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Toxicity, Growth Inhibition Effect, Oxidative Stress Induced in *Spodoptera littoralis* by Zinc Sulfate and Its Histological Alternation of The Reproductive Organs

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ABSTRACT

Zinc sulfate is a promising insecticide qualifying for disrupting the growth, development and reproduction of treated insect pests. This study was carried out to evaluate the toxicology of Zinc sulfate, its ability as an insect growth inhibitor and as a stimulant of the oxidative stress of the *Spodoptera littoralis* fourth instar larvae and the histological disruption it causes in the reproductive organs. The five zinc sulfate concentrations (1-2-3-4-5%) used in the toxicity experiment were all effective against the fourth instar larvae of *Spodoptera littoralis* with mortality percentage ranging from 53 to 95% 7 days post-treatment and the LC_{50} value is 0.9%. Zinc sulfate proved its growth inhibition ability whereas, a significant difference in the larval weight between the treated and untreated ones was noticed and the percentage of the weight reduction reached 92% of the larvae treated with the highest concentration used (5%). Vitamin C and the antioxidant enzymes (GST, peroxidase and phenoloxidase) were determined after 5 days and it was noticed that there is a decrease in Vitamin C and phenoloxidase levels (50±3.60) and (23.66±1.25) compared to that of the control (59.66±6.11) and (33.1±1.92) and great elevation in the level of both GST and Peroxidase (1336.67±56.86 and 37±2.68) compared to control one (933.3±32.53 and 28.67±1.42) respectively.

The histological examinations of male and female reproductive organs (testis and ovary) of adult *Spodoptera littoralis* treated with the zinc sulfate LC_{50} confirmed that the Zinc sulphate induced marked histopathological alterations when compared with the control group. In ovaries, necrosis, vaculation and degeneration were recorded. In testes, vaculation and atrophied follicles were observed.

INTRODUCTION

The Egyptian cotton leaf worm, *Spodoptera littoralis* (Boisd.), (Lepidoptera: Noctuidae) is a serious pest in Egypt and other countries (Hazaa *et al*., 2009). It is a destructive pest threatens several agriculture crops especially the cotton crop which consider the main resource of economy in Egypt (Ibrahim and Ali, 2018).

Zinc sulfate is odorless white powder material, non-combustible and soluble in water. It is an inorganic compound with the chemical formula ZnSO4. It is a very good fertilizer consider very essential micronutrient plants need in order to grow worthily since it is a component of many enzymes and proteins. (Cakmak, 2000). It increases the growth and development of the plants and this leads to an increase in the productivity of the crops. Plants

with zinc deficient have a reduction in carbohydrate, proteins and chlorophyll formation so the agricultural soil must provide the zinc sulfate, in addition, zinc sulfate is a naturally occurring pesticide and can be found in plants, soil, food, and water .and foliar sprays with zinc sulfate help the plant to be more tolerant to insect invasion Padhee and Mishara (1993), also it is competent to disrupting growth, development and reproduction of treated insect pests Al-Dhafar and Sharaby (2012). Zinc sulfate is a promising insecticide that proved its effectiveness in insect control and insect growth inhibition by interfering with the hormonal system of the insects causing an adverse effect on the physiological function of the insects Sharaby *et al*., 2013. It is also considered a chemosterilant agent of the accumulation of its ions in the tissues of the pupae and interfere with the biological processes of it causing retardation in the reproductive system development and also cause an adverse effect on sperms and ovaries viability El-Sabagh *et al.*, 2015.

Zinc sulfate as insect growth regulators (IGRs) have been found to render treated insects either sterile or less fecund. The IGR-treated insects may develop as morphologically deformed adults who would be non-viable or at least their reproductive capacity is reduced. The effects of IGRs on reproduction can be grouped into the following categories: reproductive behavior, oviposition, hatchability of eggs (ovicidal and embryocidal), and sterilization of adults. On the other hand, ecdysteroids have essential functions in controlling the processes involved in insect reproduction, i.e., vitellogenesis, ovulation of matured eggs and spermatocyte growth (Ghoneim *et al.*, 2014). So our aim in this study was evaluation the toxicity of zinc sulfate against fourth instar larvae of *S.littoralis*, its ability as a larval growth inhibitor, its oxidative stress and its ability to damage and destruct reproductive organs**.**

MATERIALS AND METHODS

The Test Insect:

The Egyptian cotton leaf worm *Spodoptera littoralis* were obtained from Insect Pathogen Unit-Plant Protection Research Institute-Agriculture Research Center. The larvae were fed on fresh castor leaves (Ricinus communis) and the insect culture was maintained at 25±2^{o}C and 65±5 RH with natural photoperiod El-Defrawi., *et al.* (1964).

The Test Compound:

Zinc sulphate was obtained from Techno Gene Company as powder. Five concentrations as 1, 2, 3, 4, 5 g / 100ml-distilled water were prepared.

Toxicity Experiment:

The experiment was done on fourth-instar larvae using the leaf dipping technique. Clean and dry castor leaves were submerged in each concentration for about 10 minutes, left to dry at room temperature, then offered to tested instar larvae which were allowed to feed on these treated leaves for 48h, then provided with untreated leaves daily. Fresh castor leaves treated only with distilled water were served to control larvae. 30 larvae per concentration plus 30 larvae for control were triplicate. Mortality percentages were recorded every 2 days till 7 days post-treatment and computed with the tested concentrations using the LDP line software (Bakr, 2000) to be analyzed for calculation of LC_{50} values.

The Larval Growth Inhibition Test: - The larval growth inhibition was tested compared to the control based on larval weight gain through 7 days of feeding. The larval growth inhibition was calculated using the equation of (Abivardi and Benz 1984) as the following:-

$$\text{Larval growth inhibition (\%)} = \frac{CL - Tl}{CL} \times 100$$

Where:

CL: larval weight gained in the control

TL: larval weight gained in the treatment

Biochemical Assay:

Spodoptera littoralis fourth instar larvae were allowed to feed on castor leaves treated with LC_{50} of zinc sulphate and after five days the treated larvae were collected in a clean cup and frozen at -20°C till the estimation of the enzymes.

Sample Preparation:

Treated fourth instar larvae were homogenized in distilled water (50mg/1ml). Homogenates were centrifuged at 8000 rpm for 15 min at 5°C in a refrigerated centrifuge. The deposits were discarded and the supernatants were kept at -20°C till use.

Vitamin C (Non-enzymatic antioxidant):

Vit C or ascorbic acid content was determined according to the method of A.O.A.C., (1975), based on measuring the de-colorization of 2,6-dichlorophenol-indophenol dye solution by the presence of ascorbic acid at 518nm.

Antioxidant Enzymes Assay: -

1- **Peroxidase enzyme: -** Peroxidase activity was determined according to the procedure given by Hammerschmidt *et al.* (1982), using pyrogallol *(*0.05 M). The absorbance was recorded at 420nm.

2- **Phenoloxidase enzyme: -** Phenoloxidase activity was determined according to a modification of **Ishaaya (I971),** using catechol solution (2%) and the optical density was measured at 405nm.

3- **Glutathione S-transferase (GST): -** GST activity was determined according to the method of **Habig *et al.* (I974),** using 1-chloro2,4-dinitrobenzene (CDNB), the increment of the absorbance was measured at 340nm.

Histological Studies:

Adult males and females from zinc treatment and their control were dissected. Their testes or ovaries were immediately transferred to hot Bouin's fixative overnight. Histological preparations for these samples through a series of graded ethanol dilutions were done. Longitudinal and transverse sections at a thickness of 4µ were cut. Ehrlich's hematoxylin and eosin were used for staining (Bancroft. and Layton, 2013). The slides were examined by the common research light microscope and five random non-overlapping were captured from each slide with an attached camera. Photographs were taken with different magnifications.

RESULTS AND DISCUSSION

Toxicity Experiment:

The results illustrated in Table (1) revealed the effectiveness of all tested concentrations of Zinc Sulfate on the fourth instar larvae of *S. littoralis* and all data were recorded till seven days post-treatment. At the end of the toxicity experiment, the mortality percentages were 53, 76, 86, 91 and 95% achieved by 1, 2, 3, 4 and 5% of zinc sulfate respectively, also the toxicity regression line **(Fig. 1)** showed that the lethal concentration (LC_{50}) values were 3.9, 2.1, 1.2 and 0.9% after 1, 3, 5 and 7 days.

Table 1: Toxic effect of Zinc sulfate against fourth instar larvae of *Spodoptera littoralis***.**

Concentration (%)	Mortality Percentage (%)			
	4th instar larvae			
	1 day	3 days	5 days	7 days
1	18	32	42	53
2	32	49	64	76
3	43	59	75	86
4	50	66	82	91
5	60	75	90	95
LC_{50}	3.9	2.1	1.2	0.9

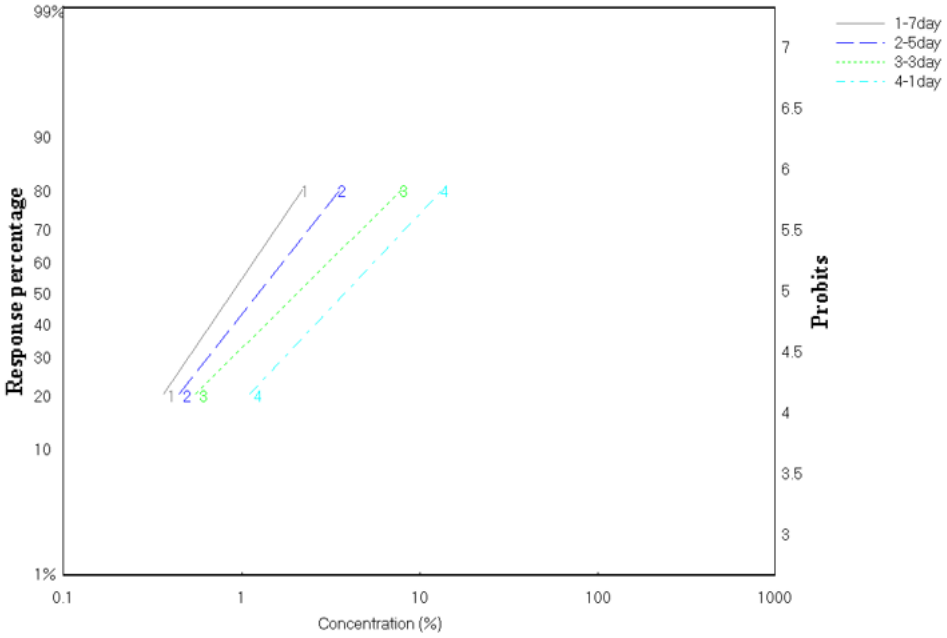

Fig 1: Toxicity regression lines of fourth instar larvae of *Spodoptera littoralis* treated with Zinc Sulfate after 1, 3, 5 and 7 days**.**

Larval Inhibition Test:

The obtained data in Table (2) and Fig (2**)** indicated the inhibition effect of Zinc Sulfate on the tested larval instar, whereas there was a great remarkable delay of the larval development and a significant difference in the weight between the treated larvae and untreated ones. The percentages of the larval weight reduction were increased gradually by increasing the concentrations. The highest larval weight reduction was recorded at a concentration of 5%. The larval weight reduction was 80, 86, 88, 92 and 92% induced by 1, 2, 3, 4 and 5% respectively.

Table 2: Growth inhibition effect of Zinc Sulfate against fourth instar larvae of *Sopodoptera littoralis*.

Treatments	Mean larval weight (mg) and weight reduction (%)	
	4th instar larvae	
	M.W (mg)	W.R (%)
Control	300	0.00
1%	60	80
2%	42	86
3%	35	88
4%	24	92
5%	23	92

A: UNtreated larvae **B: Treated larvae**

Fig 2: Strong inhibition of the larval growth of *S.littoralis* fourth instar larvae (**B**) compared to untreated one (**A**)

Biochemical Analysis:

Vitamin C as a Non-enzymatic antioxidant plus antioxidant enzymes (GST, peroxidase and phenoloxidase) were determined after five days of infection. The obtained data listed in Table (3) and represented in Fig (3) demonstrated that there is a reduction in Vitamin C and phenoloxidase levels (50±3.60) and (23.66±1.25) compared to that of the control (59.66±6.11) and (33.1±1.92) respectively, while there is great elevation noticed in the level of both GST and Peroxidase (1336.67±56.86 and 37±2.68) compared to control one (933.3±32.53 and 28.67±1.42) respectively.

Table 3: Effect of Zinc sulfate on Vitamin C, GST, peroxidase and phenoloxidase enzymes of Spodoptera littoralis fourth instar larvae**.**

Treatment	Vit C (Mean±SE)	GST (Mean±SE)	Peroxidase (Mean±SE)	Phenoloxidase (Mean±SE)
Zinc Sulphate	50±3.60	1336.67±56.86	37±2.68	23.66±1.25
Control	59.66±6.11	933.3±32.53	28.67±1.42	33.1±1.92

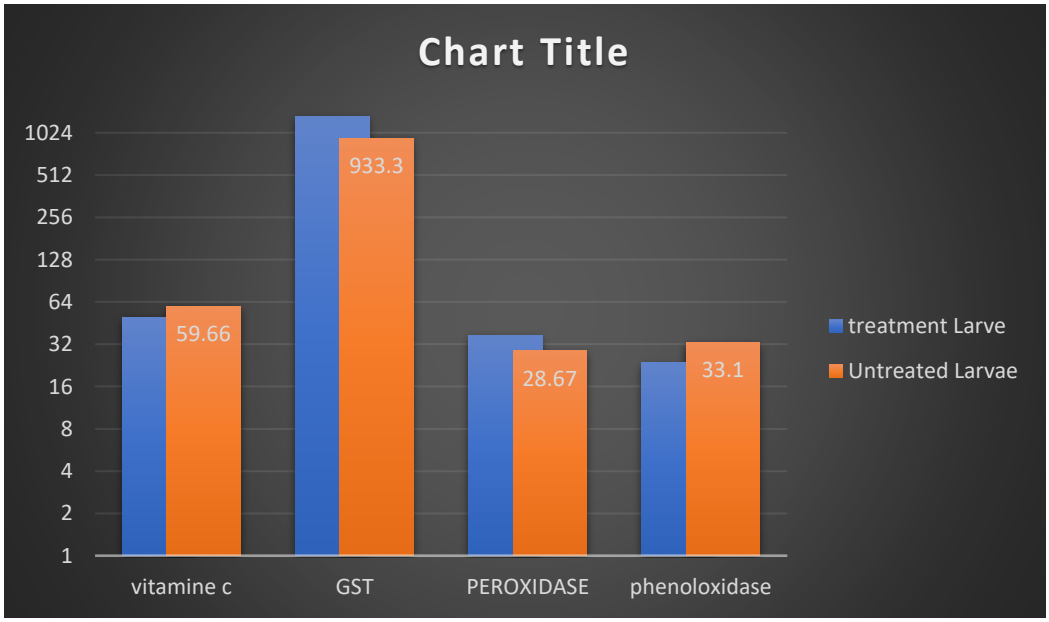

Fig. 3: Changing of *Spodoptera littoralis* enzymes activity post 5 days of treatment of fourth instar larvae with LC_{50} of Zinc sulfate

Histological Studies:

1-Female *S. littoralis*:

Normal adult female *S. littoralis* has one pair of ovaries; each consists of four convoluted polytrophic ovarioles joined by the terminal filament. Each ovariole was divided into three regions terminal filament, the zone of germanium and the zone of vitellarium. Histologically each normal ovariole has a chain of developing eggs in follicles. Each follicle consists of a growing oocyte accompanied interiorly by a few numbers of nurse cells (trophocytes), the oocyte surrounded by somewhat columnar to the cuboidal follicular epithelium, while the nurse cells are surrounded by squamous follicular epithelium (Fig. 4-A&B).

Comparing to normal control *S. littoralis*, ovaries of *S. littoralis* adult females resulting from treated larvae with zinc showed abnormal accumulation of chromatin in nurse cells nucleus and appearance of vacuoles, obvious degeneration of the wall between oocytes and complete separation between oocytes, decrease and disorganization in the number of yolk granules leading to the warp of egg chorion were also observed (Fig. 4-C). Moreover, Zinc treatment caused clumping of chromatin materials leaving space near the epithelial cells, absence of some nurse cells and semi-absorption of oocytes. The histological abnormalities included also oocyte shrinkage which left space around it and others were semi-absorbed. The follicular epithelium of some follicles became thin and slightly vacuolated while the others became greatly thickened with enlarged nuclei. Also, the ooplasm was divided into a central rounded mass separated from the main peripheral mass by a wide space. In addition, the nurse cells degenerate. Also, the oocytes became deteriorated and the epithelial cells were de-attached from the ooplasm (Fig.4-D).

2-Male *S. littoralis*:

Anatomically normal male *S. littoralis* has one pair of testes with pale creamy color and so closely apposed that they appear as a single round organ enclosed in a common membrane. Each testis leads to a vas deferens, which end at seminal vesicles. The histological examination of normal testes showed the testicular wall which surrounds the germinative (epithelial) cells. The arrangement of the germ cells in the testes shows that this structure is formed by unique and long follicles with several cysts. Each testis consists of 4 testicular follicles surrounded by epithelial cells which render the follicles and appeared as a single spherical yellow structure. Examination of a transverse section through the normal testis of *S. littoralis* showed that the testis consists of a mass of gonial cells in different stages of development and spermatogonia occurred near the periphery and inside, primary and secondary spermatocytes were present. Areas of spermatids still appeared near the centre. Most of the central area was filled with sperm bundles (Fig. 4-E).

Histological studies of testes from adult male *S. littoralis* derived from treated larvae with zinc revealed a clear reduction in cellular content and bundles of spermatocytes and spermatogonia were severely reduced in number which delayed spermatogenesis. Examination of transverse sections in the testis of male *S. littoralis* that emerged from the treatment with zinc showed damage to germ cells and vacuolation of the testes. The follicular tissue was shrinkage in many parts of the testes. The spermatogonia showed some degeneration, and some of the sperm bundles were absorbed. Also, disintegrations of spermatocytes were observed. Moreover, zinc-caused follicular tissue was clumped near the testicular wall and near the septa leaving many spaces, degeneration and destruction as of large numbers of spermatocytes that were degenerated leaving a wider vacuolated area. Some of the spermatide became liquefied. Spermatocytes necrosis or absorption was noticed (Fig.4-F).

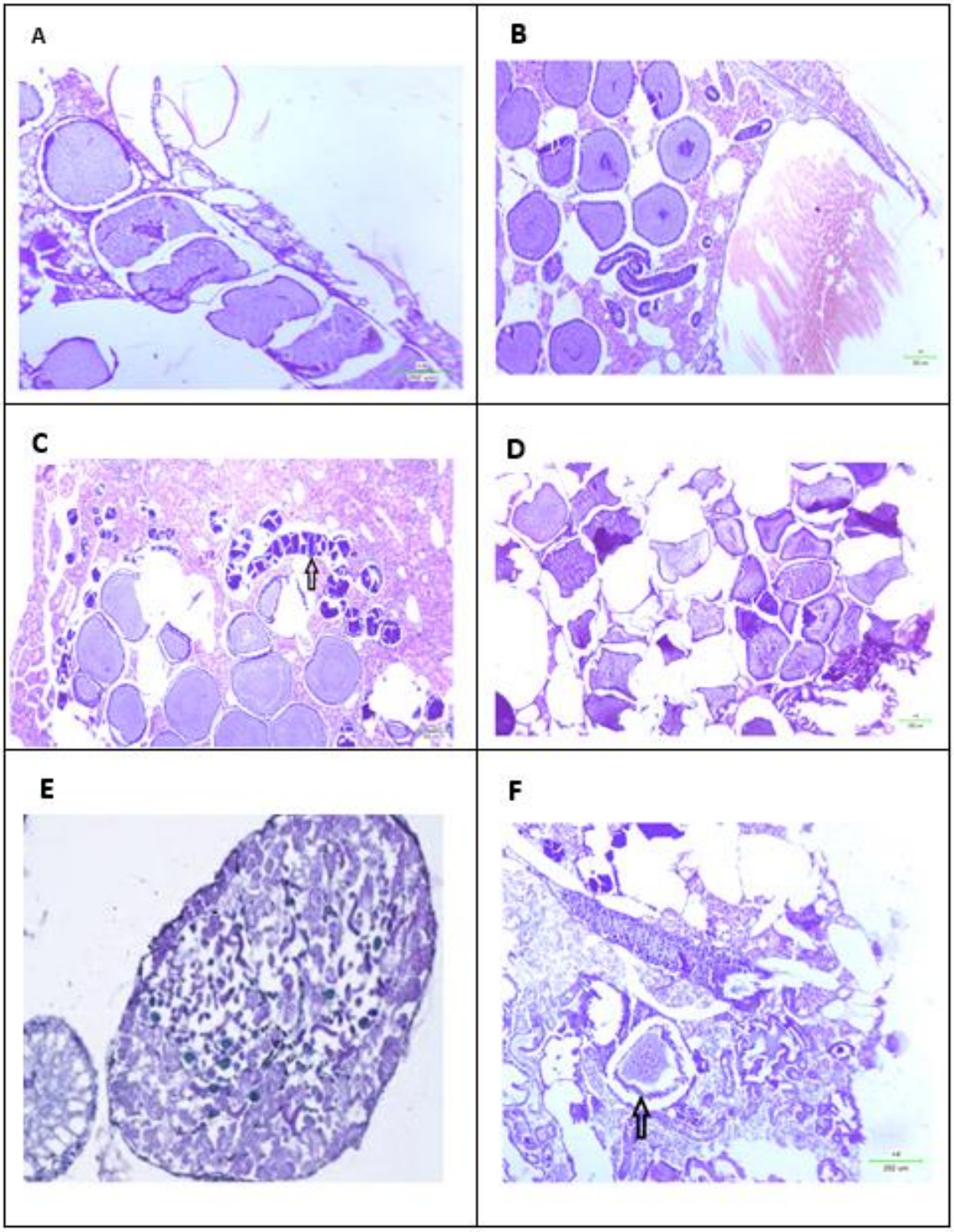

Fig 4: Malformation of *Spodoptera littoralis* adult reproductive organs after treating with zinc sulfate LC50 A) Longitudinal sections in ovariole of normal adult female *S. littoralis*, B) Sections in normal adult *S. littoralis*, showing normal ovarioles and reproductive organs, C) Sections in adult *S. littoralis* treated with zinc sulfate showing abnormal and necrotic ovarioles (arrow), D) Sections in adult female *S. littoralis* treated with zinc sulfate showing abnormal malformed ovarioles, , shrinking oocyte; deterioration; and separation, E) Sections in normal adult *male S. littoralis*, showing normal testis with testicular wall, follicles and spermatogonia, F) Sections in adult male *S. littoralis* treated with zinc sulfate showing abnormal testes showing vacuoles between follicles and degeneration of testicular wall and atrophied Spermatogonia (arrow).

DISCUSSION

We aimed this study to test the efficacy of zinc sulfate by evaluation its toxicity against the fourth instar larvae of S.littoralis, its ability as a larval growth inhibitor, its oxidative stress by measuring anti-oxidative enzyme and vitamin C and also study its ability to damage the reproductive organs histologically. Our obtained results indicated the highly toxic and growth inhibition effect of zinc sulfate against the fourth instar larvae of S.littoralis, these findings are in agreement with several authors. Sharaby. *et al*. (2013) evaluated the zinc sulfate toxicological, biological and physiological as an insect development inhibitor and recorded that zinc sulfate can be used as a growth disruptor for *S. littoralis* whereas it significantly increased both larval and pupal duration, decreased pupal weight and pupation percentages and also reduced The fecundity and fertility of females compared to control. Sell & Bodzinck (1971) mentioned that 0.2% concentration of zinc sulfate had deterrent feeding effects on Heliothis virescens newly hatched larvae, also Salama & Sharaby (1973) interpreted that the mortality of *S .littoralis* newly larvae fed on a diet supplemented with ZnSO4 at 0.1 M or higher could be related to starvation. Moawad *et al*. (2015) studied the efficiency of Zinc sulfate and some volatile oils on some insect pests of the tomato crop and reported that Zinc Sulfate alone was able to give relatively 50% protection to tomato plants from insect pests infestation and increase the whole number of plant leaves.

Approximately all insect pests have majorly been managed using synthetic pesticides while the use of these synthetic pesticides causes damage to the environment and resistance to the insecticides Rajula, *et al.,* 2020, so there is a great interest to control these pests with the most effective means that will not be dangerous to the environment and at the same time don't cause insecticides resistance Guan, *et al*., 2008, so the researchers directed to find safe and effective alternatives. Zinc sulfate is a promising source of safe insecticide, it is a natural pesticide that proved its effectiveness not only as an insecticide and insect growth inhibitor but also as a good fertilizer for good plant development and high crop production (Cakmak, 2000).

The biochemical assay investigated the oxidative effect of Zinc sulfate by determining the activities of antioxidant enzymes such as Glutathione S-transferase, peroxidase, and phenoloxidase enzyme plus the activity of Vitamin C. The data indicated an increase in the activity of both Glutathione S-transferase and peroxidase enzymes, and a decrease in Vitamin C and phenoloxidase enzymes of the fourth instar larvae treated with zinc sulfate LC_{50}. Oxidative stress can be defined as a disruption in the balance between the production of reactive oxygen species and the antioxidant defense system Betteridge 2000. Reactive oxygen species (ROS) are free radicals, responsible for the cellular functions required for different biological processes and cell growth regulation Evans, and Halliwell, 1999.

Zinc sulfate effectively caused oxidative stress by the elevation of the number of reactive oxygen species (ROS) (Koivula and Eeva 2010). In order to prevent ROS damage, living organisms have complex defense mechanisms that contain antioxidants (Howe and Schilmiller 2002). Toxicological stress induces enzymatic defense mechanisms such as the secretion of specific antioxidant defense enzymes. Dysfunction and alternation of these enzymes either by inhibition or stimulation is an indicator of oxidative stress. Our findings agree with Yanar *et al*. (2022) who study the effect of Zn, Cu, and Ni and *Bacillus thuringiensis* on the hemocyte count and the antioxidant activities of *Hyphantria cunea* (Lepidoptera: Arctiidae) larvae and showed that these metals caused increasing in superoxide dismutase, catalase, and glutathione peroxidase activities as antioxidant enzymes and also caused an adverse effect on the growth rate. Abd El-Wahab and Anwar 2014 emphasized that zinc and copper nanoparticles significantly increased the SOD activity of *S.*

littoralis. Muhammad., *et al*., 2022 demonstrated that there is altering in the antioxidant enzymes activities (SOD, GST, and CAT), a decrease in the larval body weight and changes in the gene expression of the silkworm *Bombyx mori* exposed to zinc oxide.

Regarding histological results, zinc sulphate induced marked histopathological alterations in both the female and male reproductive systems. Our findings are agreed with some authors who studied the effects of different chemicals on both male and female reproductive systems and the fertility of *S. littoralis.*

Hatem *et al.* (2007), studied the histopathological changes in the internal reproductive organs of *S. littoralis* treated with Granulosis virus (SpliGV), Spinosad and Azadirachtin on male and female internal reproductive system *S. littoralis*, which induced several alterations in the spermatogenesis development, decreased numbers of primary and secondary spermatocytes and delayed in spermatozoids development. The female coming from treatments with Azadirchtin and Spinosad showed different morphological abnormalities in ovarioles and oocytes.

Sabry *et al.* (2017), studied the inhibitory effects of sublethal concentration (LC_{25}) methoxyfenozide on the reproductive organs of *S. littoralis*. Results showed elongation in the immature stages in ovaries and longevity of adults than control, as well as reduced pupal weight, oviposition period, fecundity and hatchability percentages. Also, LC_{25} of methoxyfenozide showed general atrophy and abnormal features for ovaries and testes more than that of untreated ones. Histological disruptions were observed in ovaries as deformation in follicular shape, clumping of the chromatin material in nurse cells and disorganization of yolk and appearance of vacuoles between yolk and chorion. While in treated testes the testicular wall disappeared and vacuoles between follicles take place. Methoxyfenozide caused alterations in the amounts of both total soluble protein and total lipids and also in the activities of acid and alkaline phosphatases and phenol oxidase in the ovaries and testes of the resulting adults as compared to control adults. Sabry *et al.* (2017), suggested that the histopathological effects on the ovaries of *S. littoralis* females resulted from the effect of the treatment on the plasma membrane osmotic properties which led to dehydration and appearance of vacuoles within the oocytes, shrinkage or degeneration of the yolk and warping of egg chorion vacuolation in the cytoplasm of the nurse cells.

Hazaa *et al.* (2009), studied the effect of gamma radiation (125 Gy) on the histological and histochemical structure of the male and female reproductive systems of *S. littoralis.* The treatment caused histopathological changes in the ovaries including vacuolation, absence of nurse cells, shrinkage of oocyte tissue, clumped of chromatin material and thickness of epithelial cells in some areas. The vacuolation of the testes and absorption of sperm bundles also represent the damage to germ cells, and the disintegration of spermatocytes was most prominent in these organs. Histochemical observations showed a clear increment in the protein content of male testes while it showed a clear decrement in the female ovaries. The ribonucleic acid (RNA) showed a pronounced increase in both male and female gonads in spite of DNA showing a pronounced decrement.

The carbohydrates pass from the nurse cells to the ooplasm through the median pore and follicular epithelium while lipid is synthesized in the cortical region of the nurse cell cytoplasm. Based on these findings, it is probable that decreased yolk protein in the ovarioles of *S. littoralis* is attributed to histological alteration of nurse cells and follicular epithelium as indicated above. Moreover, the decrease of female-specific haemolymph protein (vitellogenin) may be expected. Engelman (1979) stated that the depletion of vitellogenin from the haemolymph is one of the primary causes which affect the oocytes maturation. Likewise, El-Sawaf (1971) stated that the pesticide "diptrex" decreased the synthesis of nucleic acids in the ovarioles of *Prodenia litura*. Also, El-Sawaf *et al.* (1980) stated that the ovarioles of *Musca domestica* irradiated as full-grown pupae with gamma

radiation showed a reduction of nucleic acid intensity. These reductions may be due to a deficiency in the biosynthesis of blood as an effect of irradiation or the inability of oocytes to function properly and gather the required amount for normal development. Also, inhibition in the nucleic acid contents was observed by Fadel (2000) in *Ceratitis capitata*. Protein and nucleic acids (DNA and RNA) reduction in the ovarioles of *S. littoralis* are in agreement with those obtained by El-Sawaf (1971) who concluded that in *Prodenia litura*, the follicular epithelium and extrusions from the nurse cells nucleoli play an important role in the synthesis of yolk protein.

Shalaby *et al.* (1997) recorded histopathological changes in the testes of *Pectinophora gossypiella* and *Earis insulana* previously treated as larvae with different doses of fruit oil extract of *Melia azedarach.* Also, Shaurb *et al.* (1998) stated that LC50 values of *S. terebinthifolius* extract caused histological changes in the testes of *Agrotis ipsilon* as damage to germ cells. Also, Sileem (2004) found that *Shinus terebinthifolius* leaves extracts and *Melia azedarach* fruit extracts caused several pathological alternations in the testis of the resulting moth of *Agrotis ipsilon*. Decreased protein synthesis in the testes of *S. littoralis* may reflect suppressed haemolymph protein uptake by the testes. Wilde (1964) stated that the proteins of the testes are mainly basic and consist of basic amino acids which abound in the haemolymph.

Conclusion:

In previous work we proved the ability of the zinc sulfate as a sterility agent whereas it induces 100% sterility of the *S.littorais* adults and in this work we confirmed the highly toxic effect of the zinc sulfate against *S.littorais* fourth instar larvae, high growth inhibition, ability to cause histopathological alterations in the reproductive organs which attributed to the oxidative stress induced by zinc sulfate, so we recommend using zinc sulfate as a promising insecticide for controlling the *S.littoralis.*

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