Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Silkworm stock:

Disease free layings of eri silkworm were collected from Deomoroi of Mangaldai district, Assam. The rearing of eri silkworm was performed at Seri-biotech laboratory of IASST, Guwahati under recommended condition at 25-27 °C, 75 ± 5 % relative humidity and 12 : 12 (L : D) h photoperiod. After disinfection by dipping in 2 % formalin solution for 10 min, the eggs were kept on a petridish with proper protection. The eggs were kept on observation everyday for emergence of larvae and after emergence the larvae were allowed to consume tender leaves of castor (Ricinus communis). The weighed leaves were feeded four times a day till the maturity of the larvae. The larvae were acclimatized up to 2nd instar and experiments were conducted from 3rd instar onwards.

3.1.2 Host plant for rearing:

Castor (Ricinus communis) plants of healthy type (green variety) were used to rear eri silkworm. The plants were raised from seed and planted on IASST silkworm food plant garden.

3.1.3 Chemicals and pesticides:

Sodium hydroxide, bovine serum albumin (BSA), glucose, cholesterol, maltose, sodium carboxymethyl cellulose, L-tyrosine, nitophenol, dinitrosalicylic acid, casein, 4-nitrophenylpalmitate, sodium taurocholate, sodium chloride, chloroform, glacial acetic acid,
L-alanine, NADH, lactate dehydrogenase, 2-oxoglutarate, L-3,4-dihydroxyphenylalanine, Micrococcus lysodeiticus ATCC No. 4968 cell suspension, lysozyme, agarose, low melting point agarose, trypan blue, Na₂EDTA, triton X, dimethyl sulfoxide (DMSO), ethidium bromide (EtBr), acetylthiocholineiodide (ACTCI), 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB), phosphate buffered saline (PBS) were used from Sigma Aldrich. Image-iT Live Green Caspase-3, -7 detection Kit (I35106) and Alexa Fluor® 488 annexin V Apoptosis Kit (V13241) were purchased from Molecular probes, Invitrogen. GenElute™ Direct mRNA Miniprep Kit (DMN10) was purchased from Sigma Aldrich. Reverse Transcriptase core Kit (RT-RTCK-03) and MESA GREEN qPCR MasterMix Plus for SYBR Assay were used from Eurogentec. All other chemicals used were of analytical grade purchased from Merck and Himedia.

Commercial grade of chlorpyrifos, Pyrifos-20 EC (active ingredient 20 g in 100 ml) and cypermethrin, Ustad (active ingredient 10 g in 100 ml) were purchased from Assam fertilizer house, Guwahati, Assam. Standard chlorpyrifos and cypermethrin were purchased from Sigma Aldrich.

3.2 Methods:

3.2.1 Fractionation of commercial formulations of pesticides:

A stock solution of 1 mg/ml of each pesticide standard solution was prepared by dissolving 5 mg of standard pesticides into 5 ml of hexane solution. Stock solution of 1 mg/ml of commercial formulations of chlorpyrifos was prepared by dissolving 5 µl of Pyrifos 20 EC in 995 µl of hexane. Similarly, stock solution of 1 mg/ml of
Cypermethrin was prepared by dissolving 10 µl of Ustad in 990 µl of hexane solution. Gas chromatography-Mass Spectrometry (GCMS, Shimadzu-TQ8030) has been performed to identify pesticide residues in commercial formulations. EB-5MS capillary column with length 30 m, diameter 0.25 mm and thickness 0.25 µm has been used. Helium was used as carrier gas with flow rate of 1.05 ml/min. The GC column temperature program was set as follows: 60 °C for 1 min, ramp 150 °C at a rate of 20 °C / min (4 min) and then ramped to 290 °C at the rate of 15 °C / min (held at this temperature for 5.17 min) [146]. The injector temperature was 250 °C, and all injections were made in the split/splitless mode (splitless time: 1 min). One µL of sample was injected and the mass spectrometer was operated with an ion source temperature of 230 °C and interface temperature of 310 °C. The MS scanned in the mass range m/z 40 - 600 with scan speed 2000 was used for quantitative determinations of the studied pesticides. Pesticide identification was done by comparing retention times of experimental samples with standard chlorpyrifos and cypermethrin.

**3.2.2 Observation of intoxication symptoms:**

The 5th instar larvae were exposed to various concentrations of pesticides. Behavioral changes like feeding, movement, any other abnormal changes have been observed and recorded after pesticide exposure.

**3.2.3 Determination of experimental concentrations:**

Acute toxicity in eri silkworm was determined by a semi-static method in laboratory condition as per standard protocol [147] and has been summarized as below.
**Dose preparation of pesticides:**

**Chlorpyrifos:** A stock solution of 100 mg/L of was prepared by dissolving 0.05 ml of Pyrifos- 20 EC in 99.95 ml of acetone. Subsequent dilutions in the range of 0.5-5.0 mg/L (0.5, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, 2.8, 3.0, 3.2, 3.4, 3.6, 3.8, 4.0, 4.2, 4.4, 4.6, 4.8 and 5.0 mg/L) were made with distilled water by using the formula -

\[ N_1 V_1 = N_2 V_2 \]

(Here \( N_1 = \) initial concentration, \( V_1 = \) initial volume, \( N_2 = \) desired concentration and \( V_2 = \) final volume).

**Cypermethrin:** A stock solution of 100 mg/L was prepared by dissolving 0.1 ml of Ustad in 99.90 ml of acetone. Subsequent dilutions in the range of 10 – 400 µg/L (10, 20, 40, 60, 80,100, 150, 200, 250, 300, 350 and 400 µg/L) were made with distilled water by using the formula -

\[ N_1 V_1 = N_2 V_2 \]

(Here \( N_1 = \) initial concentration, \( V_1 = \) initial volume, \( N_2 = \) desired concentration and \( V_2 = \) final volume).

**Determination of LC\(_{50}\):**

For LC\(_{50}\) value determination 50 larvae were selected randomly and separated in a rearing tray. The experiment was performed with 0.5 - 5.0 mg/L and 10 - 400 µg/L chlorpyrifos and cypermethrin concentrations respectively with 50 larvae for each concentration. 50 g of fresh castor leaves were sprayed with each freshly prepared working concentration of chlorpyrifos, cypermethrin and air dried for 10 min. The air dried leaves were fed to the pre-starved (8 h) 5\(^{th}\) instar healthy larvae. A control set was also maintained from the same stock under the same environment and fed with distilled water sprayed castor
leaves. Mortality was recorded for 24 - 96 h exposure period and the dead larvae were removed. The LC_{50} value that would kill 50 % of silkworm at 96 h was determined from obtained data by the method of Probit analysis [148].

**Determination of sub lethal concentrations:**

After LC_{50} value determination, two sub lethal concentrations of both chlorpyrifos and cypermethrin were selected for further study.

**3.2.4 Effect of pesticides on morphometric characters:**

**Larval weight, silk gland weight and percentage of silk gland in body weight:**

Body weights from 3\textsuperscript{rd} instar to 5\textsuperscript{th} instar larvae were recorded in control and experimental groups. The body weights were taken on the 3\textsuperscript{rd} day of each instar. The 5\textsuperscript{th} instar larvae were weighed 1\textsuperscript{st} to 5\textsuperscript{th} day, dissected and silk glands were removed. The weights of the silk glands of 5\textsuperscript{th} instar larvae were recorded. The percentage of silk gland (silk gland tissue somatic index, SGTSI) in body weight was calculated by following formula-

\[
\text{Percentage of silk gland in body weight (SGTSI) = Weight of silk gland / Weight of the larval body X 100}
\]

**Larval duration, pupa and larval pupal intermediate formation:**

Larval duration was counted from 1\textsuperscript{st} instar to 5\textsuperscript{th} instar in control and experimental groups. After 5\textsuperscript{th} instar, pupa formation was observed and the number of normal pupa, larval pupal intermediate was counted in both control and experimental groups.
Number of emerged moth and deformed moth:

After cocoon formation the moths were emerged from the control and treated batches. The number of moth emergence and deformed moth was counted.

3.2.5 Effect of pesticides on some metabolites:

The metabolite concentrations were studied in control, chlorpyrifos and cypermethrin exposed groups. Larval weight from 3\textsuperscript{rd} to 5\textsuperscript{th} instar larvae was taken and ten larvae of each instar were selected for metabolites estimation.

Total Protein:

Extraction of protein from silkworm:

Initially, 10\% tissue homogenate was prepared by homogenizing 1 g of tissue in 10 ml of distilled water. The tissue homogenate was precipitated by adding equal volume of 10\% trichloroacetic acid (TCA) and the solution was kept for 30 min at low temperature. The solution was then centrifuged at 6000 rpm for 10 min and the resultant supernatant was discarded. Further, the residue was dissolved in 0.1 N NaOH and 1 ml of aliquots was pipette out.

Standardization:

A stock solution of 1 mg/ml of standard protein Bovine serum albumin (BSA) was prepared. A calibration line was prepared with the values of absorbance against various working concentrations of BSA. From this line the actual concentration of protein was calculated using the experimental absorbance values.
**Estimation of protein:**

The soluble protein was determined according to the method of Lowry et al. [149]. Initially, solution A was prepared by dissolving 0.4 g sodium hydroxide in 100 ml of distilled water. Solution B was prepared by dissolving 1 g potassium sodium tertarate and 0.5 g copper sulphate in 100 ml distilled water. 2 ml of solution B was mixed with 98 ml of solution A and solution C was prepared. Thereafter, 1 ml of protein extracts from control and sub lethal concentration exposed groups were taken in the test tube. To this 5 ml of solution C was added and mixed thoroughly. The sample was allowed to stand for 10 min at room temperature for incubation. After incubation 0.4 ml of Folin phenol reagent was added and kept for 30 min for colour generation. A blue colour was developed, which was measured at 640 nm absorbance in the multimode reader (Thermo Scientific Varioskan). Protein content was calculated out from the BSA standard curve and expressed as mg/g of body weight.

**Total Trehalose:**

**Extraction of trehalose from silkworm:**

Initially 10 % tissue homogenate was prepared by homogenizing 1 g of tissue in 10 ml of distilled water. The homogenate was deproteinized with 70 % ethanol (1: 2) and then the solution was kept for 30 min at 4 °C. The solution was centrifuged at 6200 rpm for 20 min and the resultant supernatant was used for trehalose estimation.
**Standardization:**

A stock solution of 1 mg/ml of glucose was prepared. A calibration line was prepared with the values of absorbance against various working concentrations of trehalose. From this line the actual concentration of glucose was calculated using the experimental absorbance values.

**Estimation of trehalose:**

The total trehalose concentration was determined by standard Anthrone method [150]. 1 ml of clear supernatant was taken in test tube and 3 ml of anthrone reagent (2 g anthrone in 85 % of ice cold H$_2$SO$_4$) was added and vortexed briefly. The solution was then kept into boiling water bath at 100 °C for 15 min. After boiling the solution was allowed to stand for 5 min at room temperature. The absorbance was measured at 620 nm in multimode reader (Thermo Scientific Varioskan) and trehalose content was calculated out from the trehalose standard curve and expressed as mg/g of body weight.

**Total Lipid:**

**Extraction of lipid from silkworm:**

10 % tissue homogenate was prepared by homogenizing 1 g of body tissue in 10 ml of 2:1 v/v chloroform: methanol mixture. The homogenate was centrifuged at 4000 rpm for 20 min. The resultant supernatant was used for lipid estimation.

**Standardization:**

A stock solution of 1 mg/ml of standard cholesterol was prepared. A calibration line was prepared with the values of absorbance against various concentrations of cholesterol.
From this line the actual concentration of lipid was calculated using the experimental absorbance values.

**Estimation of lipid:**

The lipid concentration was determined by Sulphovenilin method of Barnes and Blackstock [151]. 1 ml resultant supernatant was taken in a test tube. The solution was then evaporated in a warm water bath and the process was continued until drying. After complete evaporation, the tubes were allowed to stand for 3 min. 2 ml of 98 % H₂SO₄ was added to the test tubes after cooling and placed in warm water bath for 10 min. Thereafter, 5 ml of 1 % phospho-venilin was added to the solution and incubated for 15 min at 37 °C. The absorbance was taken at 540 nm in multimode reader (Thermo Scientific Varioskan) and lipid content was calculated out from the cholesterol standard curve and expressed as mg/g of body weight.

**3.2.6 Effect of pesticides on digestive physiology:**

To study the effect of pesticides on digestive physiology, activity of some digestive enzymes were assayed in control, chlorpyrifos and cypermethrin exposed groups of 5th instar larvae.

**Crude enzyme preparation:**

Crude enzyme preparation was performed by adopting standard protocol of Blakemore et al. [152]. The whole gut of 5th instar larvae was isolated from control and pesticides exposed groups. The isolated gut was rinsed three times in 1 X phosphate buffer saline (pH 7.4) and divided into foregut, midgut and hindgut. The different gut sections were
homogenized in 500 µl of sodium acetate buffer (0.1 M, pH 5.6) on ice. The homogenate was centrifuged at 15,000 rpm for 15 min at 4 °C and the supernatant was used for enzyme assay.

*Amylase activity assay:*

A stock solution of 1 mg/ml of maltose was prepared. A calibration line was prepared with the values of absorbance against various working concentrations of maltose. From this line the actual concentration of produced maltose was calculated using the experimental absorbance values.

Amylase enzyme activity was measured by standard protocol [153, 154]. 1 % glucose was used as substrate and 20 µl of glucose solution was mixed with 200 µl sodium acetate buffer (0.1M, pH 5.6). 50 µl of crude enzyme was added to the reaction mixture and the mixture was incubated at 37 °C for 30 min. After incubation, 90 µl of dinitro salicylic acid solution was added and the mixture was boiled for 15 min. The reaction mixture was allowed to cool at room temperature. At the end of the incubation period maltose was produced as end product. Maltose production was detected at 620 nm by taking the absorbance using multi-mode reader (Thermo Scientific Varioskan) and the enzyme activity was measured as µg maltose produced per 30 min.

*Cellulase activity assay:*

A stock solution of 1 mg/ml of glucose was prepared. A calibration line was prepared with the values of absorbance against various working concentrations of glucose.
From this line the actual concentration of produced glucose was calculated using the experimental absorbance values.

Cellulase enzyme activity was measured by standard protocol [153, 154]. For cellulase assay, 20 µl of the substrate sodium carboxymethyl cellulose was mixed with 200 µl sodium acetate buffer (0.1M, pH 5.6). 50 µl of crude enzyme was added to the reaction mixture and incubated in a water bath at 37 °C for 30 min. Further, 90 µl of dinitro salicylic acid solution was added and the mixture was boiled for 15 min. Glucose was produced as the end product and glucose production was detected at 540 nm by taking the absorbance using multimode reader (Thermo Scientific Varioskan). The enzyme activity was measured as µg glucose released per 30 min.

**Protease activity assay:**

A stock solution of 1 mg/ml of L-tyrosine was prepared. A calibration line was prepared with the values of absorbance against various working concentrations of L-tyrosine. From this line the actual concentration of produced L-tyrosine was calculated using the experimental absorbance values.

Protease activity was assayed by using standard protocol of Faulk et al. [155]. For protease enzyme activity, 1 % casein was used as substrate. 30 µl of crude extract was mixed with 600 µl of 1 % casein and incubated for 30 min at 37 °C. The reaction was stopped by adding 270 µl of 20 % TCA (v/v). The reaction mixture was kept for 20 min in ice bath followed by centrifugation at 5000 rpm for 10 min and absorbance was measured at 280 nm. L-tyrosine was produced as end product and the enzyme activity was measured as µg L-tyrosine released per 30 min.
**Lipase activity assay:**

A stock solution of 1 mg/ml of nitrophenol was prepared. A calibration line was prepared with the values of absorbance against various working concentrations of nitrophenol. From this line the actual concentration of produced nitrophenol was calculated using the experimental absorbance values.

Lipase activity was assayed by using standard protocol of Faulk et al. [155]. For this study, 4 nitrophenylpalmitate (0.35 mM) in Tris-HCl (0.5 mM, pH 7.4) was used as substrate. Further, 30 µl of the crude enzyme extract was mixed with the substrate, 6 mM sodium taurocholate and 1 M NaCl. The reaction mixture was incubated for 30 min at 37 °C. The absorbance was measured at 400 nm. Nitrophenol was produced as end product and the enzyme activity was measured as µg nitrophenol generated per 30 min.

**Alanineaminotransferase (ALT) activity assay:**

ALT assay was performed following the method of Inagaki et al. [156]. Ten µl of the crude enzyme supernatant was mixed with 550 µl of the reaction mixture (0.5M L-alanine, 0.5 mM NADH, 1.3U/ml lactate dehydrogenase and 0.9 mg/ml of BSA) and vortexed. Later, 50µl of 180 mM 2-oxoglutarate solution was added to the reaction mixture and incubated for 90 min at 30 °C. Absorbance was measured at 339 nm and the activity was expressed in unit (1U defined as change in absorbance of 0.001 per min per µl of enzyme sample).

**Gut structure:**

To study the effect of pesticides on gut structure the 5th instar larvae of control and pesticide exposed groups were dissected and the gut was removed. The removed gut was
sectioned and fixed in Carnoy’s fluid (absolute alcohol: chlorpform: glacial acetic acid = 6:3:1). The histological slides were prepared by the standard protocol of dehydration and paraffin embedding [157]. After fixation, the tissues were passed through a series of graded alcohol in Histopathology assembly machine (Leica TP 1020, No. - 0548-3) as follows-

70 % alcohol (1 h) → 90% alcohol (1 h) → 100% alcohol (1h, two changes at the interval of 30 min)

Then the tissues were cleaned using xylene, which removed the alcohol and other dehydrating agents, which were not miscible with paraffin. Then the tissues were ready for impregnation in wax. Paraffin wax was put in wax bath stations of Histopathology assembly machine and maintained temperature at 60 °C. The gut sections were embedded in melted paraffin for 4 h, two changes per two hours. After embedding, paraffin blocks of gut sections were prepared by block preparation unit of Histopathological assembly. Thereafter, block was fixed on the metal block holder and sectioned into 0.5 micron. After that tissue section was kept on slide and was floated on water bath (temp 60 °C). Then the section was put in middle of the slide and kept in slide warmer.

Thereafter, slides were stained using eosin-heamatoxylene counter staining procedure. The staining procedure was followed as follows–

Xylene (1 min) → absolute alcohol (1 min) → 90 % alcohol ( 2 min) → 70% alcohol (2 min) → 50 % alcohol (2 min) → 30 % alcohol (2 min) → washed in tap water haematoxylene solution (15 min) → dipped in acid water → dipped in alkali water washed in distilled water → 30 % alcohol (5 min) → 50 % alcohol (5 min) → 70 %
alcohol (5 min) → eosin solution (5 min) → 90% alcohol (2 min) → absolute alcohol (2 min, 2 changes) → xylene (2 min, 3 changes) → mounted with DPX

The mounted slides were observed under microscope in 10 X and 40 X resolution in phase contrast microscope (Zeiss Axio Cam ERC 5S).

3.2.7 Effect of pesticides on immunomodulation:

**Phenoloxidase activity assay:**

Phenoloxidase enzyme activity was studied by following the method of Ashida and Soderhall [158]. Ten µl of 5th instar hemolymph samples were taken from control, chlorpyrifos and cypermethrin exposed groups. The hemolymph was mixed with 20 µl of 1 X PBS and centrifuged at 5000 rpm for 5 min at 4 °C for removal of hemocytes. The supernatant of hemolymph plasma were used for phenoloxidase enzyme assay. Thereafter 10 µl hemolymph plasma solution was mixed with 200 µl of 10 mM L-3, 4-dihydroxyphenylalanine (L-DOPA) and incubated for 30 min at room temperature. After incubation, absorbance was measured at 490 nm and phenoloxidase enzyme activity was expressed in unit (1U defined as change in absorbance of 0.001 per min per µl of enzyme sample).

**Lysozyme activity assay:**

Lysozyme enzyme activity was measured by following standard protocol of Azambuja et al. [159] and Drayton et al. [160]. Hemolymph was collected from both control and pesticide exposed groups. Herein, 50 µl of cold 1 X PBS was added to 10 µl of hemolymph and centrifuged at 5000 rpm for 10 min. After centrifugation, the supernatant
was collected and 30 µl of supernatant was added to 200 µl of Micrococcus lysodeikticus ATCC No. 4698 cell suspension. The reaction mixture was incubated at room temperature for 10 min. Thereafter, 200 µl of aliquots were taken in the microplate reader and the change in absorbance was measured at 450 nm at 1 min interval for 30 min. The enzyme activity was expressed in unit (1U defined as change in absorbance of 0.001 per min per µl of enzyme sample). A lysozyme standard was used simultaneously along with the hemolymph samples to confirm the progress of the assay.

**Total and differential hemocyte count (THC and DHC):**

Total and differential hemocyte count was performed in both control and pesticide exposed 5th instar silkworms at regular time intervals (24, 48, 72 and 96 h). The total number of hemocyte was counted by following the standard method. Tauber-Yeager reagent was prepared by adding 0.5 ml of 10 % acetic acid and 3 ml of 0.01 % gentian violet to 20 ml of distilled water [161]. Hemolymph was drawn to 0.5 mark in WBC pipette and diluted by Tauber-Yeager reagent. Thereafter, the cell number was counted by Neubauer hemocytometer by the following formula

\[
\text{Hemocytes} / \text{mm}^3 = \frac{\text{Hemocytes in five 1 mm squares} \times \text{Dilution} \times \text{Depth factor of chamber}}{\text{Number of square counted}}
\]

Dilution = 20

Depth factor = 10

Number of square counted = 5
For DHC, hemolymph samples were diluted to 5 times with 1X PBS buffer (pH 7.4) initially, smeared uniformly on slides and air dried. After drying the slides were stained with 10 % giemsa stain for 10 min. The stained slides were washed with 1X PBS (pH 7.4) for two times, air dried and mounted with DPX. 100 cells per slide (3 replications) were taken into account for both control and pesticides exposed groups.

3.2.8 Genotoxicity of pesticides:

Induced genotoxicity of sub lethal doses of chlorpyrifos and cypermethrin on silkworm was studied by different assays as follows –

Alkaline single cell gel electrophoresis (SCGE) (comet assay):

The genotoxicity was assessed by adopting the procedure of Singh et al. [162]. For comet assay 10 larvae of 5th instar control and pesticide exposed groups were selected. A positive control has to be set to compare the induced toxicity of pesticide with known genotoxic agents. Herein, \( \text{H}_2\text{O}_2 \) was used as positive control and 10 µl of 10 % \( \text{H}_2\text{O}_2 \) was injected to the silkworms. Hemolymph sample from each group was collected by cutting one of the prolegs.

Cell viability was tested by standard Trypan-blue exclusion method [163] and samples with 95 % cell viability were processed for single cell gel electrophoresis (SCGE).

Fifty µl of 1 % agarose in double distilled water (DDW) was smeared on one end frosted slide and air dried. The second base layer coat was given by placing 200 µl agarose (1 % in PBS, pH-7.4) on the slide. About 20 µl cell suspension were mixed with 80 µl low melting point agarose (1 % in PBS) and layered in the previously coated slide. Further, a third layer of 100 µl of 1 % low melting point agarose was applied and covered with a cover
glass. After solidification of the gel, the slide was immersed in lysing solution (2.5 M NaCl, 100 mM Na$_2$ EDTA and 10 % dimethyl sulfoxide in DDW and pH was adjusted to 10). Triton-X-100 (1 %) was freshly added and kept overnight at 4 °C. The slides were placed in electrophoresis buffer (10 N NaOH, 200 mM EDTA, 10 % dimethyl sulphoxide) for DNA unwinding for 20 min. Electrophoresis was carried out at 120 mV for 20 min at 4 °C in dark condition. The slides were immersed in the neutralizing solution (400 mM Tris, pH 7.5) for 20 min. The slides were stained with 75 µl ethidium bromide and the excess stain was removed by rinsing the slides in DDW.

DNA damage was visualized by observing the slides on 40 X fluorescence microscope (Leica DMI3000B). The control hemocytes with intact round nucleus refereed as head of the comet. The treated cells had a head along with a tail. Total length, tail length and head length was calculated by ImageJ (NIH) software. Further, head, tail percentage and tail moment was calculated by using the following formula –

- Head percentage = Head length / Total length ∗ 100
- Tail percentage = 100 – Head percentage
- Tail moment = Tail length ∗ Tail percentage/100

**Apoptosis assay:**

Induction of apoptosis in pesticide exposed groups was studied by active caspase detection and annexin V assay.

**Caspase detection assay:**

Hemolymph was collected in ependroff tubes by cutting one of the proleg of 5$^{th}$ instar larva. The collected hemolymph was centrifuged at 4000 rpm for 5 min. The
hemocytes were precipitated in the bottom of the tubes and the cells were suspended in PBS buffer. The cells were transferred to a glass slide and a uniform smear was prepared. The slide was then incubated at 27 °C for 15 min in an incubator (Labtech LBI-150 E, Korea). A 30-fold dilution of 30 X FLICA (florescent inhibitor of caspase) reagent working solution was prepared with PBS and mixed well. A sufficient amount of FLICA reagent was added to cover the cells and the cells were incubated for 60 min under dark condition. After incubation, the solution was removed and the cells were gently rinsed with PBS. A 1000-fold dilution of the 1 mM Hoechst stain and 5 mM propidium iodide stain was prepared in PBS. Sufficient amount of the stains were added to cover the cells and incubated for 10 min under existing condition. The cells were washed twice with 2 mL of 1 X wash buffer. The cells are fixed by preparing a 10-fold dilution of apoptosis fixative solution to 1 X wash buffer by adding 1 part apoptosis fixative solution to 9 parts 1 X wash buffer. The slides were then mounted using one drop of the diluted fixative and stored at 2–6 °C in dark condition for up to 24 h. The cells were observed under a fluorescence microscope (Leica DMI3000B) using excitation/emission filter of FLICA at 488/520 nm, Hoechst at 350/461 nm and propidium iodide at 490/635 nm.

Annexin V assay:

Annexin V assay was performed by Alexa Fluor® 488 annexin V Apoptosis Kit with Alexa® Fluor 488 annexin V and PI (Catalog nos. V13241, Molecular probes, Invitrogen). Hemolymph was collected from 5th instar control and pesticide exposed larvae by cutting one of the prolegs. 50 µl of hemolymph was taken on a slide and a smear was prepared. The slide was air dried for 10 min. Thereafter 5 µL Alexa FluorR 488 annexin V
and 1 µL 100 µg/ml PI working was added to the slide. The slide was incubated at room temperature for 15 min. After the incubation, 400 µl of 1 X annexin-binding buffer was added, mixed gently and the samples were kept on ice. The slide was analyzed as soon as possible in fluorescence microscope (Leica DMI3000B) at 530 / 488 nm.

3.2.9 Effect of pesticides on acetylcholinesterase enzyme:

In vitro assay:

Extraction of crude enzyme:

The crude enzyme was extracted from 5th instar larvae. The head was dissected out from larvae, which was followed by separation of head and body tissue. The head and body tissues were weighed and 5% homogenate of head and 20% body tissue homogenate were prepared in 1 M Sodium phosphate buffer containing 0.2% Triton X (v/v, 0.2 ml Triton X in 99.80 ml PBS). The extracts were then centrifuged at 10,000 rpm for 20 min at 4°C and the clear supernatant was used as enzyme source.

Enzyme inhibition assay:

Enzyme inhibition was assayed by adopting standard protocol [164, 165] with slight modification and IC_{50} (inhibition concentration that inhibits enzyme activity by 50%) value was calculated. 100 mg/L stock solution of chlorpyrifos-20 EC and cypermethrin-10 EC was prepared in acetone. For chlorpyrifos, 10 working concentrations were prepared ranging from 0.5 - 5.0 mg/L. For cypermethrin, the range of 0.5 – 20 µg/L was prepared. 100 µl of supernatant of enzyme extract was added to the well of microplate. To this solution 50 µl of PBS was added, which was followed by addition of 1.25 µl of 10 mM of 5,5’-Dithiobis (2 nitrobenzoic acid) (DTNB). The solution was incubated at room temperature for 5 min.
After incubation 100 µl of substrate acetyltiocholineiodide (10 mM) was added to initiate the reaction. To determine the inhibitory effect of pesticides 50 µl of each pesticide concentration was added to the reaction mixture. The content was then mixed and absorbance was taken in 412 nm. The percentage of enzyme inhibition was derived by expressing the activity of pesticide exposed groups as percentage of activity of control group. The IC$_{50}$ value was determined by Probit regression analysis [148].

**Determination of acetylcholinesterase activity:**

Acetylcholinesterase activity in pesticide affected group was measured *in vitro* by adopting standard procedure [166]. 100 µl of supernatant of enzyme extract was added to the well of microplate. To this solution 50 µl of PBS and 1.25 µl of 10 mM of 5, 5'-Dithiobis (2 nitrobenzoic acid) (DTNB) was added. The solution was incubated at room temperature for 5 min. After incubation 100 µl of substrate acetyltiocholineiodide (10 mM) was added to initiate the reaction. An aliquot of 50 µl chlorpyrifos (10 mM) and cypermethrin (10 mM) solution was added to the solution. The reaction mixture was incubated for 37 °C prior to absorbance measurement. A reagent blank without enzyme extract (buffer + DTNB + ACTC) and a control were also maintained. The production of choline-DTNB complex (5-thio-2-nitro-benzoate) was measured at 412 nm in multimode reader (Thermo Scientific Varioskan). The relative acetylcholinesterase activity was calculated by the following formula:

\[
\text{Acetylcholinesterase activity} = \frac{\Delta A_{412} \times \text{Vol}_T \times 1000}{1.36 \times 10^4 \times \text{light path} \times \text{Vol}_S}
\]

\[
\Delta A_{412} = \text{Change in absorbance per min}
\]
Volₜ = Total assay volume (375 µl)

1.36 \times 10^4 = \text{Extinction coefficient of TNB (M}^{-1}\text{cm}^{-1})

\text{Lightpath} = \text{Microplate well depth (1 cm)}

Volₛ = Sample volume (100 µl)

**Enzyme kinetics study and determination of Km and Vmax:**

From the stock solution of 100 mM acetylthiocholineiodide (ACTCI) serial dilution was made to prepare the working substrate concentrations. For enzyme kinetics study 2 mM–100 mM substrate solutions were prepared. The kinetic parameters Km and Vmax were determined from Linewear and Burk double reciprocal plot analyzed over the range of acetylthiocholine concentrations.

**In vivo assay:**

**Exposure of eri silkworm to pesticides:**

The castor leaves (food plants of eri silkworm) were dipped with the sub lethal concentrations of chlorpyrifos, cypermethrin and air dried for 2 min. A set of 50 larvae of 3rd to 5th instar larval stage were exposed to the pesticide contaminated leaves and enzyme activity was studied in 24 h, 48 h, 72 h and 96 h interval period.

**Extraction of crude enzyme:**

The crude enzyme was extracted from 5th instar larvae. The head was dissected out from the larvae and head, body tissue was separated. The head and body tissues were
weighed and 5% homogenate of head and 20% homogenate of body tissue was prepared in 1 M Sodium phosphate buffer containing 0.2% Triton X (v/v, 0.2 ml Triton X in 99.80 ml PBS). The extracts were then centrifuged at 10,000 rpm for 20 min at 4 °C and the clear supernatant was used as enzyme source.

**Residual Acetylcholinesterase enzyme activity study:**

Residual activity of acetylcholine esterase was studied by standard method [164, 165]. 100 µl of the supernatant was added to the well of microplate. To this solution 50 µl of PBS was added, which was followed by addition of 1.25 µl of 10 mM of 5,5′-Dithiobis (2 nitrobenzoic acid) (DTNB). The solution was incubated at room temperature for 5 min. After incubation 100 µl of substrate acetylthiocholineiodide (10 mM) was added to initiate the reaction. The reaction mixture was incubated for 37 °C prior to absorbance measurement. A reagent blank without enzyme extract (buffer + DTNB + ACTC) and a control were also maintained. The production of choline-DTNB complex (5-thio-2-nitrobenzoate) was measured at 412 nm in multimode reader (Thermo Scientific Varioskan). The percentage residual enzyme activity was calculated by using the following formula:

\[
\text{Percentage residual activity} = \frac{\text{Absorbance of the sample}}{\text{Absorbance of the control}} \times 100\%
\]

**In silico interaction of pesticide molecule with acetylcholinesterase enzyme:**

**Molecule preparation:**

The structures of the pesticides (chlorpyrifos and cypermethrin) were downloaded from pubchem database (CID: 2730 and CID: 2912). Both the structures were optimized by adding gasteiger partial charges. Open Babel package had been used to convert the charged
molecule into mol2 format (167). The target protein acetylcholinesterase (AChE) was downloaded from Protein data bank (PDBID: 2C5F) followed by molecular dynamics simulation studies and docking analysis.

**Energy minimization and molecular dynamics simulation:**

Classical molecular dynamics simulations were performed to ensure the stability of the target protein (AChE). GROMACS 4.6 [168] was used to carry out simulation under Linux environment. The complex was immersed in a dodecahedron-shaped box (x, y and z) and 1nm distance had been kept between the surface of the protein and box walls, followed by salvation in 3 point water model. Periodic boundary conditions (PBC) were assigned in all direction. System neutralization was done based on the Ewald equation in order to control the force field and the molecular dynamics parameter (mdp). All the files were prepared for energy minimization and equilibration of the system to run Gromacs.

Steepest descent algorithm had been applied with a tolerance level of 1500 kJ/mol/nm for energy minimization of the whole system. After convergence, the system went through NVT ensemble molecular dynamics simulations for 1000 pico second (1ns). MD simulations were carried out employing NPT in a periodic boundary condition. The simulations were extended upto 20 ns at constant pressure (1bar) and temperature (300 K) conditions.

**Molecular dynamics trajectories analysis of the target:**

The protein structure was analyzed at different time intervals (initial condition, 5 ns and 20 ns) and the deviations, potential energy variations were calculated during that time interval to check the structural deviations of the protein structure at different time frame.
The root mean square deviations (RMSD) and root mean square fluctuation (Root Mean Square Fluctuation, RMSF) were taken into account. The trajectories were visualized by VMD software package.

**Binding site prediction of acetylcholiesterase:**

Auto Ligand and Meta Pocket was used to predict the binding sites of the protein [169, 170]. By generating different fill volumes, 10 potential binding cavities were identified with a grid resolution 0.80 and further; one potential binding site was identified with a maximum volume. The volume of the potential binding site was 336.896 Å^3 and the coordinates of the active binding cavity was found to 64.4958, 65.113, 52.8311 (x, y, z). The autoligand was set at different fill points to get best possible binding site. The active site residues were selected. The active sites residues were validated by comparing the domains of acetylcholinesterase [171].

**Molecular docking of energy optimized acetylcholinesterase with chlorpyrifos and cypermethrin:**

Gasteiger charges were added to the protein structure and ligand molecule due to the scoring function of ADT (Autodock tools). Non polar hydrogen atoms were added to the PDB structure. The input receptor and ligand files were converted to ‘PDBQT’ format for docking analysis. AutoDock software package was used to carry out docking process [172]. Molegro Virtual Docker and FlexX employed to re-ensure that the binding orientation of the pesticides with the target [173, 174]. Ten ligand poses were generated based on binding affinity between the active site of the receptor and pesticides molecule. The energy terms (interaction energy, free energy of binding, torsional energy, Van der Waals interaction,
electrostatic interaction, hydrogen bond interaction) of each active pose and the receptor were calculated. The selected poses were redocked with FlexX, MolegroVirtual Docker and scores were calculated respectively. The protein-ligand complex was analyzed based upon interaction score, hydrogen bonding and covalent bonding. The binding pattern, close contacts interactions between receptor and ligand were visualized by using ADT tool, VMD software.

**Transcriptional characteristics of acetylcholinesterase gene:**

**mRNA extraction:**

The 5th instar larvae were fed with pesticide contaminated leaves. The head and fat body tissues were dissected out. The isolated tissues were stored at -80 °C. The mRNA was extracted from the tissues by using the mRNA isolation kit (GenElute™ Direct mRNA Miniprep Kit, Catalog number DMN10; Sigma). Herein, 40 mg of frozen tissue was transferred to a homogelizer vessel. 20 µL of Protinase K was mixed with 1.0 ml of Lysis solution and the tissue samples were homogenized immediately with this Protinase K/Lysis solution. After homogenization the lysate was incubated at 65 °C for 10 min to degrade the nucleases and other proteins. 64 µL of 5 M NaCl was added to the lysate. The oligo dT beads were thoroughly mixed by vortexing and inverting until homogenous. 25 µL of resuspended oligo dT beads were added to lysate-NaCl mixture and mixed thoroughly by vortexing. The lysate/bead mixture was incubated at room temperature for 10 min. Thereafter, the mixture was centrifuged for 5 min and supernatant was discarded. For a more enriched mRNA preparation 0.5 ml of lysis solution and 32 µL of NaCl solution were added to the pellet and vortex thoroughly to resuspend the pellet. The suspension was incubated at
65 °C for 5 min and again incubated at room temperature for 5 min. The suspensions was centrifuged for 5 min and supernatant was discarded. The pellet was resuspended in 350 µL of wash solution and transferred to a GenElute spin filter-collection tube assembly. The suspension was spun for 2 min and the flow through liquid was discarded. 350 µL of low salt wash solution was pipette out into the remaining of the column, spun for 2 min and flow through liquid was discarded. Another 350 µL of low salt wash solution was pipette into the columns, spun for 2 min and flow through liquid was discarded. Thereafter, the spin filter was transferred to fresh collection tube and 50 µL of preheated Elution solution (65 °C) was pipette onto the spin filter ensuring that it contacts the bead: mRNA complex. The solution was incubated at 65 °C for 5 min and after incubation spun for 2 min. To maximize the recovery of mRNA, an additional 50 µL of preheated Elution solution (65 °C) was pipette to bead: mRNA complex. The mixture was incubated for 5 min at 65 °C and after incubation spun for 2 min. The mRNA was in the flow through elute and stored at -80 °C.

**cDNA construction:**

C DNA construction was performed by using Reverese Transcriptase core Kit (RT-RTCK-03) (Eurogentec) in Applied Biosystems Thermal Cycler (2720). RT reaction mix was prepared by mixing 1 µL of 10 X Reaction buffer, 2 µL of 25 mM MgCl₂, 2 µL of 2.5 mM dNTP, 0.5 µL of primer, 0.2 µL of RNAse inhibitor, 0.25 µL of EuroScript RT and 3.05 µL of RNAse free water. To the reaction mix, 1 µL of template mRNA was added and gently mixed by inversion. The following PCR programme was maintained: initial step 10
min 25 °C, reverse transcriptase step 30 min 48 °C and inactivation of the RT enzyme 5 min 95 °C.

**Real time quantitative PCR:**

Real time qPCR was performed in Applied Biosystems SteOne™ and StepOnePlus™ Real-Time PCR Systems using MESA GREEN qPCR MasterMix Plus for SYBR Assay I dTTP (Eurogentec).

**Primer for real time PCR:** The primer pairs corresponding to acetylcholinesterase BmAChE-1 and internal reference gene actin3 are listed in Table 1 [125].

**Table 1.** Primer pairs for real time PCR

<table>
<thead>
<tr>
<th>Targeted genes</th>
<th>Primer sequence</th>
<th>Tm of primer (°C)</th>
<th>Length of product (bp)</th>
</tr>
</thead>
</table>
| Actin3         | F: 5´- CGGCTACTCGTTCACTACC-3´  
                 R : 5´- CCGTCGGGAAGTTCGTAAG-3´ | 54.9 °C  
                                              55.5 °C | 147 |
| Bm-AChE-1      | F : 5´-ATTTGAGGTTCAGGCACCCGAGA-3´  
                 R : 5´-CGCAGGAATGTGGCAGTGTTGTT-3´ | 60.6 °C  
                                              60.6 °C | 80 |

A total of 25 µL reaction was set up for qPCR: 12.50 µL of 2 X Reaction buffer, 0.75 µL of each primer, 2.50 µL of cDNA template and 8.50 µL of deionized water. The PCR conditions were: denaturation at 95 °C for 5 min, followed by 40 cycles of 3 s at 95 °C and 50 s at 55 °C. The PCR was repeated 3 times for each sample and actin3 was used as the internal reference gene.
3.2.10 Effect of pesticides on silk synthesis:

**Cocoon characters:**

The mature 5\textsuperscript{th} instar larvae were picked up from rearing trays and placed for spinning the cocoons. The cocoons were harvested after 4 to 5 days of spinning. Assessment of various cocoon parameters was made following the standard method [175]. The lengths and breadths of cocoons were measured.

The randomly selected cocoons were taken and weighed. After removing the floss, the cocoons were cut open and the dried pupae were taken out. Then the pupae were weighed and shell weights were taken by removing the pupae. Shell ratio was calculated using the following formula and expressed in percentage.

\[
\text{Shell ratio} = \frac{\text{Shell weight}}{\text{Cocoon weight}} \times 100
\]

**Fibroin and sericin contents:**

Cocoon shells were weighed, dried in a hot air oven at 70 \textdegree C and after drying cooled in desiccators. This process was repeated for several times until the weight become constant. Then 5 g of cocoon shell was taken and boiled in 0.5 \% $\text{Na}_2\text{CO}_3$ solution for 45 min. Cocoon shells were squeezed properly and again boiled in $\text{Na}_2\text{CO}_3$ for 90 min. These were again squeezed and boiled in distilled water for 20 min. After boiling the cocoon shells were washed in distilled water and dried for 12 h. Total removal of sericin was ensured by adding 0.1 \% $\text{H}_2\text{SO}_4$ in silk fiber as the removal was represented by yellow coloration [176]. The dried shells were weighed again which referred as fibroin content of the shell. The sericin content was determined by the following formula-
Sericin content = Shell weight before degumming – shell weight after degumming

The physical property of silk fibers was analyzed by following the standard method [177]. The silk fiber was degummed by using 0.3 % Na₂CO₃ (0.3 g in 100 ml of distilled water) solution and degummed fiber was analyzed.

**Thermal property:**

The DSC thermo grams were recorded with a Perkin-Elmer thermal analyzer, DSC 6000 (temperature accuracy 0.25 %, weighing precision; 0.01 %) coupled with thermo analyzer (TA) processor. Small pieces of fibers (2 to 3 mg) were kept in the aluminum sample pans of the DSC cell under nitrogen atmosphere at a rate 20 ml/min. A heating rate of 10 °C / min was maintained to get the DSC thermo grams with temperature accuracy within the temperature range of 30 - 440 °C.

**Tensile strength:**

Tensile property of the degummed silk fibers was measured using Instron 4301 (3343) universal tensile tester (interfaced with a computer) at atmospheric temperature of 25 ± 2 °C and 65 ± 2 % RH at a gauge length of 5 cm and a strain rate of 1 cm/min. The silk samples were pretreated in an environment of ideal temperature and humidity (25 ± 2 °C and 65 ± 2 % RH respectively) for 24 h. 20 specimens were tested to obtain average values of tenacity and toughness.

**Secondary structure of the silk fiber:**

Secondary structure of control and pesticide exposed silk fiber was studied by Fourier transform infrared spectrophotometer (FTIR, Perkin Elmer) in attenuated total
reflectance (ATR) mode in the spectral region of 500-4500 cm\(^{-1}\). For each measurement, 32 interferograms were co-added and transformed at a resolution of 4 cm\(^{-1}\). All spectra were measured by absorbance method at room temperature. The spectra were analyzed with Origin 6.0 software.

**Morphological structure of fiber by Scanning Electron Microscope (SEM):**

The morphological structures of control and pesticide affected silk fibers were studied in Scanning Electron Microscope (SEM) using gold sputtering carried out by Carl Zeiss Sigma VP with incident electron beam energy of 5 kV.

**3.2.11 Statistical analysis:**

Probit analysis was performed with Biostat 2009 software package with 9 replications for LC\(_{50}\) value calculation (n=50). The other experiments were performed in triplicates and results were presented as mean ± standard deviation (n=10). One way analysis of variance (ANOVA) was carried out for statistical analysis at \(P < 0.05\) level of significance and means were compared by *post hoc* Tukey’s test. The graphical representations were performed using Microcal Origin version 6.1 [178].