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# Effects of Dipel 2X and Littovir bioinsecticides on larvae of the cotton leafworm *Spodoptera littoralis* (Lepidoptera: Noctuidae)

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## Abstract:

*Bacillus thuringiensis* (Bt) and Nuclear Polyhedrosis Virus (NPV) are effective microbial control agent for controlling numerous species from different insect orders. The cotton leafworm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) is a polyphagus insect pest which infests many crops in Egypt. Toxicity of Dipel 2X (*Bacillus thuringiensis Kurstaki*) and Littovir (SpliNPV) was evaluated on *S. littoralis* 2nd instar larvae in the laboratory. Larvae were selected by the two bioinsecticides at sublethal concentrations of $LC_{70}$, $LC_{50}$, $LC_{25}$, $LC_{10}$ and $LC_5$. All selected larvae were died after seven days of treatment except those of $LC_5$ treatment. The offspring (F1 progeny) of $LC_5$ treatment was selected with two tested formulations at $LC_5$ concentration. Dipel 2X treatments caused slight depletion in the total protein content of the treated larvae whole body tissues and their gut tissue compared with untreated larvae. $LC_{70}$ and $LC_{50}$ treatments produced significant decrease (35.7and 27.8%, respectively) in protein of treated parent larvae. $LC_{70}$ treatment of Dipel 2X induced a significant elevation (39.7and 27.3%) of body tissues ($Na^+/K^{+)}$ ATPases and gut Proteases, respectively. The vigorous histopathological effects of Dipel 2X on midgut tissue of treated larvae were observed in $LC_{70}$ and decreased in $LC_{50}$ and lower in $LC_{25}$.These effects were hyperplasia of epithelial cell layer, appearance of apoptotic bodies, rupture of the bruch border microvilli, activation of stem cells, contraction of the muscle layer, extensive fragmentation and separation of mucosal epithelium from the basement membrane and the gut lumen was filled with debris of disrupted cells. Littovir caused insignificant decrease in total proteins of larval tissues with $LC_{70}$, $LC_{50}$ and $LC_{25}$ treatments but $LC_{10}$ and $LC_5$ induced increase in protein concentration of treated larvae. This content was increased in gut tissue of all treated larvae. High concentrations of Littovir produced insignificant reduction in ATPases activity in bodies of treated larvae, while lower concentrations elevated this activity which reaches to significant value (31%) in tissues of $LC_5$ treated larvae. All treatments inhibited the activity of midgut Protease in treated larvae and this effect was pronounced (40.1 and 32.3%) in $LC_{70}$and $LC_{50}$ treatments. $LC_{70}$ treatment of littovir caused hyperplasia and destruction of the midgut epithelium, appearance of apoptotic bodies, rupture of the bruch border microvilli and vacuole formation. These effects decreased with $LC_{50}$ and $LC_{25}$ treated larvae. Dipel 2X at $LC_5$ treatment of 1st generation (F1) larvae caused slight reduction in total proteins and ATPase in larval bodies but Littover increased proteins and significantly elevated (31%) ATPase in treated larvae. Insignificant increasing in gut proteins and Protease were detected and, slight changes in midgut tissues were observed in larvae treated with the two tested bioinsecticides. Results of this study concluded that the ($Na^+/K^{+)}$ ATPase and Protease played an important role in toxicity of *Bacillus thuringiensis* subsp. *Kurstaki* and Nuclear Polyhedrosis Virus (SpliNPV) and tolerance of *S. littoralis* larvae to their effects.

## Introduction

The cotton leaf worm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) is one of the most harmful pests in Egypt and many other countries of the world. This insect infested over 112 plant species belonging to 44 families. The larval stage of *S. littoralis* is known as a notoriously leaf eater accepting almost all herbaceous plant (Hill, 1975). Cotton leaf worm control program was based mainly on use of insecticides, which created some problems such as insecticides-resistance, environmental pollution and hazard to natural enemies and beneficial insects (Toscano *et al.,* 1974 and Abbas *et al.,* 1996). Applying new types of insecticides, originated from natural agents or products that disrupt the physiological processes of the target pest, could be useful alternatives in the integrated management approach (Parsaeyan *et al.,* 2013). Microbial biopesticides are environmentally safe, self-perpetuating in nature and specific to target pests. Among the microbial biopesticides, bacterial, virus and fungal products occupy a special space in managing several pests (Bidyarani- Devi *et al.,* 2016).

Bacterial insecticides were the earliest developed and the most widely used microbial pesticides in the world. During sporulation, different strains of this gram-positive bacterium produce crystalline parasporal inclusion bodies, composed of one or more toxic proteins known as δ-endotoxins, with a high level of specificity against different species of lepidopteran, dipteran, and coleopteran insects (Ho¨fte and Whiteley, 1989). The mode of action of the largest group of Cry proteins, can be divided into four main steps: (i) The cry toxins are ingested and solubilized of the inclusion body to release the cry proteins in their protoxin form, (ii) Gut protease processing of these protoxins to the active toxins, (iii) Binding of the active form to specific receptors in the midgut of the insect, (iv) Oligomerization, membrane insertion, and pore formation of the active toxin, eventually leading to cellular lysis of the insect midgut epithelium (Gill *et al*., 1992; Knowles, 1994; Schnepf *et al*.,1998; de Maagd *et al*., 2001 and Bravo and Soberón, 2008). The *Bt* crystal inclusion, toxicity is dependent on a complex process that requires multiple steps, these include solubilization of the crystal proteins, proteolytic processing of the protoxin to the active form, high affinity binding with the midgut receptor, and the irreversible insertion of the toxin in to the gut membrane (Jenkins *et al*., 2000).

Alterations in any of these steps could result in the development of resistance to one or several Cry proteins in each insect population (Ferré and Van Rie, 2002 and Heckel *et al*., 2007). *Bacillus thuringiensis* (*Bt*) is the most common bacterial insecticides which specific to larvae of lepidopteran pests (Peng, 1992). The application of *B. thuringiensis* in Egypt started at 1960 against young larvae of the cotton leafworm *S. littoralis* (El-Husseini, 1981). In 1980 many cry specific toxin genes cloning was subsequently performed, led to *Bt* uses development as well as in transgenic plants and manifested to be an alternative to the chemical pesticides.

Viruses are sub microscopic, intracellular and obligate pathogenic entities with nucleic acid and protein. These viruses are often genus or species specific and highly virulent to their hosts. Entomopathogenic viruses are currently used as alternatives to traditional insecticides. Several groups of viruses having a potential in controlling many insect pests. Among these groups of viruses, Nuclear Polyhedrosis Virus (NPV) which belongs to the family baculoviridae was exploited widely as a microbial control (Herniou *et al*., 2012). Its use should not be generalized because each pest has its own case. In specific cases, viruses proved very effective in managing populations of certain pests as Lepidoptera and Hymenoptera (El-Husseini,

2006). Baculoviral preparations are made up based on viral polyhedral, composed of virions embedded in a matrix of polyhedrin protein, highly resistant to the ecological factors and recently utilized alone and in combined with *Bt* called, recombinant viruses. The virus enters the nucleus of infected cells and reproduces until the cell is assimilated by the virus and produces crystals in the fluids of the host and death. These crystals will transfer the virus from one host to another (Chiu *et al*., 2012). NPV forming polyhedra like occlusion bodies kills most important crop pests such as *Helicoverpa armigera* (Hübner) and *Spodoptera litura* (Fabricius) (Lepidoptera:Noctuidae) (Bidyarani-Devi *et al*., 2016). Fact is that the NPVs infect and replicate in different host tissues (fat bodies, hypoderm, trachea, and blood cells). In sawflies (Symphyta: Hymenoptera), NPVs infect only the midgut tissue. Such differences influence greatly the number of virions produced per infected host individual, affecting both the dynamics of horizontal transmission in nature and the economics of commercial virus production (Van Driesche and Bellows, 1996). Asri *et al.* (2013) reported that the polyhedral occlusion bodies of *S.litura* Nucleopolyhedrosis Virus (SpltMNPV) infected various organs i.e. lumen, peritrophic membrane, midgut epithelial layers, trachea, blood vessels, malpigian tubules, muscles and adipocytes but not the cuticle.

The laboratory techniques of experimental biochemistry and histopathology were used to elucidate the tissue changes as a result of insect pathogen interaction (Kellogg, 1903). Insect adenosinetriphosphatase (ATPases) and gut proteases were affected by bioinsecticide treatments. ATPases are a class of enzymes that catalyze the decomposition of ATP into ADP and a free phosphate ion. This dephosphorylation reaction releases energy, which the enzyme (in most cases) harnesses to drive other chemical reactions that would not otherwise occur. This process is widely used in all known forms of life. Some such enzymes are integral membrane proteins and move solutes across the membrane, typically against their concentration gradient. These are called transmembrane ATPases. ATP-binding cassette (ABC) transporters, a large class of transmembrane proteins, are widely found in organisms and play an important role in the transport of xenobiotics. Insect ABC transporters are involved in insecticide detoxification and *Bacillus thuringiensis* (*Bt*) toxin perforation (Wu *et al.*, 2019). Proteases are defined as peptide hydrolases and include all enzymes that hydrolyze peptide bonds (Beynon and Bond, 1993). Proteinases refer to a specific class of proteases and are synonymous with the term endopeptidases, which cleave internal bonds in a peptide. Most of the proteases that degrade *Bt* insecticidal crystal proteins (ICPs) are proteinases. ICPs were degraded by proteases from a variety of sources, including those endogenous to the bacterium, those purified from animals and plants, or those found in insects. Proteases in the bacterium function in protein metabolism during sporulation; in some cases, they hydrolyze ICPs. Insect proteases are implicated in *Bt* toxin specificity, mode of action and insect adaptation to *Bt* (Oppert, 1999). Wu *et al*. (2016) stated the insect midgut proteases generally activating Cry toxins. Proteases are the most important protein digestive enzymes in the midgut of insects. The histopathological investigation of midgut epithelial cells in treated insect showed signs of apoptosis manifested as shrinkage of the cells and the presence of condensed chromatin with some vacuolization. This process is considered as the proper mechanism of cells against pathogens and toxic compounds (Dougherty *et al*., 2006 and Sakr, 2007).

This work aims to investigate the effects of bioinsecticides (Dipel 2X and Littover) on ATPase and protease enzymes and histology

of midgut in treated larvae of the cotton leafworm *S. littoralis*.

**Materials and methods**

**1. Insects:**

The lab strain of the cotton leaf worm *S. littoralis* was collected as egg masses from fields of Giza Governorate, Egypt. Eggs were kept in glass jars until hatching under laboratory condition (25±2°C, 65±5%RH. and photoperiod of 16 hrs. and 8h.D). The larvae were fed on castor bean plant leaves (*Ricinus communis*) until pupation and adult emergence. The newly emerged adults were mated inside glass jars supplied with a piece of cotton wetted with 10% sugar solution as feeding source and branches of tafla plant *(Nerium oleander*) were added to each jar as an oviposition site (Hatem *et al.*, 2011). The insects were reared without any exposure to insecticides for seven years in Central Agricultural Pesticides Laboratory, Agricultural Research Center, Giza, Egypt.

**2. Insecticides:**

**2.1.** Dipel 2X 6.4% WP ($32\times10^3$ spores/mg) product of Valent BioSciences company–Canada. Dipel 2X is a biological insecticide produced to control lepidopteran larvae and prepared as dry flowable formulations containing live spores and endotoxin of *Bacillus thuringiensis*, subsp. *Kurstaki* in the form of a water dispersible granule

**2.2.** Littovir SC ($5x10^{11}$ polyhedral inclusion bodies - PIB /liter) product of Bienvenue Chez Andermatt Biocontrol company-Switzerland. Littovir SC is a formulated insecticide specified to *S.littoralis* larvae and classified as order nucleopolyhedrovirus (SpliNPV) and Family baculoviridae.

**3. Bioassay:**

Seven serial aqueous concentrations of Dipel-2X and Littovir were prepared by water dilution. Castor bean plant leaves were dipped in each prepared concentration and left to dry in air then they placed in petri dishes. Almost 280 larvae of the cotton leafworm *S. littoralis* newly molted 2nd instar were used in the initial bioassay with 4 replicates. Treated castor bean leaves introduced to feed larvae for two days then they provided with clean castor bean leaves. Control Larvae were fed on leaves dipped in tap water. Larval mortality was scored daily. Mortality percentages were recorded after four days and corrected by Abbott's formula (Abbott, 1925). Data were analysed by probit analysis (Finney, 1971) to estimate the sublethal concentrations. Other 200 larvae were selected with each of determined $LC_{70}$, $LC_{50}$, $LC_{25}$, $LC_{10}$ and $LC_5$ of two tested bioinsecticides. The offspring (F1 progeny) from the previous $LC_5$ treatment was selected with different concentrations of the two tested bioinsecticides. The survivals from selected parent and $LC_5$ of F1 2nd instar larvae were collected for biochemical investigation and hiotopathological examination.

**4. Biochemical assay**s:

**4.1. Preparation of samples:**

Hundred milligrams of the untreated, $LC_{70}$, $LC_{50}$, $LC_{25}$, $LC_{10}$ and $LC_5$ treated parent and $LC_5$ treated (F1) larvae were homogenized in 1ml of sodium phosphate buffer (pH=7) for 3 minutes using a manual teflon homogenizer surrounded by a jacket of crushed ice and centrifuged for 30 min at 10000 rpm / min at $4^oC$ by a cooler centrifuge (Mikro – 22R, Germany). The supernatant was transferred to new tubes and frozen at ($-20^oC$) for biochemical analyses. Total protein content and the activity of adenosinetriphosphatase (ATPase) enzyme of control and treated larvae were determined by spectrophotometer (Unico UV 2100 spectrophotometer U.S.A).

**4.2. Determination of total protein contents:**

Total protein content in the whole-body homogenate of the untreated, treated parent and F1 larvae was determined based on Biuret test (Henry, 1964), using Kit purchase from dp international laboratory. A mixture of 1.0 ml of the total protein reagent (0.2N sodium hydroxide, 18mM/L sodium

potassium tartrate, 12mM/L potassium iodide and 6mM/L cupric sulfate), 20µl of each sample and 20µl of deionized water then incubated for 5 minutes at 25°C. Read and recorded the absorbance at wavelength of 546 nm versus the reagent blank as reference and standard. The total protein concentration in the whole-body homogenate of control and treated larvae was represented as mg/gm body weight of insects.

**4.3. Determination of adenosinetriphosphatase (ATPase) activity:**

ATPase catalyzed the conversion of ATP to ADP. During this conversion, one molecule of phosphorus was liberated. The inorganic phosphorus liberated was assayed (Fiske and Subbarrow, 1925). The specific activity of sodium and potassium dependent on ATPase in the whole-body homogenate of untreated, treated parent and F1 larvae was determined according to the method of Shiosaka *et al.* (1971) with some modifications. Reagent 1 consisted of 6 mM MgCl2, 6 mM ATP, 50 mM Tris, 100 mM NaCl and 20 mM KCl. Reagent 2 was trichloroacetic acid (T.C.A., 10%) and reagent 3 was ammonium molybdate consists of 1.5 gm ammonium molybdate, 40 ml distilled water (d.w.) and 20 ml sulphuric acid 10 M completed to 80 ml d.w. Reagent 4 was 0.5 gm Metol in 50 ml sodium bisulfate 3%. Reagent 5 was the standard phosphate solution consists of 0.022 gm KH2PO4, 100 ml d.w and few drops of chloroform. The procedure was: 1ml of d.w., 100 µl of sample and 1ml of reagent (1) were added to test tube then incubated at 45°C for 10 min and added 0.5 ml T.C.A., then incubated in ice for 30 min and centrifuged at 3000 rpm for 15 min, 200 µl of supernatant was put to 1.8 of T.C.A., then mixed and centrifuged at 3000 rpm for 5 min, three tubes were prepared as sample (S), standard (ST) and blank (B), then pipetted 200 µl of ammonium molybdate solution and metol into each tube, 0.9 and 1ml of T.C.A in (ST) and (B) tubes respectively, 100 µ of standard phosphate solution in (ST) tube, and 1ml of sample into (S) tube, finally each tube was measured spectrophometry at 680 nm after 30 min. The standard curve was prepared by using serial dilutions of KH2PO4 solution (0.2µm-1µm) and the activity was represented as ml mo1 / min / mg protein.

**4.4. Determination of gut protease activity:**

The untreated, treated parent and F1 larvae were dissected and the gut tissues were isolated. Then gut tissues were homogenized and prepared for protein and protease analysis. The activity of gut protease enzyme was determined spectrophotometry according to the method of Brik *et al.* (1962) with some modification by Wang *et al.* (2007). The procedure was, 20 µl of midgut homogenate was mixed with 150µl 1% azocasein in 50mM NaHCO3-Na2CO3 buffer, (pH 11) and incubated at 28°C for 2hrs. The digestion was stopped and indigestive azocasein was precipitated by addition of an equal volume of trichloroacetic acid (T.C.A., 10%), followed by incubation at room temperature for 1hrs. The precipitated proteins were removed by centrifugation at 18000 rpm for 10 min, the supernatant was collected and mixed with an equal volume of 1M NaOH, and then its optical absorbance was measured at 450 nm. Control reactions were identical except for the absence of gut extracts. The rate of proteolysis of azocasein was expressed as change in absorbance / min / mg protein.

The total protein content and enzyme activities of all samples are reported as mean ± standard error and statistically analyzed using Excel Microsoft Office and Student's t-test Program. Differences were considered significant at $p< 0.05$ level.

**5. Histopathological assay:**

The survival from untreated and treated parents ($LC_{70,}$ $LC_{50}$ and $LC_{25)}$ and 1st generation ($LC_5$) larvae were fixed in 10% formalin solution for quick larvae killing and tissue fixing. About ten larvae were bulked in

vials and kept in refrigerator till cutting. The fixed specimens were trimmed, washed and dehydrated in ascending grades of alcohols and cleaned in xylene. The larvae were imbedded in paraffin wax and serial sections of 4- 6 microns were made by microtome and mounted on glass slides and stained with haematoxylene and eosin and counterstained in alcoholic solution and prepared for examination by light microscope and photography (Durry and Wallington, 1980).

**Results and discussion**

**1. Bioassay:**

Toxicity parameters (concentration of insecticide and response of treated insects) of the two biological insecticides were illustrated in Tables (1 and 2). The value of $LC_{70}$, $LC_{50}$, $LC_{25}$, $LC_{10}$ and $LC_5$ of *B. thuringiensis*, sub sp. *Kurstaki* (Dipel 2x) reached to 36600, 18400, 7640, 7000 and 3450 spores/ml for parent 2nd instar larvae of *S.littoralis* and $LC_5$ value of 1st generation (F1) was decreased to 2150 Spores/ ml. The same values of Nucleopolyhedrovirus NPV (Littovir) were $2.38x10^8$, $1.45x10^8$, $7.75x10^7$, $7.71\ x10^7$, $4.40\ x10^7$ and $3.13\ x10^7$ PIB/ml for parents and 1st generation (F1) larvae, respectively. These data showed that Dipel 2x was more toxic to *S. littoralis* 2nd instar larvae than Littovir. The parent and their offspring (F1) larvae submitted insecticide doses dissimilar between the two generations, where the parents tolerated the dose that was equal $LC_{70}$ while the 1st generation (F1) was more susceptible and the dose declined to $LC_5$. The mortality rate was arising quickly and noticeable timely and was obligated according to the biological degradation affected by insecticide stressful and refer to the pest population at the subsequent generation had weak tolerance to this type of insecticide formulations. The two insecticide concentration values refer to the definite number of spores and virion that can kill the larvae of *S. littoralis* and the selection methods by the two formulations was not steepest but always decreased to smallest number of spores and virions responsible for killing. General observations on treated *S. littoralis* 2nd instar larvae were taken during the selection period with two pathogens. Treated larvae with higher concentrations of Bt produced morphological abnormalities appeared shortly after treatment and mortality become higher. After few days larvae died and remaining inactive ingoing prolonged moulting and body color changed. Abnormalities and growth disruption were taking place at lower concentrations, failure moulting and incomplete shaped were performed, infected larvae had small bodies and large heads. Feeding of larvae on virus revealed no symptoms during the first three days of treatment, but in the 4th day, infected larvae appeared more slowly, whitish, weak cuticle, body swelling than healthy larvae, body turn to liquid and death of treated larvae within few days. All treated larvae with the two bioinsecticides were dead except some of those treated with $LC_5$ which could grow to pupae and adult moths and produce 1st generation (F1) larvae. These larvae (F1) were more susceptible to the two tested pathogens and all selected larvae were dead after few days except those treated with $LC_5$.

Other researchers were detected the same results as, Jakka *et al*. (2014) mentioned that no differences in susceptibility to XenTari WG and Dipel ES pesticides were detected. No re-entry interval spraying is needed, and resistance development is slower than other chemicals. Time of application depend on the insect early stages. Hallad *et al.* (2011) recorded the highest mortality of *S. litura* was 80.3,72.6 and 64.2% for 2nd, 3rd and 4th instar larvae which treated with leaf disc feeding method by 80 Ppm of cry toxin. Polanczyk *et al*. (2000) reported that treatment of *S. frugiperda* 2nd instar larvae with suspensions of *Bt aizawai* (HD 68) and *Bt. thuringiensis* (4412) which containing 3 x $10^8$ cells/ml, produced 100% and 80.4% mortality of treated larvae, respectively. Abd-El Wahed *et al.* (2011) evaluated the potency

of commercially formulated biological insecticide; protecto (*Bt* var. *kurstaki*), viruset (Nuclear Polyhydrosis Virus SLNPV) and their mixture profect against *S. littoralis* 2nd instar larvae. Protecto is more effective than others, viruset was more effective on 4th instar larvae than profect. $LC_{90}$ and $LC_{50}$ for viruset were $1x10^6$ and $1x10^3$ PIBs/ml, respectively. Benuzzi *et al*. (2012) mentioned that timing of application and standard dosage of Littovir (SpliNPV) 200 ml/hectar reduced the hatching of *S.littoralis* larvae at repeated sprays in 8-day intervals. No residues were recorded on the crop and safe on mammals, beneficial insects and can be in uses in organic farming. Bt toxins and Baculoviruses are invertebrate-specific pathogens cause alterations in the physiology, metabolism and morphology of insects. Feeding of larvae on the two bioinsecticides produced symptoms which observed by Sharaby and El-Bendary (2017) who mentioned that the spore δ-endotoxin complex of *Bt* var aizawai effect on *S. littoralis* larvae. Toprak *et al*. (2005) reported that after inoculated of *S. littoralis* larvae with SpliNPV doses of 3000 and 20,000 OBs/ml (for neonates) and $10^6$ and $3x\ 10^6$ OBs/ml (for third instar), the symptoms appeared as; slowness of movement, vomiting ,diarrhea, complete paralysis and death. A unique dissimilar symptom and killing efficiency of multiple nucleopolyhedrovirus- SfMNPV-6th isolates were detected by Vieira *et al*. (2012) as no liquefaction in integument of *S. frugiperda* larvae and took a long time to kill than the high virulent isolate, SfMNPV-19.

**Table (1): Distinct concentrations of Dipel 2X and Littovir submitted to *Spodoptera littoralis* 2nd instar larvae (parents).**

| Insecticides | $LC_{70}$ (95% FL) | $LC_{50}$ (95% FL) | $LC_{25}$ (95% FL) | $LC_{10}$ (95% FL) | $LC_5$ (95%FL) | Slope ± SE | χ2(df) |
|---|---|---|---|---|---|---|---|
| **Dipel 2x 6.4% WP ($32\times10^3$ Spores /mg)** | 36600 (27300-53400) | 18400 (12600-24800) | 7640 (3770-11430) | 7000 (3200-10400) | 3450 (1190-6080) | 1.76± 0.297 | 1.2 |
| **Littovir SC ($5x10^8$ PIB /ml)** | $2.38X10^8$ ($1.9X10^8$-$2.5X10^8$) | $1.45X10^8$ ($1.15X10^8$-$1.8X10^8$) | $7.75X10^7$ ($5.5x10^7$-$6.9x10^7$) | $7.71X10^7$ ($4.7x10^7$-$6.1x10^7$) | $4.40\ X10^7$ ($3.7x10^7$-$5.2x10^7$) | 2.46± 0.312 | 0.634 |

**FL: Fiducial Limits**

**Table (2): Distinct concentration of Dipel 2X and Littovir submitted to *Spodoptera littoralis* 2nd instar larvae (1st generation F1).**

| Insecticides | $LC_5$ (95% FL) | Slope± SE | χ2(df) |
|---|---|---|---|
| **Dipel 2x 6.4% WP ($32\times10^3$ Spores /mg)** | 2150 (590-4210) | 1.76± 0.297 | 1.2 |
| **Littovir SC ($5x10^8$ PIB /ml)** | $3.13\ x10^7$ ($2.5x10^7$-$4.4x10^7$) | 2.46± 0.312 | 0.634 |

**FL: Fiducial limits.**

**2. Biochemical assays:**

**2.1. Total protein content:**

Total protein content is one of the major biochemical components necessary for an organism to develop, grow and perform its vital activities. Protein are important for individual level fitness associated traits such as body size, growth rate and fecundity and at higher levels of organization they have been linked to population dynamics, life histories and even biological diversification (Fagan *et al.*, 2002). Data in Table (3) illustrated the effect of $LC_{70}$, $LC_{50}$, $LC_{25,}$ $LC_{10}$ and $LC_5$ (for parents) and $LC_5$ (for F1) of Dipel 2X and Littovir treatments on total protein content in the whole-body homogenate of *S. littoralis* 2nd instar larvae. The protein concentration reached to 0.227 ±0.052, 0.146 ±0.075, 0.164 ±0.036, 0.177 ±0.064, 0.192 ±0.031, 0.201 ±0.016, 0.216 ±0.027 (mg/ mg B.W.) in the whole-body tissues of untreated (control) and Dipel 2X treated larvae, resp. These values were 0.208 ±0.044, 0.211 ±0.042, 0.217 ±0.033, 0.234 ±0.047, 0.263 ±0.023, 0.291 ±0.011 (mg/ mg B.W.) in Littovir treated larvae, respectively. These results revealed the presence of significant depletion (35.7and 27.8%) in total proteins of treated parent larvae with $LC_{70}$ and $LC_{50}$ of Dipel 2X compared with control larvae. Insignificant decrease in proteins of treated larvae was detected in other treatments of Dipel 2X and high concentrations of Littovir. Insignificant increase (3.1 and 15.9%) in larval proteins was recorded with $LC_{10}$ and $LC_5$ treatments of Littovir, but a significant value (28.2%) was presented in tissues of F1 larvae treated with $LC_5$ of virus.

Nath *et al.* (1997) mentioned that protein depletion in tissues may constitute a physiological mechanism and might play a role in compensatory mechanisms under insecticidal stress to provide intermediates to the kerbs cycle by retaining free amino acid content in insect tissues. Abd El-Aziz (2000) reported that infection of the cotton leafworm, *S. litioralis* larvae with *B. thuringienses var. kurstaki* (Dipel 2X) produced high significant reduction in the protein content. $LC_{50}$ treatment of *P.gossypiella* larvae with Bt and NPV caused insignificant increasing (13.9 and7.3%) in the total protein of treated larvae than untreated ones (Radwan *et al.,* 2018).

**2.2. ATPases activity:**

ATPases are membrane bound enzymes, the role of membrane lipids and their micro-environmental changes at physical and chemical levels may be responsible for the differential response observed at the level of ATPases activity in the insect populations. Membrane ATPases assist transport, reabsorption of metabolites and nutrients. These enzymes are secondary careers of ions and non-electrolytes (Lechleitner and Phillips, 1988 and Fogg *et al.,* 1991). ATPases are essential for the transport of glucose, amino acids, and other organic molecules. Any impairment in their activity will affect the physiology of the insect gut. These enzymes are in the midgut, malpighian tubules, muscles, and nerve fibers of the lepidopteran insects (Nathan, 2013). In lepidopterans, the perineurium serves as a diffusion barrier for polar cardenolides and provides an active barrier for non-polar cardenolides, and the P-glycoprotein-like transporter mediates the efflux of cardenolides in the nerve cord, thereby preventing interaction of these toxins with the susceptible target site in Na+/K+ ATPase (Petschenka *et al.,* 2013).

The effects of Dipel 2X and Littovir treatments on the activity of Na+/K+ ATPase enzymes in the whole-body tissues of *S.littoralis* larvae were shown in Table (3). The activity of ATPases in untreated larvae reached to 7.71 ±0.822 µ mol/ mg protein/ min, but this activity was insignificantly elevated (16.5 and 20.4 and 15.1 and 24.0%) with $LC_{50}$ and $LC_{25}$ of Dipel 2X and $LC_{25}$ and $LC_{10}$ of Littovir treatments for parent larvae, respectively. A significant elevation (39.7 and 31.0%) in ATPase enzymes was

recorded in Bt $LC_{70}$ and virus $LC_5$ treatments of parent larvae compared with control. Insignificant inhibition of enzyme activity was presented in other treatments of parents and F1 larvae.

Dermauwa and Van Leeuwen (2014) mentioned that the hydrolysis of ATP during substrate transport is followed by quantification of the liberated inorganic pyrophosphate. The transported compounds such as inhibitors controlled this ATPase activity. Nathan *et al.* (2004) reported that the rice leaffolder *Cnaphalocrocis medinalis* (Guen´ee) (Lepidoptera: Pyralidae) larvae exposed to bacterial toxin were exhibited reduction in ATPase activities. The ATP content of toxin-treated tissue of the spruce budworm, *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae) is inversely proportional to the amount of toxin added and incubation with δ-endotoxin protein for 60 min and revealed massive outer membrane disruption and subsequent loss of cytoplasmic constituents, accompanied by swelling of the nuclear membrane (JohSon, 1981). Castagnola and Stock (2014) reported the binding of Bt to ATP reported to disrupt ion channels in the midgut neuromuscular junctions that causes paralysis by the release of K+ from the midgut into the hemolymph or from intracellular sources such as K+ channels and disrupt ion transport across a membrane and named Bt receptors. The midgut plasma membrane V-ATPase of the tobacco hornworm *Manduca sexta* L. (Lepidoptera: Sphingidae) was occurred in the apical cell membranes of goblet cells, and down-regulated during moulting and starvation. The decrease in transcript levels lead to minimizes V-ATPase biosynthesis, inactivation and save cellular energy (Wieczorek *et al.,* 2000). Mikhailova (1989) stated the *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) cells exposed to baculovirus vector SUR1 and Kir 6.2 infection, ATP-sensitive K-channels was sensitive to ATP, glibenclamide and diazoxide, and anywhere blocked by ATP but insensitive to sulphonylureas.

**Table (3) : The total protein content and Na+/K+ ATPase enzyme activity in the whole-body tissues of untreated and treated *Spodoptera littoralis* larvae.**

| Bioinsecticide concentration | Dipel2x | | | | Littovir | | | |
|---|---|---|---|---|---|---|---|---|
| | Total protein concentration (mg/mg B.W.) | | ATPase activity (µmol/mg protein/min) | | Total protein concentration (mg/mg B.W.) | | ATPase activity (µmol/mg protein/min) | |
| | Mean ± S.E | Change % | Mean ± S.E | Change % | Mean ± S.E | Change % | Mean ± S.E | Change % |
| **Untreated** | 0.227 ±0.052 | 0.0 | 7.71 ±0.822 | 0.0 | 0.227 ±0.052 | 0.0 | 7.71 ±0.822 | 0.0 |
| **$LC_{70}$** | 0.146 ±0.075 | (-) 35.7* | 10.77 ±0.614 | (+)39.7* | 0.208 ±0.044 | (-) 8.4 | 7.52 ±0.572 | (-)2.5 |
| **$LC_{50}$** | 0.164 ±0.036 | (-) 27.8* | 8.98 ±0.353 | (+)16.5 | 0.211 ±0.042 | (-) 7.1 | 7.38 ±0.384 | (-)4.3 |
| **$LC_{25}$** | 0.177 ±0.064 | (-) 22.0 | 9.29 ±0.467 | (+)20.5 | 0.217 ±0.033 | (-) 4.4 | 8.87 ±0.276 | (+)15.1 |
| **$LC_{10}$** | 0.192 ±0.031 | (-) 15.4 | 7.68 ±0.482 | (-)0.4 | 0.234 ±0.047 | (+) 3.1 | 9.56 ±0.355 | (+)24.0 |
| **$LC_5$** | 0.201 ±0.016 | (-) 11.5 | 6.55 ±0.753 | (-)15.1 | 0.263 ±0.023 | (+) 15.9 | 10.10 ±0.437 | (+)31.0* |
| **F1) $LC_5$ (** | 0.216 ±0.027 | (-) 4.9 | 5.98 ±0.677 | (-)22.4 | 0.291 ±0.011 | (+) 28.2* | 7.19 ±0. 922 | (-)6.7 |

**Each value represents the mean of three replicates ± S.E (+) =Increase, (–) =Decrease, S.E = Standard Error at P < 0.05**
*** = Significant**

## 3. Proteases activity:

Insect midgut proteases are original targets for insecticidal agents such as Bt Cry toxins and viruses (Terra and Ferriera, 1994). Data in Table (4) revealed that the total protein content (0.205 ±0.052 mg/mg gut) of gut

tissue was low than in the whole-body tissues of untreated *S.littoralis* larvae with insignificant value (9.7%). The concentration of gut proteins was slightly decreased ( 16.6, 9.8 and 5.4%) in parent larvae treated with $LC_{70}$, $LC_{50}$ and $LC_{25}$ of Dipel 2X than control larvae. The remaining treatments of Dipel 2X and all Littovir caused insignificant increase in gut proteins of treated larvae. Rashed *et al*. (2012) mentioned that after 24 and 48 hrs. of treatment the pink bollworm, *P. gossypiella* 3rd instar larvae with $LC_{50}$ of Dipel 2X and Abamectin, the total protein content of gut tissue was decreased by 33.1, 33.5% and 39, 26.3%, respectively.

A set of protease enzymes allows *S.littoralis* to feed on different host plants, so that the activity of protease enzymes in gut tissue of control larvae was 181.20 ±4.752 (mM/mg protein/ min) and increased in bacterial treatments till significant value (27.3%) with $LC_{70}$ treatment of parent larvae. Littovir caused slight reduction in gut proteases of treated larvae which reach to significant value (40.1 and 32.3 %) with $LC_{70}$ and $LC_{50}$ (Parent) treatments. $LC_5$ treatment of two pathogens caused a slight increase in activity of F1 treated larvae gut proteases. The same results were detected in *S. littoralis* gut by Keller *et al.* (1996) who found that the degradation rate of δ –ednotoxin was consistent with the increase of protease activity during larval development. Trypsin proteases in *S. litura* larvae midgut were involved in the Vip3Aa toxin activation (Song *et al.,* 2016). Oppert (1999) found no differences in midgut proteinase activity from susceptible and *Bt kurstaki*-resistant strains, but *Bt subsp. Entomocidus,* had lower soluble gut proteinase activities. Bt resistance mechanisms may include changes in gut pH, or modifications in proteases and changes in solubility, differences in the degree of protoxin activation, enhanced toxin or receptor-toxin degradation. Wilkins (2017) stated that changes in proteases activity are insecticide resistance evidenced and play role in maintaining healthy cells and elevated protease activity leave more degradation products (amino acids) of intracellular proteins. The specific activity of total protease, chymotrypsin and elastase enzymes was significantly decreased with increasing of *Btk* concentrations (Fathipour *et al*., 2019). The ingestion of *S. exigua* to sub-lethal doses of two *B. thuringiensis* toxins, Cry1Ca and Vip3Aa, and nucleopolyhedrovirus (SeMNPV) caused high transcriptional activation of genes encoding antimicrobial peptides (AMPs) and lysozymes in the midgut (Crava *et al.,* 2015).

**Table (4) : The total protein content and total protease enzymes activity in the gut tissues of untreated and treated *Spodoptera littoralis* larvae.**

| Bioinsecticide concentration | Dipel2x | | | | Littovir | | | |
|---|---|---|---|---|---|---|---|---|
| | Total protein concentration (mg/mg gut tissues) | | Protease activity (mMol /mg protein/min) | | Total protein concentration (mg/mg B.W.) | | Protease activity (mMol /mg protein/min) | |
| | Mean ± S.E | Change % | Mean ± S.E | Change % | Mean ± S.E | Change % | Mean ± S.E | Change % |
| **Untreated** | 0.205 ±0.052 | 0.0 | 181.20 ±4.752 | 0.0 | 0.205 ±0.052 | 0.0 | 181.20 ±4.752 | 0.0 |
| **$LC_{70}$** | 0.171 ±0.075 | (-) 16.6 | 230.64 ±4.625 | (+) 27.3* | 0.246 ±0.016 | (+) 20.0 | 108.51 ±3.621 | (-) 40.1* |
| **$LC_{50}$** | 0.185 ±0.036 | (-) 9.8 | 219.88 ±6.112 | (+) 21.4 | 0.231 ±0.067 | (+)12.7 | 122.75 ±3.954 | (-) 32.3* |
| **$LC_{25}$** | 0.194 ±0.064 | (-) 5.4 | 211.58 ±3.877 | (+) 16.8 | 0.218 ±0.036 | (+)6.3 | 150.51 ±5.222 | (-) 16.9 |
| **$LC_{10}$** | 0.213 ±0.031 | (+) 3.9 | 201.45 ±5.566 | (+) 11.2 | 0.224 ±0.033 | (+) 9.3 | 162.93 ±4.363 | (-) 10.1 |
| **$LC_5$** | 0.234 ±0.016 | (+) 14.2 | 194.67 ±2.451 | (+) 7.4 | 0.209 ±0.064 | (+) 2.0 | 177.24 ±2.883 | (-) 2.2 |
| **F1) $LC_5$ (** | 0.226 ±0.027 | (+) 10.2 | 188.54 ±3.621 | (+) 4.1 | 0.216 ±0.027 | (+) 5.4 | 184.12 ±6.455 | (+) 1.6 |

**Each value represents the mean of three replicates ± S.E (+) =Increase, (–) =Decrease, S.E = Standard Error at P < 0.05**
*** = Significant**

**4. Histopathological examination:**

The digestive system in insects consists of three parts, foregut (stomodeum), midgut (mesontron) and hindgut (mesontron). Mid gut is the important part which produces digestive enzymes and, absorbs the digested food (Terra and Ferriera, 1994). In lepidopteran insects the midgut is composed of 3 major laminations: peritrophic membrane, mucosa, and musculature. The peritrophic membrane is chitinous forms and envelops the food particles, along the midgut. The mucosa is consisting of midgut epithelium and stem cells embodied by group closely packed, spherical nuclei with thin chromatin surrounded by a thin layer of clear cytoplasm. The epithelial cells are responsible for absorption and secretion of enzymes (columnar cells), ion homeostasis (goblet cells), and the epithelia (regenerative or stem cells). The columnar and goblet cells have a brush border of microvilli in outside and spherical vesicular nucleus with a large vacuole at its distal end, or they have a larger oval-shaped nucleus in the middle of the cell and a smaller vacuole above the nucleus (Chi *et al.,* 1975 and Schünemann *et al.,* 2014).

The photomicrograph of transverse section in midgut of untreated *S.littoralis* larvae revealed that the gut wall has normal muscular tissues, epithelial cells (regenerative, goblet and columnar cells), brush border membrane and peritrophic membrane (Figure, 1). Treatment of larvae with $LC_{70}$ of Dipel 2X produced serious effects on midgut tissues as the epithelial cells of the midgut appear to be swollen (hyperplasia is increase in the number of cells and mass of tissue) and ruptured, rupture of the bruch border microvilli, appearance of apoptotic bodies, activation of midgut stem cells, contraction the muscle layer, extensive fragmentation and separation of mucosal epithelium from the basement membrane and the lumen was filled with debris of disrupted cells. $LC_{50}$ of Dipel 2X caused disarrangement and degeneration of the columnar cells, appearance of apoptotic bodies, enlargment of vacuoles, contraction of the circular muscle bands, rupture of the bruch border microvilli and the lumen was filled with debris of disrupted cells. $LC_{25}$ treatment of Dipel 2X induces epithelial lifting, appearance of apoptotic bodies, rupture of the bruch border microvilli, vacuoles formation and the lumen was filled with debris of disrupted cells. Treatment of F1 larvae with $LC_5$ of Bt increased vacuole numbers, swelling, and disarrangement of epithelial cells and rupture of the bruch border microvilli (Figure, 2). $LC_{70}$ treatment of Littovir caused hyperplasia and destruction of the midgut epithelium, appearance of apoptotic bodies, rupture of the bruch border microvilli and vacuoles formation. $LC_{50}$ of Littovir showing increase in number of stem cells, degeneration of most columnner cells, appearance of apoptotic bodies and rupture of the bruch border microvilli. $LC_{25}$ of Littovir shown hyperplasia of cells lining mid gut and cytoplasmic vacuolization. Mid gut of treated F1 larvae with $LC_5$ of Littovir produced cytoplasmic vacuolizatio (Figure, 3).

The histopathologicl effects of Bt and virus on midgut tissues of treated insect larvae were investigated by other researchers. Galluzzi *et al.* (2012) investigated that some cell death phenomenon as apoptosis is a mechanism included in cell death program conferring to pathogenic infection, firstly this cells activate enzymes whereas key proteins are cleaved by cysteine proteases, known as caspases, that degrade the cells own nuclear DNA and cytoplasmic proteins, break up into fragments, called apoptotic bodies and contained portions of the cytoplasm and nucleus. The dead cell and its fragments are rapidly gobbled. Dougherty *et al*. (2006) mentioned that the midgut epithelial cells showed signs of apoptosis manifested as shrinkage of the cells and the presence of condensed chromatin with some vacuolization. This process is considered as the proper mechanism of cells against

pathogens and toxic compounds (Sakr, 2007). Escriche *et al.* (1988) stated that *Bt.* crystal proteins Cry1Ca, induce pore formation in midgut cell membranes of *S. littoralis*, made brush border membrane vesicles permeable to KCl (osmotic swelling) but Cry1Aa, Cry1Ab, and Cry1Ac did not. Rouis *et al*. (2007) recorded the histopathological changes in midgut of olive pest insect, *Prays oleae* (Bernard) (Lepidoptera: Praydidae) , larvae by Bt, included vacuolization of the cytoplasm, hypertrophy of the epithelial cells and nucleus, brush border membrane impairment, disintegration of vesicle formation in the apical region of cells toward the midgut lumen, and leakage of cytoplasm material in the lumen proved that proteolytic processing of cry toxins is responsible for activation, and in insect resistance. Physiological hyperplasia can cause by hormones induce increases in the functional capacity of a tissue when needed, or compensatory hyperplasia, induce increases tissue mass after damage (Sanderson *et al*., 2017). Cry34Ab1 and cry35Ab1are proteins derived from *B. thuringiensis* together comprise a binary insecticidal toxin with specific activity against the western corn rootworm, *Diabrotica virgifera virgifera* (LeConte) (Coleoptera: Chrysomelidae). Clear symptoms of intoxication were observed for proteins tested, including swelling and sloughing of enterocytes, constriction of midgut circular muscles, stem cell activation, and obstruction of the midgut lumen (Bowling *et al*., 2017). Sanjaya *et al*. (2010) reported the histological change in *S. litura* exposed to SlNPV at serial concentrations was peritrophic membrane eternally affected by the longer time of immobilisation of the virion. Pachiappan *et al*. (2018) found that mulberry Leaf webber, *Diaphania pulverulentalis* (Hampson) (Lepidoptera: Pyralidae) infected with NPV showed virus virions scattered throughout the granular virogenic stroma and accumulated peripherally.

Results of this study concluded that the Na+/K+ ATPase and Protease played an important role in toxicity of *Bacillus thuringiensis* subsp. *Kurstaki* and Nuclear Polyhedrosis Virus (SpliNPV) and tolerance of *S littoralis* larvae to their effects.

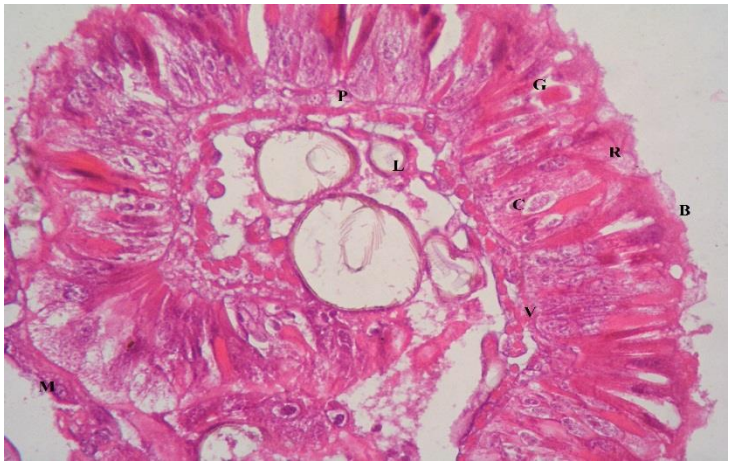
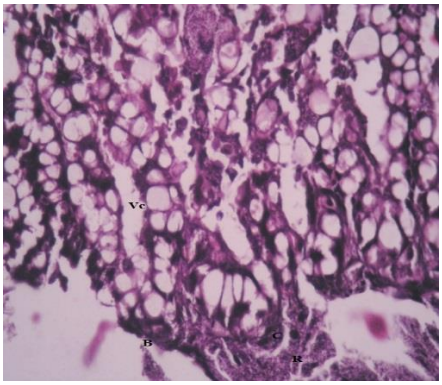
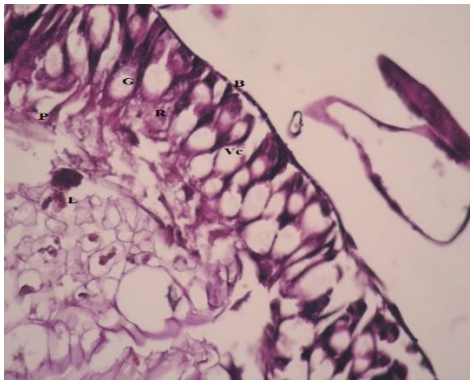


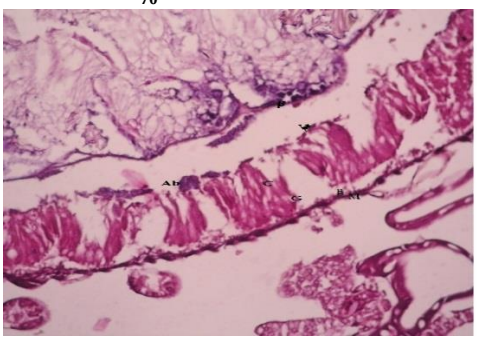

**Figure (1): Photomicrography of transverse section in midgut of untreated *Spodoptera littoralis* larvae (stained with Hematoxylin and Eosin, H + E 200X). B:Basement membrane, C:Columnar, G:goblet cells, M:Muscular tissue, P: peritrophic membrane, R:Regenerative cells, and V:Microvilli.**



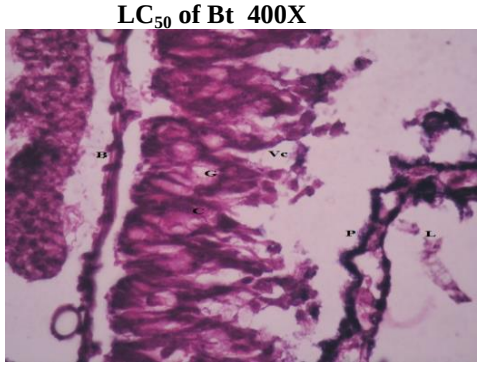

$LC_{70}$ of Bt 400X




$LC_{50}$ of Bt 400X

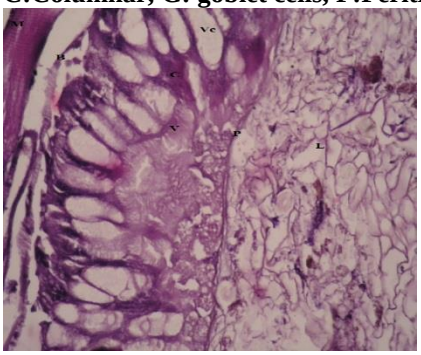



$LC_{25}$ of Bt 200X

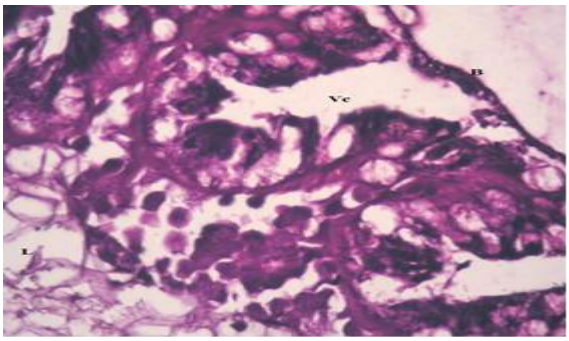



$LC_5$ of Bt (F1) 400X

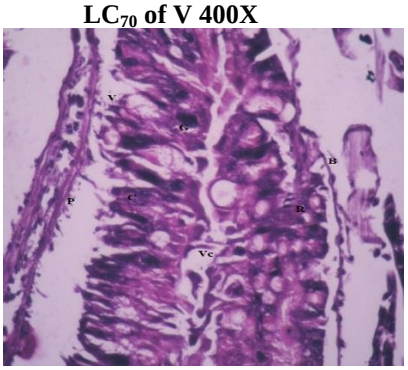
**Figure (2): Photomicrography of of partial transverse section in midgut of treated *Spodoptera littoralis* larvae with Dipel 2X (stained with Hematoxylin and Eosin, H + E). Ab: Apoptotic bodies, B: Basement membrane, C:Columnar, G: goblet cells, P:Peritrophic membrane R:Regenerative cells, V:Microvilli and Vc:Vacuole.**



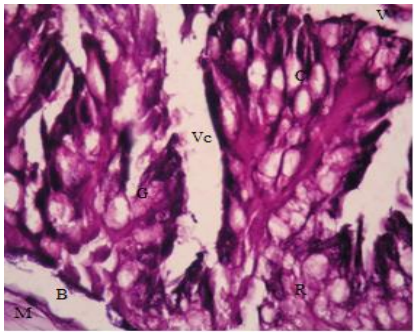

$LC_{70}$ of V 400X




$LC_{50}$ of V 400X




$LC_{25}$ of V 400X




$LC_5$ of V (F1) 400X

**Figure (3): Photomicrography of partial transverse section in midgut of treated *Spodoptera littoralis* larvae with Littovir (stained with Hematoxylin and Eosin, H + E). B:Basement membrane, C:Columnar, G: goblet cells, P:Peritrophic membrane, R:Regenerative cells, V:Microvilli, and Vc:Vacuole.**

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