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Larvicidal, Biochemical and Histological Effects of Rhizospheric Actinobacterial Secondary Metabolites on *Leucinodes orbonalis* Larva

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Abstract: Non-pathogenic actinobacterial strains were isolated from rhizospheric soil sample collected from brinjal fields. The secondary metabolite of the isolated strain developed an acute stress in the lepidopteran larva *Leucinodes orbonalis*. The lethal toxicity (LD_{50}) was observed at 75 ppm. The sublethal exposure (30, 35 and 40 ppm) of actinobacterial secondary metabolites cause severe biochemical changes in the larval tissues. Larval biochemical levels were greatly reduced in the treated groups due to the energy demand developed by secondary metabolites exposure. This condition was evidenced by the tissue damaging enzymes (AST, ALP, ACP and LDH) levels which significantly ($P<0.01$) increased in treated larva as compared to the control larva. Histological analysis of gut of treated larva gut showed increased vacuolation with muscle degeneration. The exposure of secondary metabolites of the isolated strain altered cell membrane integrity which resulted in the enhanced tissue damaging enzyme with altered histoarchitecture of the larva gut.

Keywords: Actinobacteria, *Leucinodes orbonalis*, Lepidopteran, Biochemical, Gut histology

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Introduction

Actinobacteria are the soil-dwelling and saprophytic microbes usually present either at the deep (2 m) ground or surface soil. It is under the largest taxonomical lineages of bacterial domain (Ludwig *et al.*, 2012; Bahrami *et al.*, 2022b). It promotes translocation of nutrients from soil into the plants which act as the plant growth regulators with antimicrobial properties (Fenton

et al., 1992; Jiang *et al*., 2021; Zhang *et al*., 2022). Isolated active compounds were used as biostatic and biocidal agents (Balakrishnan *et al.*, 2017; Bahrami *et al*., 2022a).

The phytophagous insect *Leucinodes orbonalis* (Class: Lepidoptera) is considered as a serious pest of the brinjal plants. *L. orbanolis* decreases up to 60% of the yearly productivity of brinjal plants (Alam *et al.*, 2003; Tiwari and Singh, 2015). Pest control mechanism along with various biological byproducts should satisfy the need of the farmers as either cost effective or target specific (Rausell *et al.*, 2000).

Insect pest management (IPM) reported the importance of pesticides against the pest (Saruhan *et al*., 2014). Pesticidal non-targeted species interaction is considered as a serious issue due to the bioaugmentation of the chemicals in the biosystem. Usage of chemicals in pesticides cause serious hazards to the ecosystems (water, land and air) (Abedi *et al*., 2014). Elbert and Nauen (1998) reported the impact of chemical pesticide on the various strains of lepidopteran pest. Utilization of the chemical based fertilizers and pesticides on the fields developed serious damages to the soil nutrient composition, water holding capacity, altered soil and water pH, developed resistance and mutations to the exposed insects, genetic variability of the plant, accumulation of toxicants in the plant which resulted in the bioaugmentation of chemicals into the organisms. Mabrouk *et al*. (2001) suggested the importance of an alternative and sustainable method of fertilizers such as biofertilizers and biopesticides.

The aim of this study was to isolate efficient Actinobacterial strain against the pesticides from rhizosphere soil collected from brinjal field and also to characterize the efficacy of Actinobacterial Secondary metabolites against the lepidopteron pest, *L. orbonalis* (Guenee). The larvicidal activity of secondary metabolites (isolated from the selected actinobacterial) on *L. orbonalis* was evaluated in terms of biochemical and histological studies.

Materials and Methods

Experimental design

Rhizospheric soil treated with fertilizers were collected from brinjal cultivated field in Paithur village (11°30'54.71"E, 78°33'19"E), Attur Taluk (Salem district, Tamil Nadu) One kilogram of surface soil was collected from sampling site and transported to the laboratory (Department of Botany, Jamal Mohamed College) for air dry and sieve process. Based on the primary and secondary screening (Helen Diana and Syed Jahangir, 2019), the isolated actinobacteria and their secondary metabolites were obtained.

Synthesis of extra cellular products

A loopful of the selected actinobacterial strain was cultured in mineral medium on an orbital shaker for 7 days at 120 rpm and centrifuged in 4°C at 8000 rpm (Helen Diana and Syed Jahangir, 2019). Supernatant was collected and mixed with equal volume of Ethyl acetate:Diethyl ether in a separating funnel. The organic layer of Ethyl acetate:Diethyl ether was collected individually, then aspirated, pooled and evaporated at room temperature for further studies.

Larval mortality studies

Leucinodes orbonalis Guen. is a monophagus and phytophagus insect which belongs to the order Lepidoptera, commonly known as eggplant borer, brinjal borer, fruit and shoot borer. Their host is *Solanum melongena* L. (brinjal). Brinjal fruits procured from Attur agricultural market and the 3rd and 4th instar larva were collected. Lethal concentration of isolated actinobacterial strain secondary metabolites was tested on *L. orbonalis* larvae (Fig. 1). Various concentrations (50, 70, 90, 110, 130 and 150 ppm) of isolated strain secondary metabolites were impregnated with brinjal fruits and the instars of *L. orbonalis* (n=12) were exposed for 96 h. The impregnated sample was replaced by new sample at everyday morning.

Dead larva were removed from the testing vessel every day during experimental period (4 days). The lethal concentration (LC_{50}) of the

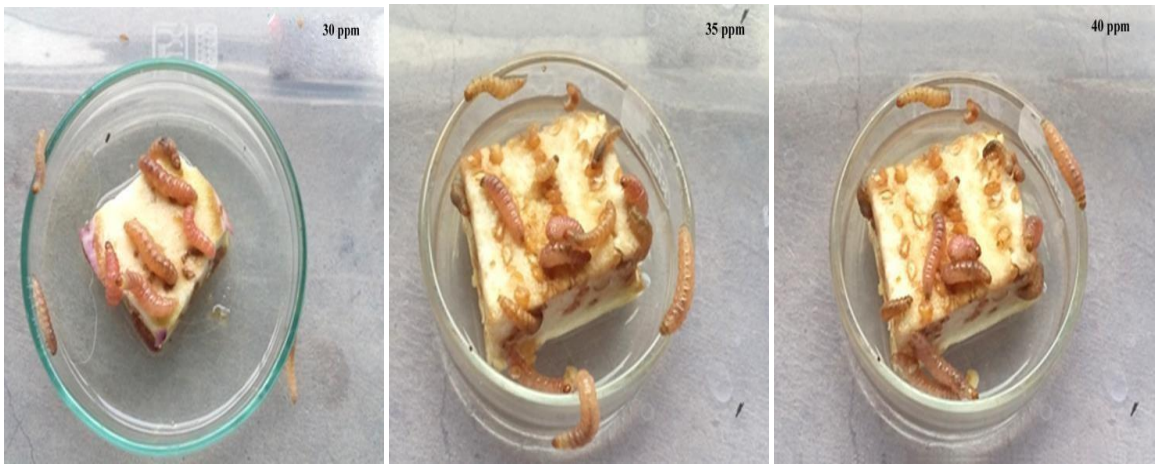


Fig. 1: Actinobacterial extra cellular products treated larvae (n=12).

secondary metabolites were identified by regression and probit analysis. Based on LC_{50}, three different concentrations (30, 35 and 40 ppm) were determined.

Biochemical analysis

After treatment (4 days), the larvae were collected and homogenized with 0.09% saline, supernatant was collected by centrifugation (15 min, 1200 rpm). Tissue biochemical parameters such as total cholesterol, total glucose, albumin, and globulin (total protein) were analyzed by standard methods (Lowry *et al*., 1951; Zak *et al*., 1954; Trevor *et al*., 2008). Tissue damaging enzymes such as alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH) and alkaline phosphatase (ACP) levels were analyzed by standard procedures (Srikanthan *et al*., 1955; King *et al*., 1965; Estiarte *et al*., 2008).

Data were statistically analyzed by SPSS 17.0 version software and subjected to homogenous subset with Duncan post hoc Tukey test for significance between the control and treated larvae.

Histological analysis

For light microscopic studies, four larvae from each group were fixed (formalin), washed and processed through 30%, 50%, 70%, 80%, 90% and 100% alcohol series. Samples were dehydrated and cleared in xylol and paraffin wax used for embedding. By using ultramicrotome, sections were cut at 5 µm and stained with Hematoxylin and Eosin (Al-Mehmadi and Al-Khalaf, 2010). Sections were mounted and observed under Olympus microscope and photomicrographs were taken.

Results and Discussion

Lethal dose concentration and Experimental Dose

During experimental period (4 days), each day the mortality of the instars was observed for control (without secondary metabolites) and treated groups. Actinobacterial metabolites effect on the instars were evalauted by LC_{50} (probit method). LC_{50} of actinobacterial strain was determined as 75 ppm (Fig. 2) based on the regression equation $Y=26.30+11.61X$ ($R^2=0.974$). It has been reported that larva of *Hyphantria cunae* treated with biopesticides showed increased mortality rate in the experimental duration (3-7 days) and significantly increased lethality with increased concentration of pesticide (Saruhan *et al*., 2014). Lethality was studied for the biopesticide exposed larva of mosquitoes *Anopheles senphensi*, *Aedes aegypti*, *S. albofavus*, *S. rochei*, *S. albus* and *S.*

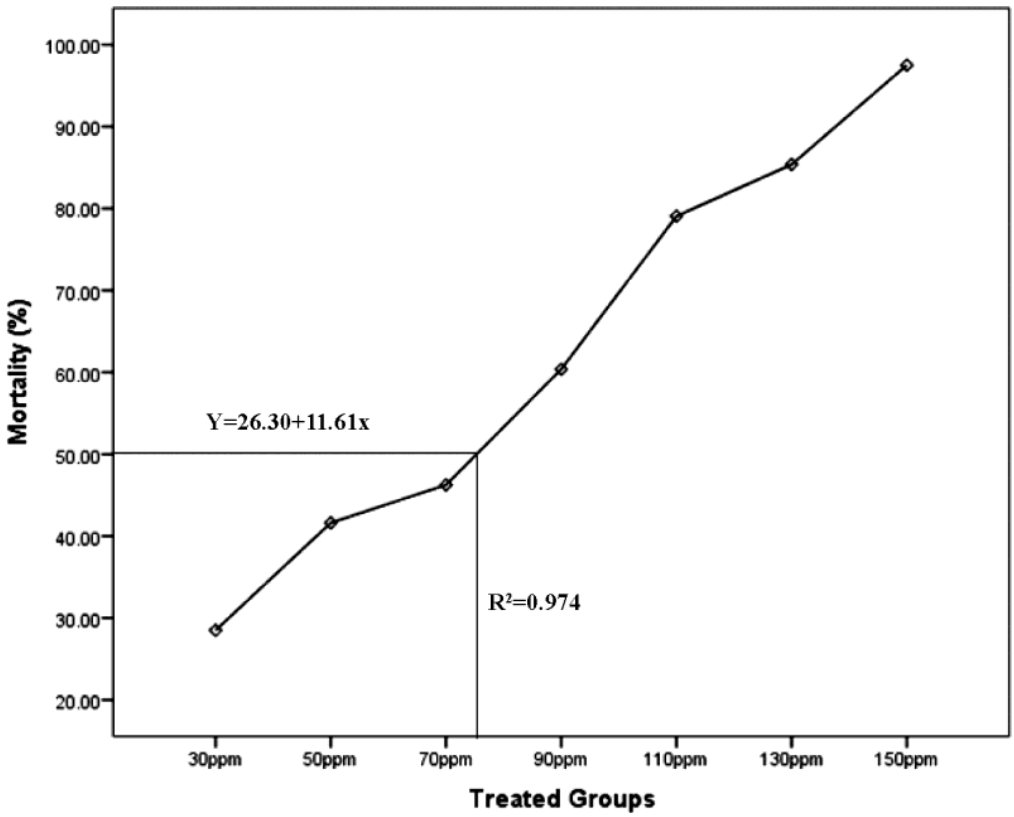


Fig. 2: Lethal concentration of secondary metabolites of actinobacterial strain for larva of *Leucinodes orbonalis*.

griseus (Balakrishnan *et al*., 2017). Primary and Secondary screening of actinobacterial strains were the essential step for the larvicidal and biocontrol studies (Swamidoss *et al*., 2022).

Biochemical analysis

Actinobacterial strain secondary metabolites exposed groups showed significantly decreased ($P<0.01$) total glucose levels as 6.81±0.03, 6.10±0.05 and 5.10±0.07 mg/dl after treatment with 30 ppm, 35 ppm and 40 ppm, respectively as compared with glucose level of control group (Table 1). Tukey post hoc Duncan Test with homogenous subsets (a-d) between the treated groups were analyzed. When compared to control group, the metabolite exposed groups showed decreased glucose concentrations in larvae to meet the stress developed in the larvae system due to the actinobacterial metabolite exposure. Similarly decreased glucose concentrations were noticed in mosquito larva by Ranjit and Dash (1994), El-Sheikh (2002) and Assar *et al.* (2012).

In treated groups, significantly decreased cholesterol concentrations were observed as 11.05±0.01, 9.83±0.07 and 7.63±0.07 mg/dl after treatment with 30 ppm, 35 ppm and 40 ppm, respectively as compared to control group (16.21±0.03 mg/dl). Larval epidermal region stores fat for various metabolism. Actinobacterial secondary metabolites exposure developed a stress in the larval system, which utilizes fat for the tissues repairment. Increased utilization of tissue cholesterol levels by the larva with increased concentration of the metabolites caused reduced cholesterol levels in the treated group larvae.

Control larva protein level was 1.02±0.02 g/dl whereas it was 0.90±0.06, 0.71±0.05 and 0.59±0.03 g/dl, in 30 ppm, 35 ppm and 40 ppm, respectively in exposed larvae. Significantly reduced total protein levels observed in treated groups, which resulted due to the assimilation of high amount of total protein to produce more energy intermediated by catabolic reaction and enzyme synthesis. Significantly reduced albumin

Table 1: Actinobacterial secondary metabolites effects on the tissue biochemical and enzyme (Mean±SE) profile of *Leucinodes orbonalis* instars (n=12)

Parameters	Control	Actinobacterial strain (Secondary metabolites)		
		30 ppm	35 ppm	40 ppm
Biochemical analysis (Mean±SE)				
Total Glucose (mg/dl)	11.09±0.05^{d}	6.81±0.03^{c}	6.10±0.05^{b}	5.10±0.07^{a}
Total Cholesterol (mg/dl)	16.21±0.03^{d}	11.05±0.01^{c}	9.83±0.07^{b}	7.63±0.07^{a}
Total protein (g/dl)	1.02±0.02^{d}	0.90±0.06^{c}	0.71±0.05^{b}	0.59±0.03^{a}
Albumin (g/dl)	0.56±0.05^{c}	0.48±0.05^{b}	0.39±0.06^{a}	0.31±0.03^{b}
Globulin (g/dl)	0.44±0.03^{d}	0.34±0.03^{b}	0.29±0.05^{a}	0.20±0.03^{c}
Enzyme analysis				
AST (U/L)	91.21±0.55^{a}	121.45±0.74^{b}	170.01±0.11^{c}	201.50±0.66^{d}
ALT (U/L)	79.98±1.66^{a}	99.01±1.88^{b}	110.31±2.75^{c}	129.98±2.43^{d}
ALP (U/L)	41.22±0.18^{a}	78.98±0.22^{b}	89.20±0.54^{c}	101.11±0.22^{d}
LDH (U/L)	76.72±8.00^{a}	94.11±2.11^{b}	122.78±8.05^{c}	134.12±0.24^{c}

P<0.01, Superscript letters (a-d) – Homogenous subsets

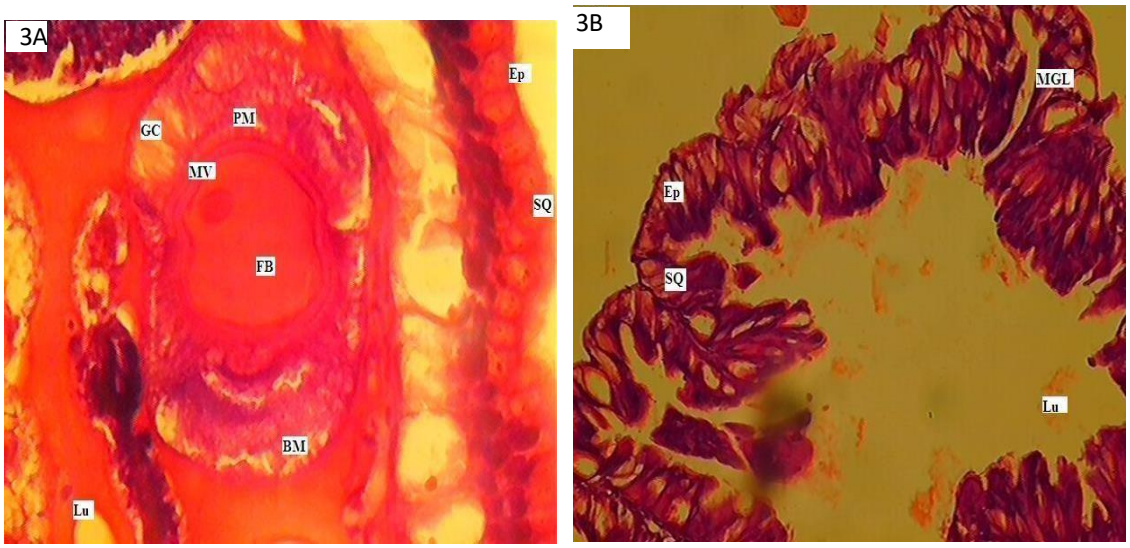
and globulin levels (Table 1) were observed in treated larval tissues due to the release of enzyme and free amino acids into the circulatory fluid haemolymph. When compared to control larva total protein as 1.02±0.02 g/dl, the treated larva showed significantly (P<0.01) reduced concentrations of total protein. Tissue damaging enzymes such as AST, ALT and ACP were increased in tissues and blood plasma due to the stress developed by the toxicant exposure. The increased ALT levels due to the enhanced metabolic activities in the treated larva. ANOVA results showed significance between the mean differences of control and treated groups.

Edward and Jenner (2001) reported the free aminoacid and protein levels in circulating fluid representing the improved detoxification in the treated tissues, eventually leads to the release of lactate dehydrogenase enzyme from the affected tissues. Decreased levels of protein and glucose concentrations with high AST and ALT levels were observed in the dipteran insects treated with Cyromazine (0.1 ppm) (Assar *et al*., 2012).

Enhanced ALP levels gradually increased the moulting process by increasing Ecdysone and lysosomes (Radford and Misch, 1971). Enhanced activity of AST related with high protein levels in experimental groups denoted that instar (3rd and 4th) larvae immediately moulted into the pupae with deformities but 5th instar formation was absent (Pant and Morris, 1972; Sidhu, 1982; Dominika *et al*., 2010).

Histological studies

In larva, the midgut constitutes the largest part of the digestive system where the digestion takes place. Histological sections of midgut, malpighian tubules and their neighbouring organs or parts of larva (400x magnification) were mainly focused here to study about the effect of exposure of actinobacterial secondary metabolites through feed. Mid gut cross section of control larvae of 4th instar larva (Fig. 3A) showed epidermis (Ep) with squamous cells (SQ) present in the outer layer. Brush border membrane composed of numerous

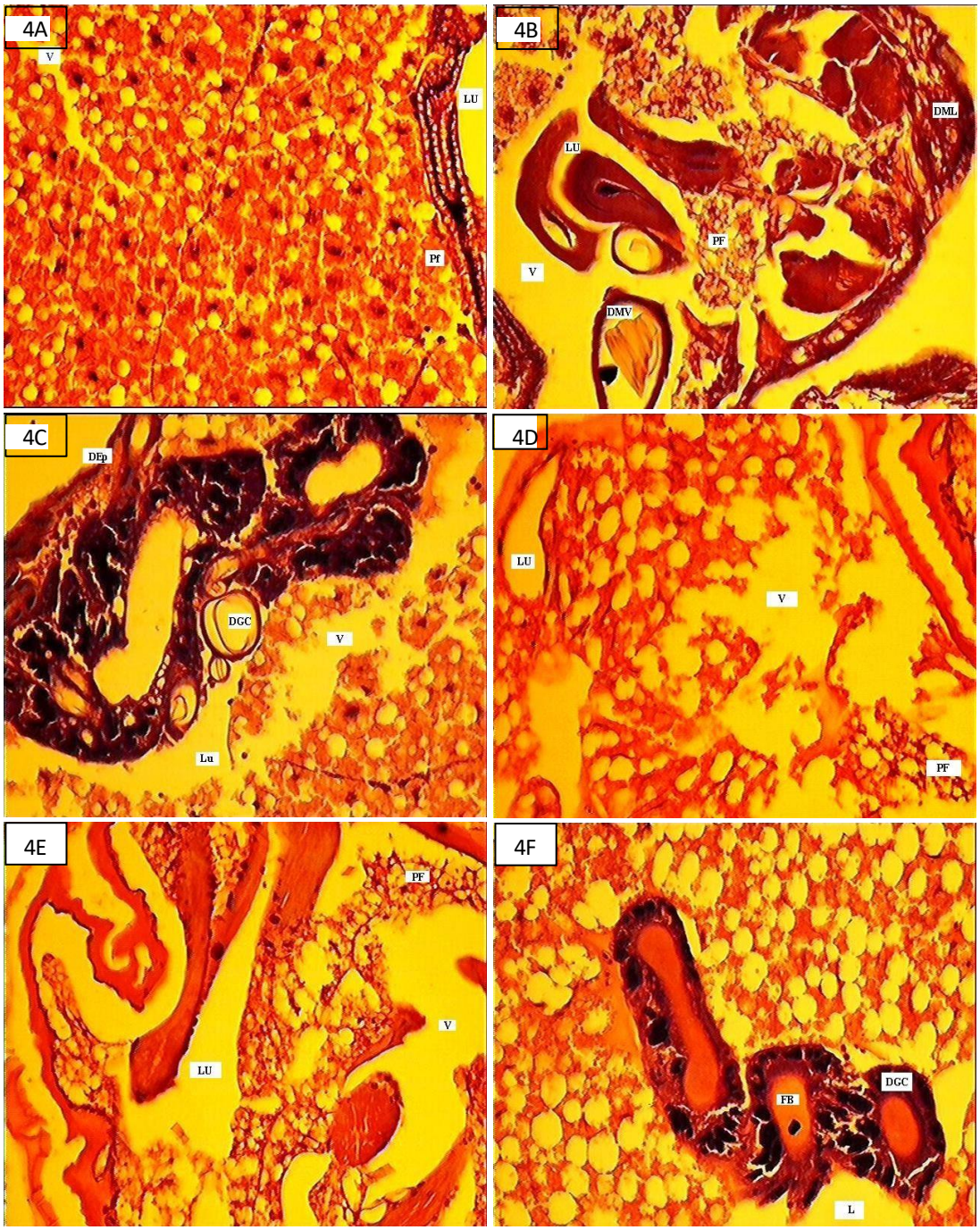


Figs. 3A, B: Cross section observations of mid gut and epidermis of 4th instar *L. orbonalis* control larva (400x).

and regularly placed microvilli (MV) observed in gut lumen (Lu) filled with food bolus (FB). The gastric caecum (GC) showed a well-preserved layer of epithelial cells and innerside of the caecum, the peritrophic membrane (PM) and basement membrane (BM) were observed. In control larva, the malpighian tubules walls composed of a single layer of cuboidal epithelial cells with prominent basal lamina and their apical membrane showed numerous short and irregular projections external invaginations with properly ordered epidermal (Ep) layers with elongated squamous (SQ) cells (Fig. 3B) with lumen enters into the malpighian tubule layer (MGL). Lepidoptera insect histological studies were reported by Standlea *et al*., (1968) and Mathur *et al*. (1972) whereas it is also studied in other insect larva such as Coleoptera (Vasques *et al*., 1988), Diptera (Patil *et al*., 1984) and Hymenoptera (Caetano and Overal, 1984; Arab and Caetano, 2002).

Figure 4A shows degenerated microvilli (MV), denatured gastric caecum (DGC) with altered food bolus (FB) portions, increased lumen between the gut inner lining for the treated groups. Degenerated malpighian tubule (DMT) and the nearby midgut linings with perforated (Pf) epidermal layers (Fig. 4B) were observed. Muscle tissues showed perforated (PF) in the gut regions (Fig. 4C). Figure 4D shows degeneration and fragmentation in epidermal layer with enhanced lumen (LU) in the gut and vacuolated cells. Vacuolated severely degenerated muscle layers were noticed in larva treated with 30 ppm of secondary metabolites of actinobacterial strain (Fig. 4E). Muscle tissues showed perforated (PF) gut regions and vacuolated, degenerated epidermal layers (DEp). Microvilli and gastric caecum were severely (DGC) degenerated and the Food Bolus (FB) portions were severely fragmented with high lumen (LU) portions in the nucleus (N) and gut (Fig. 4F).

Vacuolated (V) and degenerated muscle layers were observed (Fig. 5A). Muscle tissues showed perforated (PF) gut regions (Fig. 5B). Similar to our results, Al-Mehmadi and Al-Khalaf (2010) reported apical degeneration in midgut region as compared to control Dipteran larvae and also brush border, basal membrane, nucleus and cytoplasmic organelles were lysed in the gut regions. Vacuolated degenerated epidermal layers were also observed (Fig. 5C). Muscle tissues showed perforated in the gut regions, and the food bolus (FB) content severely fragmented with lumen (Fig. 5D). The epidermal layer, microvilli (MV) gastric caecum were severely degenerated (Fig. 5E). Degenerated epidermal layer, Microvilli



Figs. 4 A-F: Mid gut cross section and epidermal layers of *L. orbonalis* 4th instar larva treated with 30 ppm of secondary metabolites of actinobacterial strain (400x).

and perforated tissues were observed (Fig. 5F).

The larval mid gut was completely ruptured with perforated (PF) layers around the gastric caecum (GC) (Fig. 6A). Disoriented nucleus, vacuolated food bolus (FB) and degenerated epidermal layers were also observed when exposed to higher concentration of secondary metabolites such as 40 ppm (Fig. 6B). Degeneration such as cell shrinkage, vacuole formation, and highly degenerated muscle layer were also observed (Figs. 6C, D). Selek *et al*. (2014) reported degenerative signs like cell shrinkage, vacuole formation and apoptotic bodies in larval midgut of *Anticarcia gemmatalis* and *Bombyx mori* (Levy *et al*., 2004).

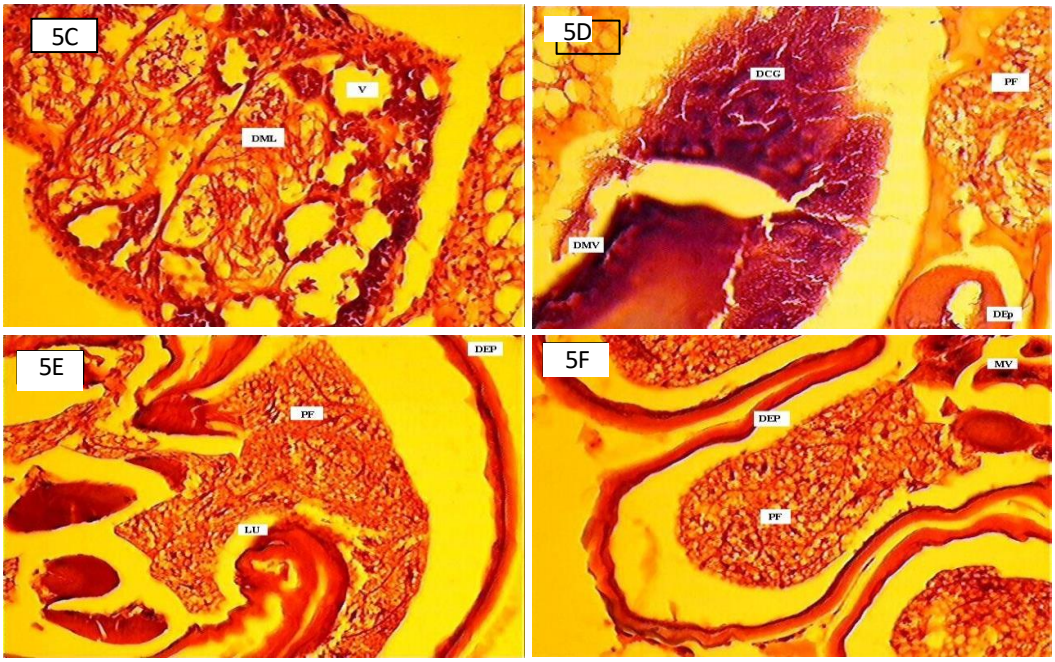
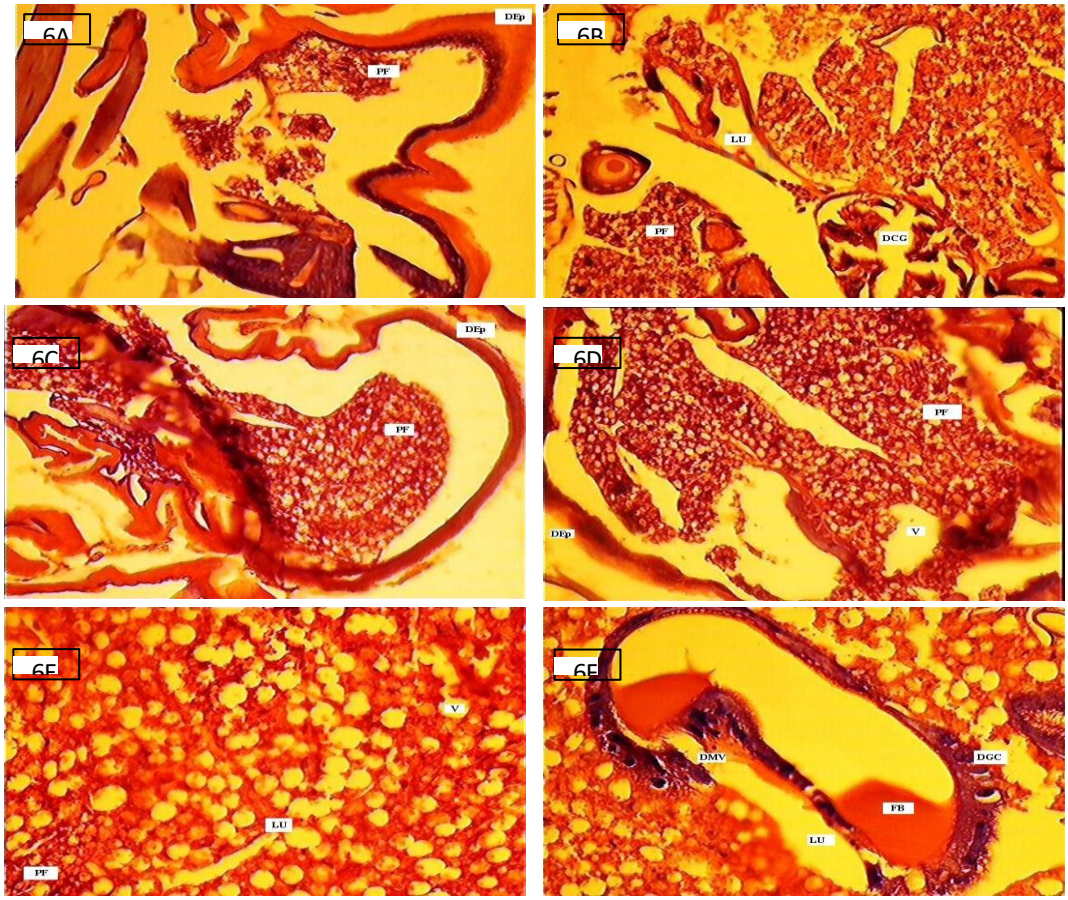


Fig. 5 A-F: Mid gut cross section and epidermal layers of *L. orbonalis* 4th instar larva treated with 35 ppm of secondary metabolites of actinobacterial strain (400x).



Figs. 6A-F: Mid gut cross section and epidermal layers of *L. orbonalis* 4th instar larva treated with 40ppm of secondary metabolites of actinobacterial strain (400x).

The degeneration of malpighian tubules and other tissues act as an evidence for the necrosis of tissues (Wigglesworth, 2003; Helen Diana and Syed Jahangir, 2019) in the insect system. Degenerated gastric caecum, degenerated epidermal and elongated lumen with perforated (PF) layers around the caecum were observed (Fig 6E). Malpighian tubules filter the hemolymph to remove the excretory products produced from metabolism into the mid gut (Jung *et al*., 2005; Cruz–Landim, 2009). Degenerated microvilli and perforated (PF) contents were observed. Food bolus (FB) content severely fragmented with increased lumen (LU) (Fig. 6F) and the muscle tissues showed perforations (PF) in their gut regions.

Conclusion

Actinobacteria is one of the common soil saprophytic bacteria isolated and identified from brinjal field. *Leucinodes orbonalis* (Lepidoptera), a phytophagous insect, cause serious damage to the brinjal field. The isolated actinobacterial strain was tested as a biocontrol agent against *L. orbonalis* 3rd and 4th instar larva. Sublethal doses of secondary metabolites of actinobacterial strains greatly influenced the biochemical composition and histoarchitecture of tissues with increased tissue damaging enzymes. The bacterial extra-cellular products (secondary metabolites) act as an efficient biopesticides against *L. orbonalis* larvae and eco-friendly to other non-targeted species.

Ethical Statement

The authors confirm that all ethical issues have been dealt with the research ethics guidelines provided by the Post Graduate and Research Department of Botany, Jamal Mohamed College, Tiruchirappalli , Tamil Nadu, India.

Author Contributions

Isa Bin Fakirulla Almaz F.: Drafting of manuscript, field work and lab assistance; *Helen Diana I*.: Work flow and instrumentation; *Tamil Kumar T*.: Work flow and instrumentation; *Syed Jahangir H*.: Final drafting of manuscript and approval. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

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