبينوساد وسجل -35.62٪ مقارنة مع الكنترول. بينما تم تسجيل تغيرات في نشاط إنزيمات (PO وGPT و(GOT وهي (40.79, 45.05 و21.43%) على التوالي مقارنة بالكنترول. بالإضافة إلى أن المعاملة بالنانو شيتوزان والسبينوساد خفضت من نسبة البروتين الكلي والكربوهيدرات الكلية (13.3 و 11.67 و 9.03 و 10.66 mg/g.b.w) على التوالي مقارنة بالكنترول (20.4 و 13.13 mg/g.b.w) على التوالي. أظهرت النتائج أن معاملة يرقات العمر التاسع من سوسة النخيل الحمراء بالنانو شيتوزان والسبينوساد أدى إلى انخفاض معنوي في تعداد خلايا الدم المختلفة وعدد خلايا الدم الكلية بالمقارنة مع الكنترول. كما ظهرت بعض التشوهات والتغيرات في أشكال خلايا الدم.

أظهرت التغيرات الهستولوجية في المعى الوسطى ليرقات العمر التاسع من سوسة النخيل الحمراء تشوهات وتدمير كبير مقارنة بالكنترول.