Chapter 5

Discussion

North east India including Assam is known as agriculture dependent state as over 70% of the state's population relies on agriculture. Recent status showed that the net cultivated area of the state is 28.10 lakh hectares (2008-09) out of total 78.43 lakh hectares [179]. Apart from agriculture practices, Assam comprises of more than 100 tea estates and its covers about 2.16 lakh hectares of total land. Nevertheless, the pest problem is a serious concern to these agricultural as well as tea growing practice causing heavy loss to the farmers. It has been reported that the average pesticide utilization in this region is 6.13 g/hac/anum and is quite low in comparison to the national level (600 g/hac/anum). However, the secondary effect of pesticides on non target organisms can not be overlooked. *Philosamia ricini* is a hard insect and reared throughout North East as household cottage industry. It is reared on stray castor plant which grows nearby the vicinity of paddy field and tea gardens. Therefore, the silkworm is to likely be exposed to pesticide contamination by spray drift or residual affect. The study has been organized to evaluate the effect of chlorpyrifos and cypermethrin on non targeted organism i.e. silkworm *Philosamia ricini* (eri silkworm) as a model organism.

Acute toxicity determination is usually considered as the initial step to assess and evaluate the toxic characteristics of any substances. It is the primary and foremost assessment of any kind of toxic manifestations. Data from acute toxicity assay serve as the basis for classification and labeling of toxic substances. It is also helpful for determination of LC$_{50}$ or LD$_{50}$ value of the compounds and dose determination in toxicity studies [180].
LC$_{50}$ or LD$_{50}$ is a common measure of toxicity that causes 50% death in exposed animals within a certain period. The chemicals with smaller LC$_{50}$ values are considered as highly toxic and vice versa. In this study acute toxicity of chlorpyrifos and cypermethrin was evaluated in 5$^{th}$ instar silkworm larvae. The young age silkworm i.e. from 1$^{st}$ to 3$^{rd}$ instar larvae are more sensitive to chemical substances even at lower dose. Therefore, generally either 4$^{th}$ or 5$^{th}$ instar larvae are selected for toxicity evaluation and also for determination of LC$_{50}$ value in silkworm species [181]. The present investigation showed a concentration-dependent increase in lethality which indicated that mortality was directly proportional to concentrations of both pesticides. The LC$_{50}$ value of chlorpyrifos in B. mori for 96 h was recorded which was found to be less toxic as compared to P. ricini [182]. Acute toxicity of cypermethrin to armyworms (Lepidoptera: Noctuidae) and Tuta absoluta was also studied earlier and various LC$_{50}$ values were recorded [183, 184]. However, the recorded LC$_{50}$ value in present study showed more toxicity of cypermethrin to eri silkworm than other lepidopteran species. Apart from these two pesticides, LC$_{50}$ values for organophosphate phoxim and pyrethroid pesticides were also evaluated in B. mori [185, 186, 147]. The variation in LC$_{50}$ values may occur for several reasons. The extent of toxicity of various pesticides depends on parameters like type and time of exposure, species, age, sex, body weight, nutritional status, temperature, hydrogen ion concentration, light, humidity etc. The variation in toxicity of organophosphates and pyrethroids among silkworm species might be due to various factors such as different silkworm species as well as physiological and environmental factors [147]. It was reported that the wild silkworms were more resistant to pesticide in comparison to domesticated one [123]. The role of genetic makeup might be attributed as an important condition in pesticide resistance mechanism of silkworm species.
The present study revealed that in comparison to chlorpyrifos, cypermethrin showed more toxicity with very less LC\textsubscript{50} value. To elucidate the mechanism of varying toxicity, attention should also be given on the mechanism of action of pesticides. Chlorpyrifos is a non-systemic organophosphate insecticide, which acts as a cholinesterase inhibitor with contact, stomach and respiratory poison action [187]. It affects the nervous system by binding to the active site of the cholinesterase enzyme and prevents the breakdown of the neurotransmitter acetylcholine in the synaptic cleft. This results the accumulation of acetylcholine in synaptic cleft which causes overstimulation of the neuronal cells, leading to neurotoxicity and eventually death [188]. However, cypermethrin acts as a fast acting neurotoxin in insects. The mode of action of this pyrethroid insecticide involves the interaction with the sodium channels in nerve cells through which sodium enters the cell in order to transmit a nerve signal. These channels can remain open for up to seconds, compared to the normal period of a few milliseconds, after a signal has been transmitted. Cypermethrin also interferes with other receptors in the nervous system. Moreover, evidence also suggests that type II pyrethroids can also interact with GABA receptors which inhibit both (3, 5 S)-t-butyl bicycle phosphorothionate (TBPS) binding at the GABA-A receptor-ionophore complex. This GABA induced chloride fluxes of central nervous system (CNS) preparation leading to adverse effect on organisms [189]. Therefore, the variation in mode of action might be the probable reason of variable toxicity of chlorpyrifos and cypermethrin on eri silkworm. However, Demetrio et al. studied the effect of cypermethrin and chlorpyrifos active ingredients and formulations on \textit{Daphnia magna} and reported the potency of acute toxicity was more in formulation than active ingredients [190].
Additionally, intoxicity symptoms like body blackening, sluggishness, vomiting and body shaking were observed in pesticide affected eri silkworm. Similar kind of phenomena was also observed in *B. mori* after exposure to various organophosphate and pyrethroid pesticides [147, 191]. This abnormality in behavior might be due to the neurotoxic effect of pesticides leading to nervous breakdown of the organism [147].

The sub lethal concentration might also induce various toxicological effects on non target organisms. Eri silkworm fed with sub lethal concentrations of chlorpyrifos and cypermethrin resulted in persistent growth inhibition at different levels during metamorphosis. During exposure to sub lethal concentrations, the organisms can sustain for a certain period of time, however their physiology and other systems might get disturbed. Srinivasa and Rao also studied the effect of chlorpyrifos and cypermethrin on *Spodoptera litura* and concluded that in phytophagus insect, exposure to insecticide treated leaves causes alteration in feeding behavior leading to conversion of both ingested and digested food into their body matter. This phenomenon eventually causes changes in their further development, metamorphosis and fecundity of resultant adults [192]. Previous study on eri silkworm also supports the present results where reduction in body weight was observed in malathion exposed larvae [193]. Perusal of literature revealed that various pesticides like phoxim, cartaphydrochloride, diflubenzuron and lufenuron etc. induced detrimental effect on growth and development of *B. mori* [194, 195]. According to Kodandaram et al. almost all insecticides initiate dose dependent changes in growth as well as metamorphosis of insects [196]. Such dose dependent inhibition of larval growth and metamorphogenesis in silkworm and other insects might be accounted due to reduced physiological changes brought about by reduced food intake [197, 198]. Pesticides have the potency to interrupt the development and
metamorphosis of silkworm and thereby produce striking disorders in the external morphology [199]. Moreover, organophosphate insecticides are known to alter juvenile hormone levels which might be the possible reason for impairment in metamorphosis process of pesticide exposed larvae [108].

Generally when the toxic pesticides are used in controlling insect pest, the various biochemical parameters of the pest is affected leading to death of the organisms or any other impairment [107]. Therefore, similar kind of alteration in biochemical constituents might be expected in non target insect species also. Monitoring the changes in key biochemical parameters lead to the recognition of toxicant induced effect [200]. Findings showed a significant decrease in biochemical constituents in the larvae after exposed to chlorpyrifos and cypermethrin. However, the impact of cypermethrin was found to be more pronounced than chlorpyrifos. The presents results were also supported by earlier studies, which reported a significant decrease in total protein, carbohydrate and cholesterol concentration in \textit{B. mori} larvae, when exposed to sub lethal doses of various pesticides [107, 201, 135, 118, 202].

Depletion of protein in tissue might be due to the compensatory mechanism of insects during insecticidal stress condition. Under normal condition the structural proteins do not mobilize for energy production but under stress condition there is a heavy mobilization of proteins. In this regard, complex protein molecules break down into simpler ones like albumins. Under these circumstances, these smaller protein molecules undergoes further hydrolysis into amino acids and enter into the trichloroacetic acid (TCA) cycle as keto acid and supply energy to the insect [107]. Another reason of protein depletion might be the cope up mechanism of insect with high energy demand which occurs due to depletion of carbohydrate level. Additionally, the overall utilization of protein might be associated with
the reduction in protein synthesis as a result of prolong stress induced by insecticide [203, 204].

Significant reduction in trehalose concentration was also observed in pesticide exposed groups. Trehalose is considered as the blood glucose of insects and acts as energy reservoir. Glucose is the energy substrate which is required for energy generation to meet the energy demand. Hence any alteration in trehalose concentration under stress condition can be expected. During pesticide stress condition, glucose is utilized for synthesis of detoxifying enzymes [205]. The hypoglycemia might be due to accelerated glycolysis or transportation of metabolite from the fat body to hemolymph in order to compensate energy crisis during stress condition. Pyruvate is the key intermediate of glycolysis cycle whereas lactate is the end product. The inter conversion of pyruvate and lactate is an important step in carbohydrate metabolism process. It is suggested that pesticide toxicity induces conversion of pyruvate to lactate. The reduction in pyruvate level implies its utilization in TCA cycle to provide energy during stress condition. The hyper and hypo trehalosemia, hyper and hypo glycaemia in the hemolymph and fat body may be the result of the accelerated glycogenolysis at the fat body. It may also be due to the transportation of these metabolites from fat body to hemolymph in their bid to meet energy crisis induced by insecticides toxicity [130]. Changes in amount of trehalose might also be due to the upset in homeostatic mechanism of insects due to insecticides exposure [206, 207, 208, 209]. Different concentrations of pesticide pyriproxifen also decreased the amount of trehalose in B. mori hemolymph and glycogen in fat bodies of Eurygaster integriceps [118, 210].

Lipids are significant molecules in biological systems and play various roles in insect physiology. It also acts as a source of metabolic energy that can be mobilized to meet
the energy requirements of the insect [211]. Insect contains circulating lipids in their hemolymph and glycerides are the principal circulating lipids [212]. Insect uses cholesterol for normal growth, development, reproduction, larval molting and metamorphosis [213]. Therefore, impairment in lipid content in insect might not be favorable for their proper growth and metabolism. In present study, both chlorpyrifos and cypermethrin induced significant alteration in lipid content in a dose dependent manner. Similar kind of result was also reported in *B. mori* hemolymph after treatment by juvenile hormone analogue and pyrifoxen [210]. Lipid turnover in insects is regulated by neuroendocrine-controlled feedback loops and therefore any disturbance in physiology as well as hormonal system due to pesticide exposure might be the cause of such kind of alteration in insects. The pesticide exposure might impair the lipid synthesis mechanism of insects, leading to the depletion in lipid concentration. Along with the physiological stress induced by pesticides, interruption in absorption system might also be attributed as a reason for reduction in cholesterol content [118].

The larvae exposed to pesticide contaminated leaves showed less feeding behavior and thereby their nutritional requirements were not fulfilled properly. The antifeedant characteristics and starvation of the larvae can induce alteration in biochemical constituents [214]. Food intake is a rough indication of available energy and a reduction in food intake will necessarily lead to a reduction in energy. This condition most likely leads to reduced growth over an extended period of time. Exposure to toxicants such as pesticides can also change a number of metabolic processes, resulting in changes of the total energy metabolism of the animal. Significant decrease in protein, glycogen and lipid contents due to absence of nutrients were also observed in *Plodia interpunctella* exposed to azadirachtin [215].
The activity of digestive enzymes is used as a parameter to study the effect of pesticides on digestive physiology. Herein, exposure to sub lethal concentrations of chlorpyrifos and cypermethrin caused a reduction in the activity of amylase, cellulase, protease and lipase enzyme. Digestive enzymes also play an important role in growth and development of insects by transforming organic food molecules into useful biomolecules. Digestive system functions to hydrolyse the ingested food materials and absorb nutrients for maintenance, survival and reproduction in insects. Insects digestive tract is a simple tubular system and can be demarcated into three different parts - fore, mid and hindgut [216]. In silkworm, mid gut digestive enzymes are mainly responsible for breakdown the complex nutrients of ingested food into simpler form. These simpler forms are easy to absorb into the body through semi permeable membrane of the alimentary canal [217]. Since significant variation in enzyme activity of midgut, was observed, therefore change in digestion process is obvious. Similar kind of reports is available describing the effect of various pesticides on digestive enzyme of different organisms including *B. mori* [107, 218, 219, 220]. Alteration in digestion and enzyme activity depends on different factors which include quantity / quality of ingested food, age, health of the larva and physical factors [88]. Inhibition of amylase enzyme activity impairs carbohydrate uptake and digestion mechanism leading to alteration of total carbohydrate level. Reduction in amylase activity during pesticide exposure might be due to the induced cytotoxic effect of pesticides on epithelial cells of the midgut, which synthesize α-amylase [221].

Similarly, proteases are important digestive enzymes which are responsible for dietary protein metabolism leading to growth and development. Decrease in protein concentration could be correlated with increase in protease activity [107]. In present study,
although protein concentration depletion was observed in pesticide exposed larvae, however, protease activity in the mid gut did not show significant alteration. The reason behind this might be the unavailability of proper substrate due to less food intake.

Lipase plays a major role in storage and lipid mobilization in insects and is also the basic components in many physiological processes like, reproduction, growth, defense against pathogens and foreign substances. Therefore, decrease in lipase activity in midgut of chlorpyrifos and cypermethrin exposed larvae of eri silkworm might affect the energy regulation process leading to growth impairment. The decrease in enzyme activity was due to unavailability of substrate resulting from insufficient food intake [88]. Moreover, feeding is extremely important for the stimulation of digestive enzyme secretion [219]. Since, larvae fed with pesticide treated leaves exhibited less feeding behavior, therefore the starvation or unavailability of substrate to form enzyme-substrate complex might be a factor for alteration in digestive physiology of the larvae. Less feeding behavior in insect after fed with insecticide might have interfered with the enzyme–substrate complex and thus affecting the peristaltic movement of gut [222, 223]. In this regard some other mechanisms can also be mentioned. For example, insecticide might also affect enzyme titers and activities [224]. Additionally, mid gut of lepidopteran insect possess endocrine cells which might responsible for local control of enzyme secretion into the gut lumen. Pesticides might affect the secretory function of these cells, leading to inhibition of enzyme activity [225, 226]. Therefore, similar inhibition of secretory function of neuroendocrine cells of eri silkworm might be expected as a result of pesticide exposure eliciting alteration in enzyme activity.

Since midgut plays a major role in absorption of digested material and defending the body against foreign invaders, it is felt necessary to investigate the damage on cell
architecture [227]. Results showed changes in midgut histology of both chlorpyrifos and cypermethrin exposed larvae. The change in histo-architecture might be related with alteration in enzyme activity. In earlier studies, effects of chlorpyrifos and cypermethrin on histo-architecture of different organs of rat were evaluated and degenerative changes due to pesticide exposure were recorded [228, 229, 127]. In *B. mori* larva degenerative effect of organophophates phoxim, dichlorvos on mid gut were reported which included abnormality in cell structure, intestinal wall cracking, damaged circular muscle epithelial cells etc. [199, 132, 230].

Alanineaminotransferase (ALT) is a transaminase enzyme and was formerly called serum glutamate-pyruvate transaminase (SGPT) or serum glutamic-pyruvic transaminase (SGPT). ALT is conserved throughout evolution in almost all organisms. Activity level of this enzyme is considered to be highly sensitive and fairly specific clinical biomarker of cytotoxicity. Therefore, ALT activity level in organisms is measured to evaluate the toxic effect induced by chemical substances [156]. Significant increase in ALT activity of both chlorpyrifos and cypermethrin exposed groups were observed in this study. Since ALT serves as a strategic link between the carbohydrate and protein metabolism, therefore alteration in these metabolism processes might be correlated with this elevation of ALT level. Moreover, ALT level might be altered during various physiological and pathological conditions. Increase in ALT activity is also caused by leakage of this enzyme from injured tissue. Another hypothesis stated that an increase in lipid peroxidation leads to an increase in ALT activity. Elevation of ALT activity in pesticide exposed *B. mori* was due to an active transportation of amino acids which provided keto acid to serve as a precursor in the synthesis of essential constituents under stress condition [107]. Significant increase in ALT
activity often suggests the existence of a physiological challenge in body which includes microorganism infections, damage to some tissues by some toxic material etc. Etebari et al. showed that activity of this enzyme increased during pyriproxifen treatment on B. mori due to their recovery properties [118]. Similarly, exposure to methyl parathion significantly increased ALT activity in greater wax moth, Galleria mellonella (Lepidoptera: Pyralidae) [231].

In particular, another concern regarding pesticide toxicity is whether pesticide exposure affects insect immunity and makes them susceptible to infectious diseases. James et al. in their review work gave an affirmative conclusion of pesticide toxicity on immune system. Present study showed the impact of chlorpyrifos and cypermethrin in eri silkworm immune system in terms of phenoloxidase, lysozyme enzyme activity as well as hemocyte abundance [135]. Similar kind of report is available indicating effect of pesticides on the immune system of different non target organisms [232, 233, 234]. Galloway and Handy focused the immunotoxic effect of organophosphate pesticides on various organisms over last 20 years which stated that the organophosphate can interfere with the immune system and exert immunotoxic effect on laboratory organisms [235]. Exposure to high doses of pesticides can cause direct damage to cells or organs of immune system and a general decrease in immune function was observed [135].

In insects, prophenoloxidase activation system comprises of an important part of the immune system. In fact, insect phenoloxidase are synthesized as zymogens called prophenoloxidase which are activated by proteolytic cleavage at a specific site in response to infection or wounding [236]. Phenoloxidase enzymes have tyrosinase-like activity which can hydroxylate tyrosine and also can oxidize o-diphenols to quinones or o-phenoloxidases
The quinones produced by phenoloxidase undergo a series of additional enzymatic and non-enzymatic reactions leading to polymerization and melanin synthesis. The final stages of this cascade are nodule formation and encapsulation against invading microorganisms [238]. The pesticide molecule might bind to the active site of the enzyme and inhibits their specificity to substrate. Moreover, pesticide molecules may also lead to conformational alteration of enzyme by binding to different positions of the enzyme and thereby inhibits enzyme activity. Studies showed that pesticide analogs could regulate phenoloxidase activity in different insect species [239, 234].

Lysozyme is a lytic enzyme which plays an important role in the immune defense system of both vertebrates and invertebrate organisms [240, 241]. Lysozyme also contributes to immune system of lepidopteran insects as it helps to clear the debris after immune response and acts as an unspecific defense mechanism. It can kill microbial cells via both lytic and nonlytic mechanisms and also plays role in activation of prophenoloxidase system by cleavage of lysine-type peptidoglycan from Gram-positive bacteria for clustering of peptidoglycan-recognition protein (PGRP)-SA [242, 243, 244]. Present study showed a significant increase of lysozyme activity upto 48 h of chlorpyrifos exposure, which was inhibited after 72 to 96 h exposure. Similarly in cypermethrin also lysozyme activity was increased upto 72 h, however at 96 h inhibition was observed. Previous works showed pesticide induced increase in lysozyme activity in non target organisms in a dose dependent manner [245, 233]. According to the studies, sub lethal concentrations of pesticides stimulate some unspecific defense mechanism in exposed organisms and act as immunotoxic agents, leading to alteration in lysozyme enzyme activity. Moreover, enzyme activity might also alter due to the physical stress induced by pesticides.
Circulating hemocytes of insect hemolymph plays important role in both cellular as well as humoral immune system. Humoral responses comprises of various factors which are related to the recognition of invading microorganisms, melanization, coagulation as well as killing factors such as antimicrobial peptides (AMPs), reactive oxygen species and reactive nitrogen intermediates. Cellular immunity consists of phagocytosis of aggressive microorganisms by hemocytes, nodule formation and encapsulation [246, 247]. Generally five basic types of hemocytes have been observed in insects known as prohemocytes, plasmatocytes, granulocytes, spherulocytes and oenocytes. Plasmatocytes and granulocytes are the important hemocytes in immune response for phagocytosis, whereas prohemocytes are the smallest and basic hemocytes that developed to plasmatocytes and granulocytes during immune response [248]. Additionally, plasmatocytes and granulocytes provide immune responses to pathogens or any foreign particles. A prominent difference in abundance of circulating hemocytes was observed in chlorpyrifos and cypermethrin exposed eri silkworm indicating an alteration in cell mediated immunity. The increase in plasmatocyte and granulocyte percentage in chlorpyrifos exposed groups signified the immune signal in response to pesticide; however decrease in prohemocyte percentage might be the reason of weak immune system in later periods. For cypermethrin, hemocyte abundance was not affected initially at 24 h of exposure; however after prolonged exposure of 96 h total hemocyte abundance was decreased with an increase in granulocyte and plasmatocyte count but decrease in prohemocyte count. Previous studies also stated that different pesticides affected hemocyte abundance and variation. For example, organophosphate pesticides increased the total hemocyte count with an increase in granulocytes but a decrease in prohemocytes and plasmatocytes. But organochlorine
pesticide exposure caused decrease in hemocyte count with a decrease in granulocytes and increase in pro hemocytes and plamatocytes [249]. The morphology and functionality of these hemocytes are affected by different environmental factors and pesticide stress might be considered as one of the reason for such alteration. In general, total hemocyte and granulocytes counts are known to increase in association with both detoxification and immune defense mechanisms [250]. Therefore, it is quite obvious that insecticides might affect hemocyte abundance and variation by their toxic effect on organisms.

Apart from biochemical and immunotoxicity, pesticides are also known to induce genotoxicity in exposed organisms. Therefore, the study investigated the probable genotoxicity of both pesticides in eri silkworm. Various tests have been developed to detect induced genotoxicity or DNA damage by different chemicals on organisms and its biological consequences in cells. These assays are commonly used as marker to assess the safety of environmental chemicals and help to explore the mechanism of action of known or questioned genotoxic agents. DNA damage is an important index in genotoxicity assessment of environmental contaminant. The damage might be caused by DNA single-strand break, DNA double-strand break, DNA adducts formation, DNA–DNA and DNA-protein cross-links which is a result from the interaction of contaminants or its metabolites and DNA. Single cell gel electrophoresis or comet assay is a highly sensitive, sophisticated and precise technique which is used as a marker to evaluate DNA damage in organisms [251]. Comet assay aids to visualize DNA damage at single cell level compared with classical cell morphology assays and useful in assessing viability of cell and cell death type, either apoptosis or necrosis [252]. The basic principle of this technique comprises of the fact that the damaged DNA can be able to migrate more easily in an electric field than the intact one.
Therefore, in case of damaged cells, the cells appear as a comet with a head region containing undamaged DNA and a tail of damaged DNA. Moreover, the severity of DNA damage is directly proportional to migration distance, more distance or tail length implies more damage in DNA [162]. Chlorpyrifos acts as a genotoxic agent by exhibiting relatively longer tail region in a dose dependent manner. Organophosphates are the potential alkylating agents and therefore protein alkylation may be directly or indirectly involved in DNA disintegration process. Moreover, the phosphorus moiety of organophosphate pesticides is a favorable substrate for nucleophilic attack, which causes phosphorylation of DNA leading to damage [253]. Additionally, it was reported that tail formation was also an indicative of apoptosis, where apoptotic cells showed nuclear fragmentation and occurred as a tail [137, 254]. Susceptibility of various doses of chlorpyriphos was also studied in mouse leucocytes and a significant DNA damage in the form of comet induction was reported [255]. The toxic action of xenobiotics like chlorpyriphos is counteracted by glutathione (GSH) and glutathione dependent enzyme systems. Reduction of cellular GSH content below the critical level prevents conjugation of xenobiotics to GSH and thereby enables them to combine covalently with DNA or RNA and cell proteins leading to cell damage [256]. Moreover, formation of GSH conjugates might deplete cellular GSH content and induce oxidative stress leading to DNA damage [257]. Depletion of GSH below a certain level by chlorpyrifos allows the enhancement of lipid peroxidation which causes induction of reactive oxygen species and contributes to the formation of DNA single or double strand breaks [258]. The present findings were in agreement with previous works where induced genotoxicity of different organophosphate in a wider range of non-targets organisms was observed [137, 254]. Moreover, pesticide inducing DNA damage on B. mori were also
evaluated and a positive correlation between pesticide concentration and DNA damage was observed [141, 145].

Likewise, sub lethal concentrations of cypermethrin induced DNA strand break in circulating hemocytes of eri silk worm was observed in a dose dependent manner. It is proposed that cypermethrin can be able to pass through the cell membrane and reaches the nucleus due to hydrophobic nature and small molecular size, which attributes the possible mechanism for its genotoxicity. Within the nucleus, cypermethrin or its byproduct might binds to the DNA by the acid moiety of the reactive groups, leading to the destabilization and unwinding of DNA [259]. Furthermore, cypermethrin possesses vinyl and dimethylcyclopropan groups which get oxidized into methyl butenol via the rearrangement of radical and formation of carbocation. Epoxidation of the vinyl or methyl butenol group further produce metabolites which cause DNA damage [260]. Earlier studies also reported cypermethrin induced DNA damage in various non target organisms [261, 262, 263]. Since, the hemocytes are considered as the key energy source of insects and play a role in the compensatory mechanism during growth period; any impairment of hemocytes in terms of genotoxicity might induce negative impact on overall growth and development of the silkworm. The damaged hemocytes would restrain the transportation, reservation, conversion process and also the elimination of extraneous substance. Moreover, it might cause difficulties in maintaining the balance of moisture content and regulating the osmosis, thereby alter the normal physiological processes of organisms [264].

Furthermore, Corcorcan mentioned that the DNA damage could considered as trigger of cell apoptosis [265]. Detection of active caspase might be a more unique, direct and sensitive indicator of apoptosis than detection of secondary process such as DNA
fragmentation or cleavage of caspase substrate [266]. Additionally, annexin V assay is considered as the most sensitive technique to detect ongoing apoptosis, as in this assay number of apoptotic cells in suspension can be determined in a fast and simple way [267]. Pesticides have the potential to induce cell apoptosis and thereby act as a mutagen. For validation of whether the DNA damage caused by chlorpyrifos and cypermethrin was due to apoptosis or necrosis, the study was extended to caspase activation as well as annexin V assay in pesticide exposed eri silkworm. Herein, molecular mechanism of DNA damage in silkworm was investigated. Results showed that chlorpyrifos and cypermethrin induced apoptosis in silkworm hemocytes and initiated a series of cell death signaling event including activation of caspase leading to DNA fragmentation. In the cytosol, a downstream series of caspase activation is considered as an important regulator of apoptosis [268]. In organisms, caspases are initially expressed as inactive procaspase but during advancement of cell death process they get activated and finally initiate a protease cascade. As a consequence of this cascade initiation, one caspase activates other caspases and once caspase gets activated it seems to be an irreversible commitment to cell death process [269]. The pesticide induced DNA damage might be considered as a trigger for apoptosis resulting caspase activation and cell death. The most likely reason behind this might be considered as cell cycle arrest or increased susceptibility of dividing cells upon pesticide exposure [270]. However, caspase activation and increase in dead cell number supported the hypothesis of disturbance in cell viability.

Likewise, annexin V assay also serves as an indicator of apoptosis which specifically binds with phosphatidyl serine residues of dead cells and produce cell death signal [269]. It is established that translocation of the membrane phosphatidylserine (PS) from the inner to
the outer leaflet of the plasma membrane is one of the earliest indication of apoptosis which is followed by loss of plasma membrane integrity and DNA fragmentation. Annexin V is a calcium dependent, phospholipid-binding protein and binds to these PS exposing membranes in a calcium dependant manner. It is usually used in conjunction with vital dyes which can be able to bind with nucleic acids, but can only penetrate through plasma membrane when membrane integrity is breached, which occurs in later stages of apoptosis or in necrosis [271]. The florescence signal of pesticide exposed group’s hemocyte during annexin V assay could be correlated with the membrane structure alteration thereby provided clear indication of necrosis. Although in previous reports, cell apoptosis and caspase activation induced by pesticides on various non targets were recorded, in silkworm this was the first report of pesticide induced cell death. In this regard our study helped to give an insight on the correlation of DNA strand break and possible mechanism of cell death in hemocytes. However, cholorpyrifos induced apoptosis was also observed in various non target organisms including Drosophila, human monocyte cell line and human placental carcinoma cells [272, 273]. Cholorpyrifos might target caspases along with other factors leading to cell death in silkworm [274]. Moreover cholorpyrifos might disturb the integrity of silkworm hemocytes plasma membrane which is followed by DNA fragmentation and as a result apoptosis signals are generated. There are earlier reports which also have suggested cypermethrin as a potential agent for inducing apoptosis on various organisms [275, 276, 277] and generation of reactive oxygen species and oxidative stress was shown to be an important apoptotic signal [278]. It was hypothesized that cypermethrin induced apoptosis in the liver of zebrafish occured through the induction of oxidative stress and activation of p53 expression, leading to the transcription of genes that encoded proapoptotic proteins [279].
Inhibition of acetylcholinesterase (AChE) is applied as a biomarker to detect and evaluate contamination exerted by anticholinesterase insecticides in organisms. These enzymes are responsible for the removal of neurotransmitter acetylcholine from synaptic cleft by hydrolysis mechanism. Acetylcholine is one of the major molecules by which nerve impulses are transmitted from nerve cell or involuntary muscle. It also serves as preganglionic and postganglionic transmitter in sympathetic nervous system and serves as an excitatory transmitter in central nervous system [280]. Acetylcholinesterase hydrolyses acetylcholine into inactive product choline and acetic acid. Therefore, acetylcholinesterase plays major role in regulation of nervous transmission by reducing the concentration of acetylcholine in the junction through. When acetylcholinesterase is inactivated by any anticholinesterase compound, the concentration of acetylcholine in the junction remains high. As a result of accumulation continuous stimulation of the muscle or nerve fiber occurs, resulting in tetany and eventually paralysis and death. Most of the organophosphates inactivate acetylcholinesterase enzyme by binding to this molecule and interfering with normal nervous system function of targeted pest as well as the non-target organisms. Present results showed an inhibition of acetylcholinesterase in the range of 3.6% – 69.04% after exposure to chlorpyrifos in different time intervals as well as in different instars which indicates toxicity of the exposed pesticides to acetylcholinesterase enzyme system. Moreover, brain acetylcholinesterase activity was more inhibited than body tissue. One metabolite or byproduct of chlorpyrifos, chlorpyrifos-oxon binds permanently to the enzyme AChE preventing this enzyme from deactivating acetylcholine in the synapse [281, 282]. Chlorpyrifos can disrupt the structure of the enzyme by attacking the active serine hydroxyl group of acetylcholinesterase. The enzyme is thus inactivated and the
hydroxyl group can no longer be hydrolysed. Therefore, acetylcholinesterase inhibition is irreversible, which finally causes neurotoxic changes including the prevention of nerve impulses transmission and interference of energy metabolism [283]. Normal functioning can return only when new molecules of acetylcholinesterase have been synthesized. The use of acetylcholinesterase activity as a biomarker of organophosphate in invertebrates was studied by various researchers. Effect of chlorpyrifos in acetylcholinesterase activity of honey bees was also taken into account and alteration in motor function was reported which could be related to the abnormality in movement, flight and foraging behavior [101]. A comparative study on the effect chlorpyrifos with other organophosphate pesticides was carried out on Daphnia magna and inhibition of acetylcholinesterase activity was reported [284]. Moreover, acetylcholinesterase activity and acetylcholine levels in brain, fat body and silk gland of B. mori exposed to organophosphate phoxim, fenitrothion and ethion were studied and AChE activity was found to be inhibited, followed by a concomitant increase in acetylcholin levels in the target tissues [107, 230].

In present study, cypermethrin exposed groups of eri silkworm showed a significant inhibition of acetylcholiesterase activity in both brain and body tissue. Almost 8 % - 70 % inhibition was observed in brain whereas in body tissue the inhibition was in the range of 5 % - 33 % only, indicating more inhibition in brain tissue. Mechanism of action of cypermethrin reveals that it opens the sodium channels in the central nervous system leading to hypo-polarization and hyper-excitation of the neurons and induces short-term neurotoxicity and modulates gamma-aminobutyric acid (GABA) level [285, 286]. Although the mode of action of pyrethroid pesticide is different, however pyrethroid induced inhibition of acetylcholiesterase activity was also reported by various researchers [287, 288,
Cypermethrin exhibited time and concentration dependent inhibition of acetylcholinesterase activity in fish species like *Labeo rohita, Channa punctatus* and inhibition of acetylcholinesterase activity in brain was more followed sequentially by gill, liver and muscle tissues [290]. Pyrethroids have an acid moiety, a central ester bond and an alcohol moiety and hydrophobic interaction of these moieties with hydrophobic aromatic surface region of acetylcholinesterase might be the reason of acetylcholinesterase inhibition [291]. Moreover, the intensity of acetylcholinesterase inhibition might also be correlated with the degree of accumulation of pyrethroids and/or their metabolites in different organs [292]. The differential distribution of different molecular forms of acetylcholinesterase as well as their mode of interaction with pyrethroid molecules might be another possible cause for various inhibition patterns of acetylcholinesterase [293]. Under normal conditions, acetylcholinesterase initially forms a complex with the substrate acetylcholine, which then acetylates the enzyme with the release of choline. Cypermethrin reacts with the acetylated enzyme, forms acetic acid which lead to formation of a precise analog to that of the normal substrate and a pesticide enzyme complex, instead of acetylated enzyme was formed [294]. Moreover, pyrethroids delay the closing of sodium channel, which allows sodium flow and as a result multiple nerve impulses rather than the usual single one occur [295]. In turn, these impulses release the neurotransmitter acetylcholine which stimulates other nerves [296]; ultimately results building up of acetylcholine within the nerve synapses leading to a variety of neurotoxic effects and decreased cholinergic transmission [297].

The half maximal inhibitory concentration (IC$_{50}$) is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function. This quantitative measure indicates how much of a particular substance (inhibitor) is required to inhibit a
given biological process. In present study, IC$_{50}$ values of chlorpyrifos and cypermethrin were 2.15 mg/L and 8.84 µg/L respectively, indicating more inhibiting potentiality of cypermethrin than chlorpyrifos. Similar type of inhibition of acetylcholiesterase activity by chlorpyrifos and cypermethrin was also studied on various organisms [298, 299, 291, 300]. The variation in IC$_{50}$ value might cause by different factors including experimental condition, substrate concentration, and enzyme source etc. However, IC$_{50}$ value provides the insight of inhibition efficiency of pesticides and there is an inversely proportional relationship exists between IC$_{50}$ value and enzyme activity inhibition capacity.

Moreover, IC$_{50}$ value determination is also essential for studying substrate-dependent enzyme kinetics parameters. Enzyme kinetics infers information about the reaction process and enzyme activity. The Km of an enzyme, relative to the concentration of its substrate under normal conditions permits prediction of whether or not the rate of formation of product will be affected by the availability of substrate. An enzyme with a low Km value is normally saturated with substrate whereas an enzyme with a high Km is not saturated with substrate. In presence of any inhibitors the apparent Km value increases as the inhibitors bind to the enzyme molecule which results raise in substrate concentration. Pesticide molecule exhibited similar kind of interaction with acetylcholinesterase either in reversible or irreversible way and thereby inhibit enzyme activity. Present results showed increase values of Km in brain and fat body of pesticides exposed groups. Therefore, the results might corroborate the Km value and enzyme-substrate (ES) hypothesis. Enzyme kinetics results also showed a non significant alteration in Vmax values in pesticide exposed groups. Vmax depends only on the maximum possible ES complex concentration which is a factor of total amount of enzyme. Apparent Vmax value depends on the type of inhibitors. In
case of mixed inhibition, the Vmax alters, because the inhibitor is capable of preventing catalysis either by binding to ES complex or enzyme. Alteration in Vmax is also observed in uncompetitive inhibition, where inhibitors bind strictly to ES complex [301]. But Vmax is unaffected by presence of competitive inhibitors. Since no significant alteration in Vmax was observed for both chlorpyrifos and cypermethrin, therefore it can be predicted that both of the pesticides bind to the enzyme only, exhibiting competitive inhibition. An apparent increase in Km was observed in organophosphate profenofos exposed earthworm indicating competitive nature of the pesticide. However, unaltered Km and decreased Vmax value of acetylcholinesterase depicted purely non-competitive inhibition of carbaryl on earthworm species [302].

The molecular structure of pesticide is an important factor to determine their reactivity with acetylcholinesterase. The molecular configurations as well as atomic composition of pesticides influence electrophobicity and hydrophobicity which are directlyrelated with inhibition of acetylcholinesterase. The compound which resembles acetylcholine binds with acetylcholinesterase faster than other analogue. In this study, the binding pattern or interaction of chlorpyrifos and cypermethrin with acetylcholinesterase was investigated by molecular docking simulation. Simulation study allowed depth analysis and interpretation of the acetylcholinesterase structure affinity relationships and accurately predict the binding interaction with the pesticide. During molecular docking, formation of the hydrogen bonds with the TRP 84 residues of acetylcholinesterase was significant due to the fact that TRP 84 is the part of the putative anionic binding site. The hydroxyl groups of TYR 121 and TYR 130 both the residues point into the ‘active site gorge’ of AChE [303]. The oxygen atom of the oxon group of chlorpyrifos bound to the TYR 130 with a strong
binding affinity and might modify the core structural arrangement of AChE active site. Chlorpyrifos also showed a binding affinity towards GLY 118 residue. This is a part of the 10 residue conserved sequence which contains three conserved glycines in a row, involving in the formation of oxyanion hole in AChE active site [304]. TYR 130, TRP 84, GLY 118 and SER 122 showed a significant binding contribution towards the docking process though there was no direct interaction found with SER 122 residue. The binding affinity of cypermethrin revealed that it formed a pi-pi interaction with PHE 290, TRP 84 and TYR 442 residues of the target molecule. A hydrogen bond was formed between the cypermethrin and GLU 199 residue of acetylcholinesterase. During the binding process PHE 330 residue was also involved as it was present in the active site gorge, which was only ~5 Å wide at a bottleneck formed by the Van der Waals surfaces of TYR 121 and PHE 330. TRP 84 contributed significantly during the binding which showed stronger affinity of cypermethrin than chlorpyrifos, however predicted mode of binding was much the same. The free energy of binding of cypermethrin with the target acetylcholinesterase showed -9.19 KCal/mole while chlorpyrifos exhibit -5.58 Kcal/mol. The results also supported by the study of binding mode prediction of organophosphates, against modeled structures of acetylcholinesterase [305]. Their results showed that monochrotophos and cypermethrin had a strong binding affinity towards the active sites of the AChE. This can be also validated by calculated IC\textsubscript{50} values, where inhibitory affect of cypermethrin was almost 250 times higher than of chlorpyrifos. Moreover, if the binding score value of the pesticides were taken into consideration similar trend of result was observed, where binding score of cypermethrin was almost 2 times higher than chlorpyrifos.
Acetylcholinesterase is encoded by *ace* gene; therefore changes in gene expression level of *ace* gene can be used as indicator of pesticide contamination. Insect acetylcholinesterase gene was first cloned from *Drosophila melanogaster*. In lepidopteran insects, two types of acetylcholinesterase gene was reported i.e. AChE-1 and AChE-2 based on genetic structures. In *B. mori*, two AChE genes (Bm AChE 1 and Bm AChE 2) have been cloned and analyzed [306]. Present study results showed an increase in Bm AChE 1 gene expression in brain and fat body in both pesticide exposed groups, however in lower dose of cypermethrin non significant decrease of AChE expression was observed. Previous study suggested that AChE 1 was evolutionary advanced and related to pesticide toxicity and resistance. Moreover, BmAChE 1 expression was more in head region as AChE is a key enzyme in the nervous system of organisms. AChE gene expression is an important part of the cholinergic neuronal systems for proper functioning of central nervous system homeostasis. Even moderate alteration in neuronal excitability may lead to apparent modulations in brain gene expression. The up-regulation might be due to the autologous feedback response of transcription to depressed cholinergic neurotransmission, leading to elevated levels of brain acetylcholine following pesticide treatment [307]. Similar phenomenon of increase in Bm AChE 1 gene expression in brain and fat body of *B. mori* was also observed [142]. Exposure to acetylcholinesterase inhibitors irrespective of pesticide class (organophosphate, carbamate etc.) resulted in up regulation of transcription of both acetylcholinesterase genes in honeybees [101]. The inhibition of acetylcholinesterase enzyme levels can be correlated to increase levels of acetylcholinesterase mRNA in the midbrain of experimental rats [308].
The success of sericulture practice mainly depends on commercial characters of cocoon and quality of silk fiber. The commercial characters of cocoon such as cocoon length, breadth, weight, shell weight, shell ratio, shape etc. are used in price fixation of commercially reared silkworm cocoon. In present study, the economic traits were found to be decreased in almost all concentrations of chlorpyrifos and cypermethrin treated groups. The variability in qualitative and quantitative characters of the cocoons mostly depends upon the food plants used for feeding the larvae [309]. Cocoon characteristics are also regulated by various factors like hormonal interactions during larval development, genetic as well as environmental factors [310]. Influence of food plants in the cocoon characteristics of eri silkworm was reported earlier [311]. Since the pesticide treated leaved fed larvae showed alteration in development and different metabolic process, therefore it might be correlated with altered cocoon characters. Moreover, silk gland growth reflects the quality and quantity of silk cocoons [312]. Therefore the reduction in percentage of silk gland might attribute to less quantity and poor quality of silk. Results from this study were in agreement with the correlation between larval weight and silk gland weight in alteration of economic traits. Commercial insecticides such as rogor, quinolphos, confidor, methylparathion, dichlorvos were also tested against B. mori and alteration in economic parameters were observed [313, 314].

It is well known that silk fiber composed of two main proteins i.e.fibroin and sericin. Fibroin is the most important part of silk fiber as it is the primary component of silk. In commercial purpose, the sericin was removed by degumming process. Fibroin and sericin contents decreased significantly in pesticide treated groups. The silk protein percentage depends widely on the silkworm species, rearing seasons, geographical location and food
habits [315, 316]. Therefore, nutritional impairment due to pesticide exposed leaves might alter the silk protein content. Since silk gland weight plays a role in silk protein, therefore decrease in silk gland weight percentage might be the reason for reducing silk protein. Similar decrease in sericin and fibroin contents was also observed in *B. mori* when treated with various concentrations of dichlorvos and applauds [87, 314].

Eri silk is found to be thermally more stable than the tasar and other wild silks [317]. The higher thermal stability of non mulberry silk is due to the presence of the - (Ala) n - sequences in the crystalline regions [318]. In this study, no significant change was observed in the thermal property of pesticide treated groups when compared with control groups. In DSC thermograms an endothermic peak was observed below 100 °C which is attributed for evaporation of water. A major endothermic peak at 356.2 °C occurred, which attributed to the decomposition of the fibroin with oriented β conformation [319]. Moreover, FTIR spectroscopy techniques help to study the molecular conformation and crystalline structure of silk protein. The position and intensity of amide bonds are sensitive to molecular conformation of fibroin. The various absorption bands of amide like amide I, amide II, aide III, amide IV and amide V are mainly responsible for molecular conformation which might be either α-helix or random coil or β-sheet structure. In case of eri silk, it was reported that peaks at 1628 cm⁻¹ (amide I, C=O stretching), 1520 cm⁻¹ (amide II, N-H bending), 1240 cm⁻¹ with shoulders at 1222 cm⁻¹ (amide III, C-N stretching), 700 cm⁻¹ (amide V, C-N torsion and N-H bending), and at 966 cm⁻¹ (skeletal vibration due to Ala-Ala) were attributed to β-sheet. Some additional peaks that occurred at 1653 cm⁻¹ (amide I), 1541 cm⁻¹ (amide II), 1269 cm⁻¹ (amide III), and at 893 cm⁻¹ (skeletal vibration) were attributed to the α-helix structure. The peak at 660 cm⁻¹ (amide V) came from the random coil structure.
Results showed no significant changes in the secondary structure of control and pesticide affected silk fibers, indicating no effect of pesticides on molecular conformation. Therefore, the unaltered secondary structure might be the reason for the similar thermal behavior of control and pesticide affected fiber [320].

Tensile behavior is one of the important parameters in the assessment of the properties and functional performance of textile fibers. Tensile parameter like stretching of fibre involves two main processes viz. bond stretching and chain straightening, before a bond contributes to the extension of a fibre, it must be oriented in the direction of the fibre axis [321]. Hence the tensile property of a fibre is dependent mostly on total amount of crystalline material in a preferred direction. A fibre possessing higher degree of crystallinity exhibits lower extensibility. Any impairment of silk gland and silk synthesis mechanism might lead to production of silk with lesser mechanical property [322]. The irregularity in silk spinning process and variation in the diameter of individual fiber resulted large variation in tensile property of silk fibre [323, 320]. Scanning electron micrography was also carried out to study finer details about the external morphology of the fibres. Although unaltered surface morphology of fiber was observed in pesticide exposed silkworm, however the diameter was reduced in comparison to control. The change in diameter might be correlated with the decrease in tensile strength, resulting low quality silk.

The present study showed the overall impact of chlorpyrifos and cypermethrin on growth and development of eri silkworm. However, cypermethrin showed more toxicity than chlorpyrifos. This severity in toxicity might be due to the mode of action of cypermethrin. Antifeedent behavior of pesticide exposed worms might have direct impact on growth as well as metabolic processes. Pesticide induced immunotoxicity and
genotoxicity were also observed leading to impairment in development process. Likewise, impairment in silk gland growth and reduction in total protein might be correlated with alteration in silk spinning process leading to deterioration of quantity and quality of silk.