



Original Research Article

Mode of action of mosquitocidal protein in the larvae and pupae of *Cx. quinquefasciatus* and biochemical and physiological changes in the mosquitoes exposed to protein

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ABSTRACT

Keywords

Pseudomonas fluorescens,
Culex quinquefasciatus,
cytochrome oxidase,
Marker enzymes,
Larvae,
Pupae.

To study the biochemical and physiological changes in the larvae and pupae of *Culex quinquefasciatus* mosquitoes exposed to the proteins, the induction of marker enzymes, MTT assay, *ex vivo* toxicity assay, Cytochrome assay and cytotoxic assays were performed. The induction of the marker enzymes were observed in the protein treated midgut cells of larvae and pupae than the untreated larvae and pupae. The induction of cytochrome C oxidase activity in the treated *Ae.albopictus* (C6/36 cell lines) than the untreated *Ae.albopictus* cell lines concluded that the binding of mosquitocidal protein leads to induction of cytochrome oxidase in the treated larvae and pupae than the untreated larvae and pupae. Binding of *Pseudomonas fluorescens* proteins to specific receptors plays an important role in their mode of action. It is concluded that the binding of mosquitocidal proteins to the midgut region of treated larvae and pupae leads to considerable increase in the marker enzymes activity and cytochrome C oxidase activity in the treated *Ae.albopictus* cell lines.

Introduction

The crystal proteins of *Bacillus thuringiensis* subsp. *Israelensis* when ingested by susceptible larvae dissolve in the alkaline environment of the larval midgut and release soluble proteins. Following their ingestion by susceptible insect larvae, these crystalline inclusions are solubilized in the highly alkaline midgut lumen and converted to active proteins by trypsin-like proteases. The activated proteins cross the peritrophic

brush border apical membrane of midgut columnar cells, and insert into the membrane. Pore formation disrupts the ionic gradients and osmotic balance across the apical membrane and eventually causes the epithelial midgut cells to lyse. This leads to a massive disruption of the epithelium and ultimately to the death of the larvae by starvation or septicemia (Knowles *et al.*, 1994). Binding of *B. thuringiensis* proteins

to specific receptors plays an important role in their mode of action (Hofmann *et al.*, 1988; Oddou *et al.*, 1993). In various insect species (in particular those belonging to the lepidopteran class), amino peptidase N (APN) is one of the two receptor proteins that are considered to be involved in protein-receptor interactions.

The physiological effects of *Bacillus sphaericus* crystal protein have been poorly documented. Lakshmi and Gopinathan (1988) reported that oxygen uptake by mitochondria isolated from *B. sphaericus*-treated *Culex pipiens*, *Culex quinquefasciatus* larvae is inhibited, as is the activity of larval choline acetyl transferase in the presence of protein. In addition, *B. sphaericus* protein decreases oxygen uptake by mitochondria isolated from rat liver (Lakshmi and Gopinathan, 1988). As the differences in susceptibility between mosquito species do not seem related to the ability to solubilise and/or activate the binary protein, this variation presumably results from differences at the cellular level. Indeed, studies report the binding of fluorescently labeled protein to the gastric caecae and the posterior stomach only in very susceptible *Culex* species. The hypothesis that a specific receptor was involved in the protein binding was confirmed by *in vitro* binding assays using ¹²⁵I-labelled activated crystal protein and midgut brush-border membrane fractions (BBMFs) isolated from either susceptible or non-susceptible mosquito larvae (Oei *et al.*, 1992).

The pathological effect of the *B. thuringiensis* Cry δ -endoproteins on susceptible insect larvae had extensive damage on the midgut epithelial cells. The cytolytic activity of a number of the Cry proteins has been demonstrated *in vitro* using a wide range of lepidopteran and

dipterans cell lines (Knowles *et al.*, 1987; Thomas *et al.*, 1983). The *ex vivo* cytotoxicity assay for assessing the insecticidal potency of the Cry4B mosquito-larvicidal proteins, and demonstrated that the trypsin-activated Cry4B protein specifically exerted its cytolytic activity towards the isolated midguts from *Aedes aegypti* larvae. The availability of this *ex vivo* system will allow further investigation of the mechanism of action and the nature of the specific receptors on the *Aedes* midgut cell surface. In this study, the specific targets of the proteins in the mosquitoes exposed to the proteins were determined and the biochemical and physiological changes in the mosquitoes exposed to the proteins were elaborated.

Materials and Methods

Production of mosquitocidal protein from *Pseudomonas fluorescens* Migula

The culture supernatant of *Pseudomonas fluorescens* was collected after 72h of growth in GPS medium and the proteins were precipitated with 30-80% ammonium sulphate. The precipitated protein was dialyzed and fractionated by gel filtration using sephacryl S300 columns (Amersham-Pharmacia, Sweden) in an FPLC system. The column was eluted with Tris-HCl, containing sodium chloride buffer at a rate of 0.5ml/min. The thoroughly prewashed fractions displayed at 280nm a peak with a retention time of 17minutes. The fraction gave a single peak when checked by HPLC analysis, indicating homogeneity of the fraction. Pure protein was subjected to 10% Native page and single band was observed with a molecular weight of 90kDa. Thus the FPLC fractionated pure protein was found to be a monomer. Two prominent bands were observed on 10% SDS PAGE and their molecular weight was found to be 55kDa

and 35kDa (Usharani and Kummarkottil, 2013).

β*-Galactosidase enzyme assay in the gut of larvae and pupae of *Cx. quinquefasciatus

The 4th instar larvae and pupae of *Cx. quinquefasciatus* were treated with the mosquitocidal proteins and midgut were dissected out as described in earlier study Usharani and Kummarkottil, 2014. The isolated midgut from the treated, untreated larvae and pupae were homogenized, centrifuged and supernatant was used for the assay. Z-Buffer (1ml) was allowed to react with 0.2ml of Ortho-nitrophenyl- β -D-galactopyranoside (*ONPG*)(4mg/ml) in dark for 5minutes. Sample (100 μ l) was added and incubated in dark for 30minutes. The reaction was arrested by adding 500 μ l of sodium bicarbonate and absorbance was read at 410nm. The β -Galactosidase enzyme was used as standard (Lycett *et al.*, 2004).

Hydrogen peroxidase assay in the midgut of larvae and pupae of *Cx. quinquefasciatus*

The midgut of the mosquitocidal proteins treated 4th instar larvae and pupae of *Cx. quinquefasciatus* were dissected out as described in earlier report of Usharani and Kummarkottil, 2014. The isolated midgut from the treated, untreated larvae and pupae were homogenized, centrifuged and supernatant was used for the assay.

Potassium phosphate buffer, 0.32ml of 0.1M, was added to 0.16ml of 30% Hydrogen peroxide and incubated in dark at room temperature for 10 minutes. To this, 100 μ l of sample and standard Hydrogen peroxidase enzyme was added and mixed well (Sanjeev *et al.*, 2004). The reaction was arrested by the addition of 5% (w/v)

Pyrogallol solution and the absorbance was read at 420nm.

Acetylcholine esterase assay in the larvae and pupae of *Cx. quinquefasciatus*

The brain tissue was isolated from the treated, untreated 4th instar larvae and pupae of *Cx. quinquefasciatus* by placing them on ice cooled microscope platform in PBS buffer (pH 7.5) and removing the outer most layer of head region with help of dissection needle. The isolated brain tissues were homogenized centrifuged and supernatant was used for the assay. Potassium phosphate buffer, 152 μ l of 0.1M, was taken in the control and activity wells of micro plate. To this, 1 μ l of 75mM concentration of substrate, acetylcholine iodide, was added. 5-thio-2-nitrobenzoate (DTNB) (5 μ l) was added to both the control and activity wells and incubated at room temperature for 10minutes. The absorbance was read at 405 nm and taken as Blank reading (Venkateswara *et al.*, 2005). To this, 20 μ l of samples and standard acetylcholine esterase enzyme (1U/ml) was added, and the absorbance was read in the beginning and after five minutes at 405 nm.

Biochemical changes in the *Ae. albopictus* cell lines after treatment with the protein Cytochrome oxidase assay

Ae. albopictus cell lines (C6/36) were grown in Dolbeccos eagle's medium with 10% fetal calf serum in 25cm² tissue culture flasks. Confluent monolayer were washed in 5ml by low speed centrifugation (1000xg) and re-suspended at a cell concentration of 3 \times 10⁶ cells/ml in Tissue Culture medium without fetal calf serum. The mosquitocidal protein was added to the cell lines at a concentration of 7.5 μ g/ml and incubated for 1h. The cell lines without the protein were maintained as control. The control and

treated cell lines were harvested by centrifuging at 600xg for 10 m. The pellets was recovered and washed with PBS and once again resuspended with five times the volume of solution A (0.25 M sucrose, 20 mM Hepes – KOH, 1 mM EDTA, 1 mM DTT and 0.1mM PMSF). The cells were homogenized in the solution A and spun down at 750xg for 10 minutes. The supernatant was collected and centrifuged at 10000xg for 15 minutes. The supernatant was discarded and the crude mitochondrion recovered in the pellet was resuspended in the solution A.

The isolated intact mitochondria (40µl) were aliquoted in triplicate in 96 well plates. A volume of 150µl of reduced cytochrome C substrate was added to each well and the plate was assayed in a plate reader, reading at every 1m at 550nm for 10seconds. As 10x cytochrome oxidase oxidizes cyt C, the optical density of the solution declines at 550nm. The plate reader calculates the maximum slope of the plot of optical density (Timothy *et al.*, 2003). The protein concentration for the mitochondrial homogenate was determined by the method of Lowry *et al.* (1951).

Western blot assay of membrane fractions of *Ae. albopictus* cell lines

The membrane fractions were prepared from the control and treated *Ae. albopictus* cell lines (1.25×10^5 cells/ml) by washing the cells with TBS (10mM Tris HCl pH 7.5 and 150 mM NaCl). The washed cells were suspended in Tx 1000 and the homogenate was spun down at 4000xg for 30 minutes.

The supernatant was incubated at 37°C for 10m and spun down out at 60xg for 15 m. The pellets were suspended in TBS. The protein concentration was estimated by a

modification of the Lowry *et al.* (1951) method using bovine serum albumin as a standard. The cytoplasmic membrane fractions were subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane using a MiniTrans-Blot electrophoretic transfer cell (Bio-RAD) with transferring buffer (190 mM glycine, 20% methanol and 25 mM Tris-HCl, pH 8.3) at constant voltage for overnight (Laemmli 1970; Towbin *et al.*, 1979). The membranes were washed thrice with phosphate buffered saline containing 0.05% Tween20 and treated with BSA for 2h to block non-specific binding sites.

After blocking, the proteins immobilized on the membrane were incubated for 5h with 1: 500 dilution of the polyclonal antibodies developed against the mosquitocidal protein. The membrane was washed thrice with PBS-0.05% Tween20 and the immunocomplexes were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (whole molecule) as the secondary antibody for 2h. Color was developed with TMB/H₂O₂ as the substrate and the reaction was stopped after 1h with water (Salas-Benito *et al.*, 1997).

SDS-PAGE analysis of mitochondrial fraction to determine binding of protein

The mitochondrial fraction from the control, treated larvae and pupae of *Cx. quinquefasciatus* was suspended in 4x lamelli sample loading buffer [0.125 M tris/Hcl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercapto ethanol, 0.01% (w/v) bromophenol blue]. The samples were boiled for 5minutes, separated by 10% SDS –PAGE to find out whether the binding of protein enhances the cytochrome oxidase IV complex subunit (Srikrishnaraj *et al.*, 1995).

Analysis of specificity of toxicity of alkaline metalloprotease on cell lines Cytotoxicity assay

Ae. albopictus cell lines (C6/36) were grown in Dolbecco's eagle's medium with 10% fetal calf serum in 25cm² tissue culture flasks. Confluent monolayer were washed in 5ml by low speed centrifugation (1000xg) and re-suspended at a cell concentration of 3×10⁶ cells/ml in TC medium without fetal calf serum. The mosquitocidal metabolite was added at every 20 minutes intervals for 1h. The cell lines without mosquitocidal metabolite were kept as control. Cell viability was measured using a hemocytometer counting chamber. The LC₅₀ and LC₉₀ values were recorded (Davidson *et al.*, 1987).

Immuno fluorescence toxicity assay

Ae. albopictus cell lines (C6/36) were grown in Dolbecco's eagle's medium. The cells were centrifuged at 1000 rpm for 10 min. The resulting pellet was placed on two separate slides and air dried. The slides were then fixed with 3% para formaldehyde in PBS containing 0.5% BSA for 5 min at room temperature. The slides were rinsed with PBS twice and incubated with the polyclonal antibodies developed against the mosquitocidal protein. The other slide was kept as control. Both the treated and control slides were washed thrice with PBS containing 0.05% Tween20 and then incubated with the FITC conjugated antimouse antibodies, they were washed thrice with PBS and mounted in DPX and observed under fluorescence microscope (Davidson and Titus, 1987)

Ex-vivo cytotoxicity assay of protein to isolated midguts of *Cx. quinquefasciatus* larvae

Cytotoxicity was assessed through a cell

viability test using the CellTitre96[®] Aqueous kit (Promega Corporation, Madison, USA), which is based on the quantitative determination of the conversion of a tetrazolium compound to a formazan product that is directly proportional to the number of living cells. The assay was performed in a 96-well flat-bottomed microtitre plate containing 70µl of the dissecting buffer with isolated midguts. After adding 10µl of the protein samples (3-6µg) and 20µl of the CellTitre96 solution, formation of the reduced formazan product in each well was monitored at 490 nm. Three independent experiments were carried out for protein sample (Barusrux *et al.*, 2003).

Methylthiazole tetrazolium (MTT) assay of protein treated *Ae. albopictus* cell lines C6/36

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxy phenyl) -2-(4-sulfophenyl) -2H-tetrazolium (MTS) dye reduction assay in which viable cells convert MTS into a coloured formazan product (A492). The 400µl of 1.25x 10⁵ cells/ml stock *Ae. albopictus* cell lines C6/36- (1.25 x 10⁵ cells/ml) were plated in 96-well micro titre plates and incubated with 10µl of mosquitocidal protein in one set of three wells.

The another set with 10µl MTT reagent for 24h at 37°C. The cells were viewed periodically for the appearance of punctate, intracellular precipitate using an inverted microscope. The 100µl of detergent reagent was added to all wells, including control wells after the visibility of purple precipitate under microscope. The plate was covered and kept in the dark at 18–24°C for at least 2h to overnight. The plate cover was removed and measures the absorbance of the wells, including the blanks was read at

570nm. Results presented are means of eight wells \pm SD, and each experiment was repeated at least three times.

In vitro* digestion of gut extracts of larvae and pupae of *Cx. quinquefasciatus

The guts from 50nos of 4th instar control larvae and pupae were pulled out, and the peritrophic membranes were removed. The guts were then washed in 0.5ml of PBS and re-suspended in 0.2ml of PBS. The guts were sonicated for 60seconds in a 1.5ml Eppendorf tube and the protein concentration was determined by the method of Lowry *et al.* (1951). About 5 μ g of the crude gut extract was added to 10 μ g of the mosquitocidal protein. Trypsin digestion was carried out by adding 1 μ g of trypsin to 10 μ g of mosquitocidal protein. The mixtures were then incubated for 1h at 37°C and the products were analyzed on a 10% SDS-PAGE. Bioassay was carried out in two tubes to determine the toxicity by introducing 5 pupae/ larvae in 2ml of water. To one tube 10mg/ml of the mosquitocidal protein and to the other tube trypsin treated protein was added and monitored for mortality for 1h (Thirumaran *et al.*, 1992).

Results and Discussion

The induction of the marker enzymes β -galactosidase (Figure 1a,b,c), hydrogen peroxidase (Figure 2a,b,c), acetylcholine esterase (Figure 3a,b,c) were studied to find out the biochemical changes on the protein exposed 4th instar larvae and pupae of *Cx. quinquefasciatus* from the various tissues / organs. Since the P value of liner regression analysis is <0.0010 at 95% confidence intervals, the results of β -Galactosidase, hydrogen peroxidase, acetylcholine esterase assay of larvae and pupae is statistically significant.

The target of the active mosquito cidal protein was identified using *Ae. albopictus* (C6/36 cell lines) exposed to the mosquito cidal metabolites and quantitative analyses of enzymes from intact mitochondria were studied. The results of cytochrome oxidase assay showed that there is an induction of cytochrome C oxidase activity in the treated *Ae. albopictus* (C6/36 cell lines) than the untreated *Ae. albopictus* cell lines (Figure 4a, b). The results of Western blot assay of membrane fractions of *Ae. albopictus* cell lines revealed that the binding of mosquitocidal protein to the 90kDa of *Ae. albopictus* cell lines (Figure 5). The results of qualitative binding assay of mosquitocidal protein to the cytochrome oxidase by SDS-PAGE analysis of mitochondrial fraction showed induction of ~18 kDa protein of cytochrome oxidase IV subunit in the treated larvae and pupae compared to that of untreated larvae and pupae (Figure 6).

The mechanism of action and receptor binding of a dual specificity of alkaline metalloprotease using insect cell line culture (C6/36) was studied. The mosquitocidal proteins incubated with C6/36 cell lines of *Ae. albopictus* caused rapid rounding of cells followed sequentially by swelling, bulging of membrane vesicles, vacuolation and finally lysis. Cell death was found to be induced by increasing the concentration of the purified protein (7 μ g/ml–15 μ g/ml), and showed the characteristics of apoptosis (Figure 7). The results of assay of toxicity through detection of immunofluorescence did not show fluorescence in control cell lines, where as in treated cell lines incubated with FITC conjugated anti-mouse antibodies showed bright fluorescence. The bulging of membrane vesicles and vacuolization of cells were observed in treated cell lines (Figure 8).

The trypsin digested gut extract of mosquitocidal protein exposed larvae and pupae were analysed on 10% SDS Poly acrylamide gel. The trypsin resistant bands were observed in the both larval and pupal gut extract. It showed that the trypsin did not cleave the 55kDa and 35kDa into smaller peptides (Figure 9). The midguts were isolated from larvae of *Cx. quinquefasciatus* for the assessment of mosquitocidal protein toxicity via cell viability assay. After incubating the dissected midguts with the protein samples, the number of viable epithelial cells was determined by a colorimetric method that monitors the quantity of water-soluble formazan products by photometric measurement at 490 nm. Changes in A490 were assumed to be directly proportional to the number of viable cells (Figure 10). The decline of A490 was due to the degradation of formazan that is possibly caused by oxidative agents from the dissected tissues. Each reaction curve was analyzed by nonlinear regression using Graph Pad PRISM version 2.0. A statistical analysis was carried out by using one-way analysis of variance (ANOVA) tests and the probabilities <0.05 were considered as significant. The MTT assay revealed 45% viability after 24 h after infection (Figure 11). Compared with uninfected control cells, there was a significant decrease in the viability of mosquitocidal protein exposed cell lines at 24h. Probabilities <0.05 versus untreated cell lines C6/36 were considered as being significant.

In the present investigation, specific target of the protein, biochemical and physiological changes in the protein exposed 4th instar larvae and pupae of *Cx. quinquefasciatus* were studied. The protein treated midgut cells of larvae and pupae showed induction of β galactosidase activity. This may be due to the irreversible

binding of protein to the mid gut cells leading to constitutive expression of lacZ gene, which codes for β galactosidase enzyme. In general, a wide range of carbohydrases are present in insects. The gut of insects like cockroach contains amylases α galactosidase and β galactosidase (Sabine *et al.*, 2006). β galactosidase utilizes lactose as a substrate by cleaving the anomeric carbon and glycosyl oxygen to form galactose and glucose. β galactosidase is a reporter gene and regulated by lac Z under the control of Lac operon (Seeber *et al.*, 1996). The protein treated midgut cells of larvae and pupae showed the induction of β galactosidase activity and it could be due to the binding of protein to the midgut cells of treated larvae and pupae leading to increase in the β galactosidase enzyme activity.

Similarly, hydrogen peroxidase activity in the midguts of treated larvae and pupae were studied after staining with TMB (Tetramethyl benzidine) peroxidase – specific chromopore and hydrogen peroxide as substrates. This histochemical study revealed that the treated midgut cells were stained very strongly than the control cells. This could be interpreted in such a way that the binding of mosquitocidal proteins to the mid gut epithelial cells leads to induced peroxidase enzyme activity, which subsequently leads the generation of hydrogen peroxide to promote apoptosis. It has been reported that the *P. berghei* invasion of *An. stephensi* midgut cells induces expression of nitric oxide synthase followed by increased peroxidase activity. To confirm the induction of peroxidase activity in response to malaria infection, the activity was determined in the mid gut homogenates by performing histochemical staining and spectrophotometric assay using TMB (Tetramethyl benzidine) peroxidase – specific chromopore and hydrogen peroxide as substrates. The parasite infection resulted

in a marked increase in the peroxidase activity when homogenates from mid guts that had been fixed with a mixture of glutaraldehyde and formaldehyde were used in the assay than the control midgut cells (Sanjeev *et al.*, 2004). Similarly, in the present study also, mid guts of treated larvae and pupae were stained with TMB (Tetramethyl benzidine) peroxidase – specific chromophore and hydrogen peroxide as substrates.

The activity of marker enzyme acetylcholine esterase was increased significantly in the brain tissues of treated larvae and pupae than the midgut region of treated larvae and pupae. The enzyme activity was decreased considerably after five minutes interval. In general, acetylcholine receptors belong to the cyst-loop super family of (GABA) gated chloride (Cl⁻) channels consisting of five sub units. Each sub units possess a large N-terminal extra cellular domain that includes the components forming the acetyl choline binding site. The acetyl choline acts as a neurotransmitter and hydrolyzed by the enzyme acetylcholine esterase. The cuticular invasion of mosquitocidal protein to the central nervous system leads to increase in acetyl choline esterase activity in the treated larval and pupal brain tissues. This elucidates the biphasic effect of mosquitocidal proteins in such a way that the first phase of protein effect of increased levels of acetylcholine esterase is due to the inhibition of neuronal transmission while in second phase, the acetylcholine esterase activity was decreased; due to the neuronal degeneration of tissues. The acetylcholine esterase enzyme activity in the mid gut of treated larvae and pupae was decreased than the control larvae and pupae. This could be due to the fact that the mosquitocidal protein

acts as a potential inhibitor to the enzyme. The difference of acetylcholine esterase activity in the brain and mid gut region of treated larvae and pupae is depended on the function of the tissues involved (Zhen *et al.*, 2004; Gao *et al.*, 2007).

The induction of cytochrome C oxidase activity in the treated *Ae. albopictus* C6/36 cell lines than in the untreated cell lines indicates that the protein might have regulated mitochondrial ETC complex IV. The protein would have mediated the enhancement of mitochondrial biogenesis in the treated cell lines under the stress condition as reported by Luciakova *et al.* (1999). As a confirmation, the results showed induction of ~18 kDa protein of cytochrome oxidase IV subunit in the treated larvae and pupae as compared to that of untreated larvae and pupae. It has been reported that the proteases enhances the mitochondrial biogenesis (Nagy *et al.*, 2004). The C6/36 cell lines incubated with the mosquitocidal proteins with caused rapid rounding of cells followed by swelling, bulging of membrane vesicles, vacuolation of cells and finally cell lysis. At lower concentrations, the protein caused no morphological change and the cells were viable. Cell death induced by increasing the concentration of purified protein had the characteristics of apoptosis. The fluorescence was not observed in control cell lines where as in treated cell lines, incubated with FITC conjugated antimouse antibodies showed bright fluorescence. The bulging of membrane vesicles and vacuolization of cells were observed in treated cell lines, indicating that the FITC conjugated antibody reacted with the protein which bound to the specific receptor on surface of the cell lines.

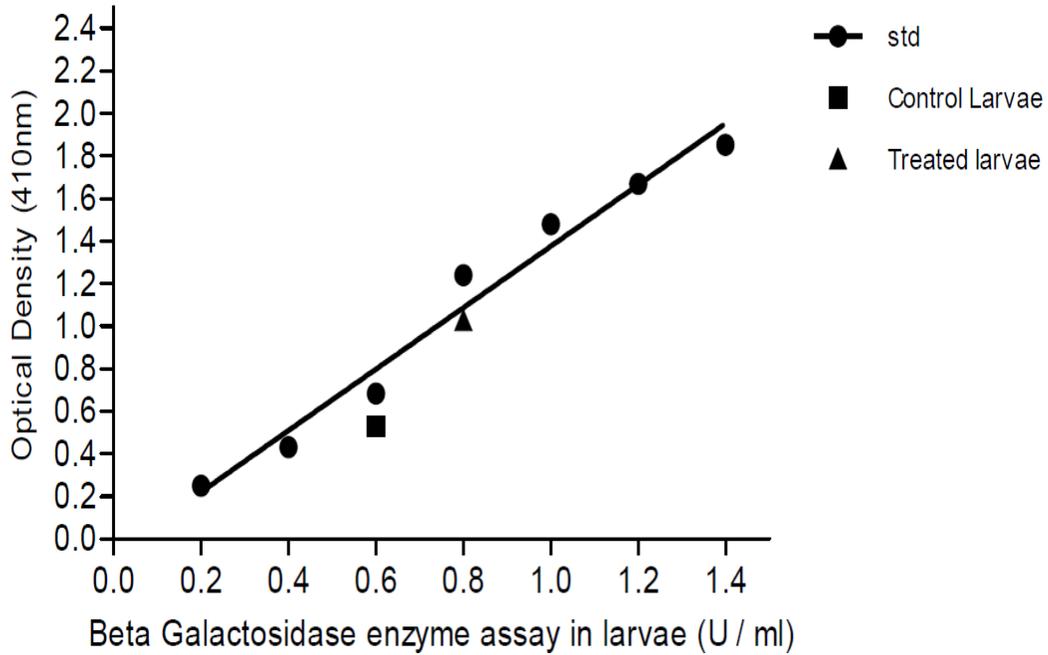


Figure.1a β galactosidase enzyme assay in the larvae *Cx. quinquefasciatus* (U / ml)

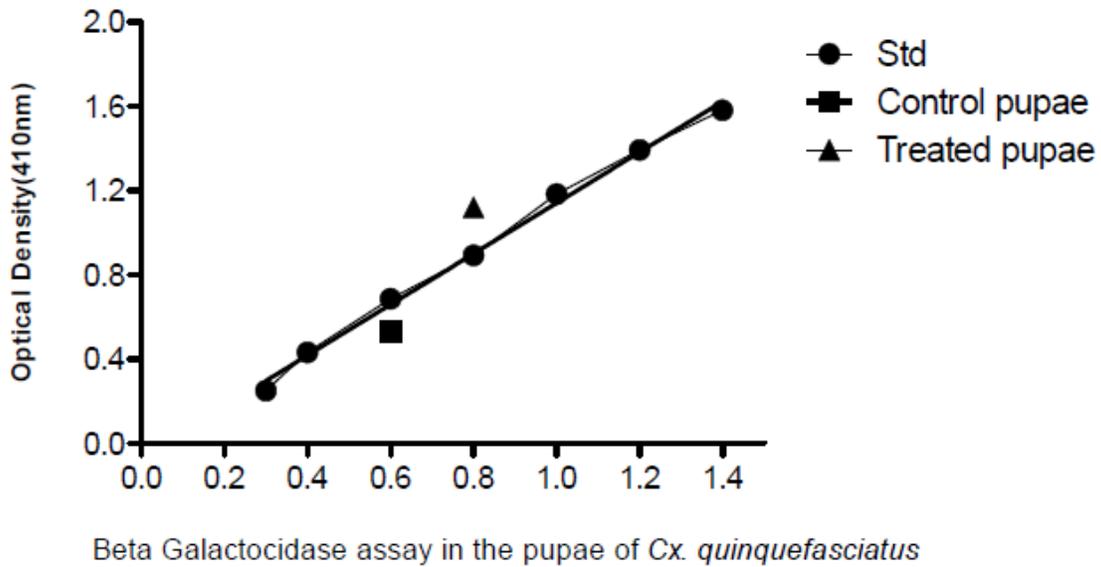


Figure.1b β galactosidase enzyme assay in the pupae of *Cx. quinquefasciatus* (U/ml)

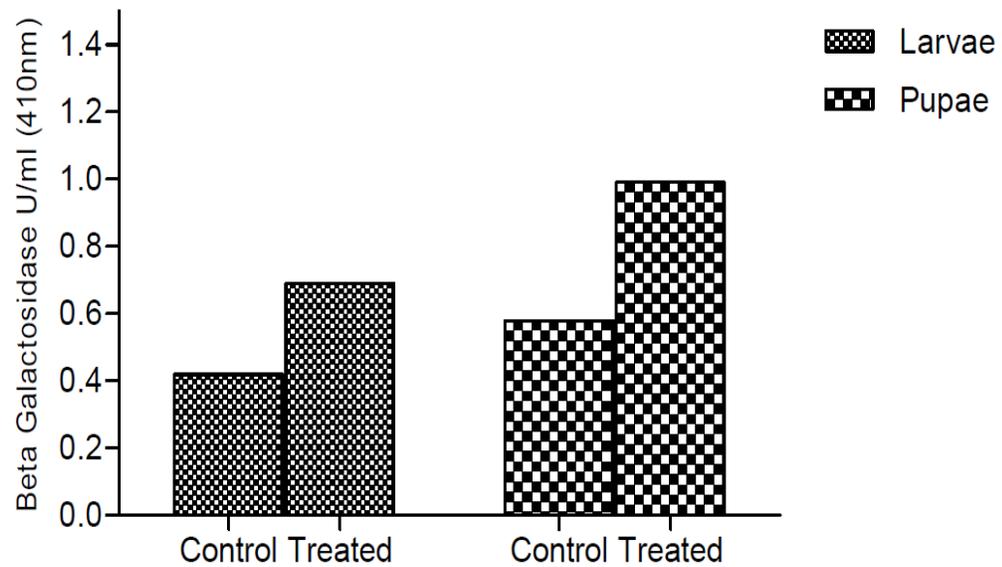
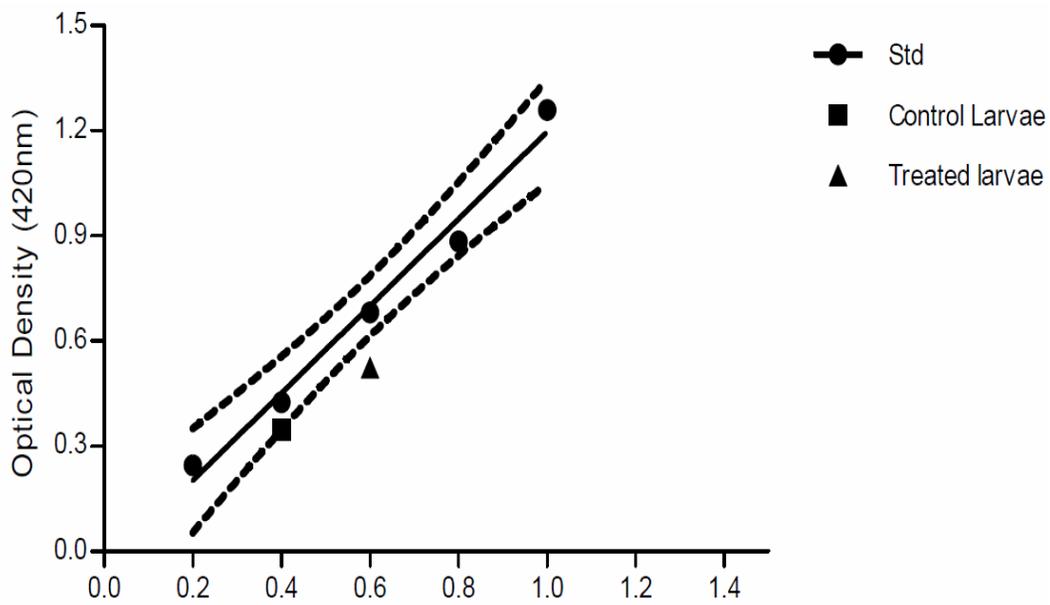
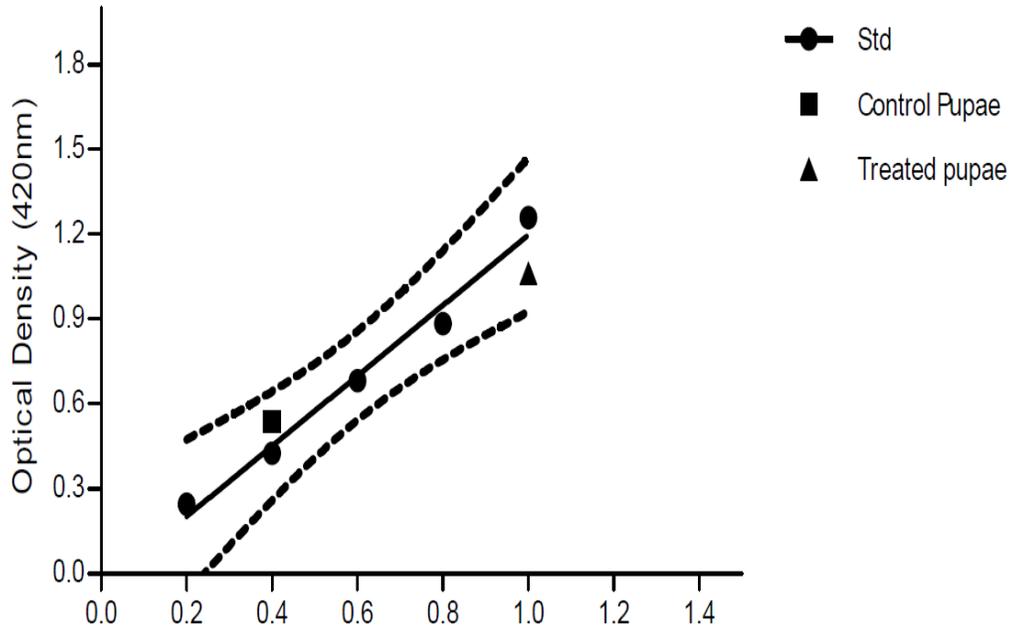


Figure.1c Comparison of β galactosidase assay in the control and protein treated larvae and pupae of *Cx. quinquefasciatus*



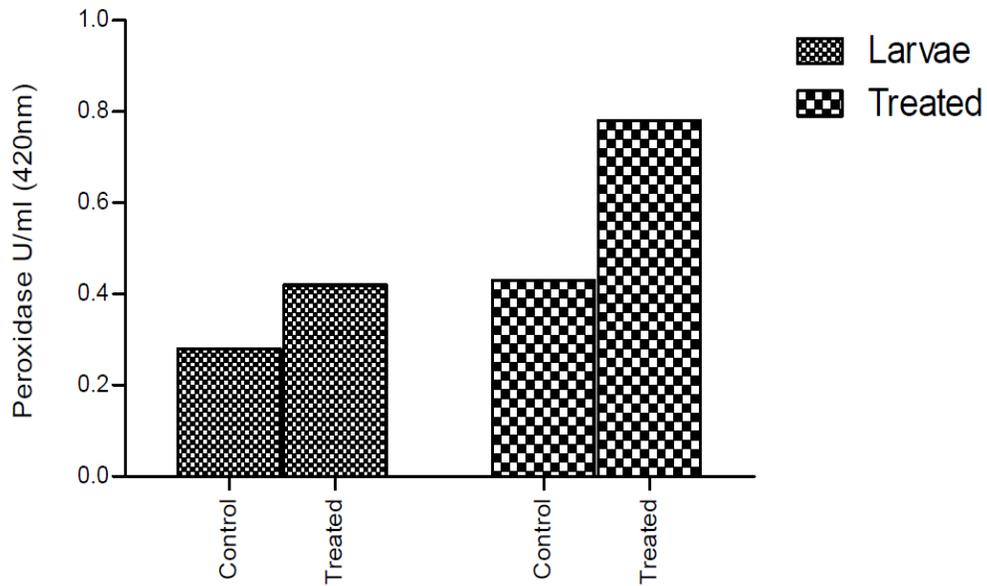
Peroxidase enzyme assay in larvae of *Culex quinquefasciatus* (U/ml)

Figure.2a Peroxidase enzyme assay in the larvae of *Cx. quinquefasciatus*



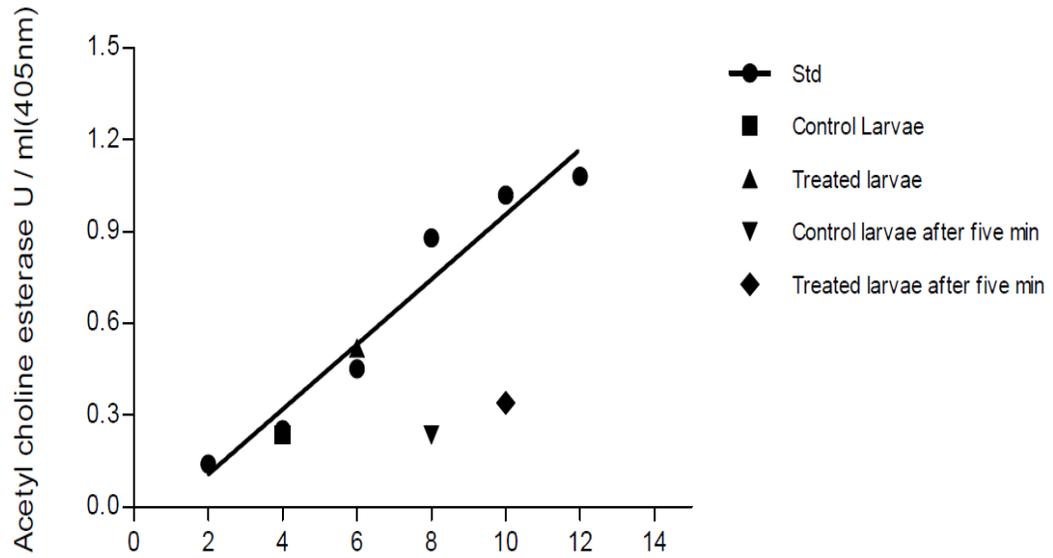
Peroxidase enzyme assay in pupae of *Culex quinquefasciatus* (U/ml)

Figure.2b Peroxidase enzyme assay in the pupae of *Cx. quinquefasciatus*



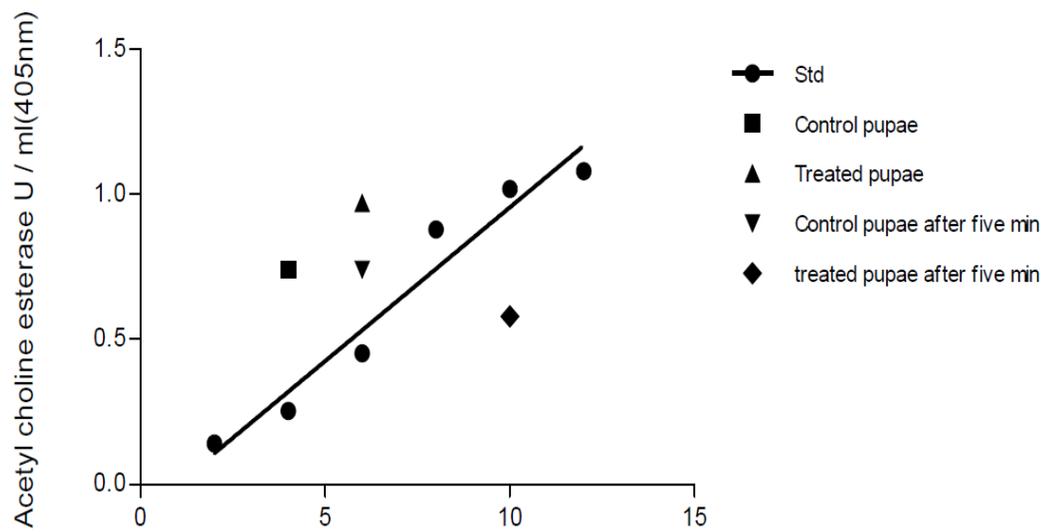
Peroxidase enzyme assay in the larvae and pupae of *Culex quinquefasciatus*

Figure.2c Comparison of peroxidase enzyme assay in the control and protein treated larvae and pupae of *Cx. quinquefasciatus*



Acetylcholine esterase assay (Brain tissue) in larvae of *Culex quinquefasciatus* (U/ml)

Figure.3a Acetylcholine esterase assay in the brain tissue of larvae of *Cx. quinquefasciatus* (U/ml)



Acetylcholine esterase assay (Brain tissue) in pupae of *Culex quinquefasciatus* (U/ml)

Figure.3b Acetylcholine esterase assay in the brain tissue of pupae of *Cx. quinquefasciatus* (U/ml)

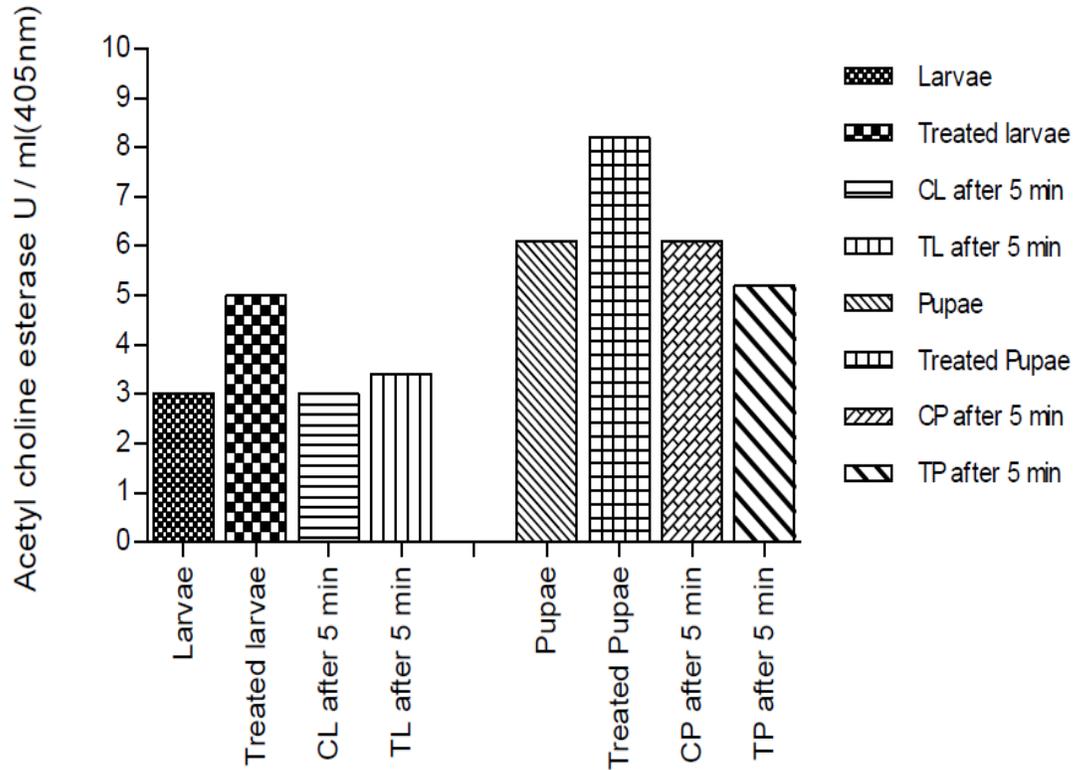


Figure.3c Acetylcholine esterase assay in the brain tissue of larvae and pupae of *Cx. quinquefasciatus*

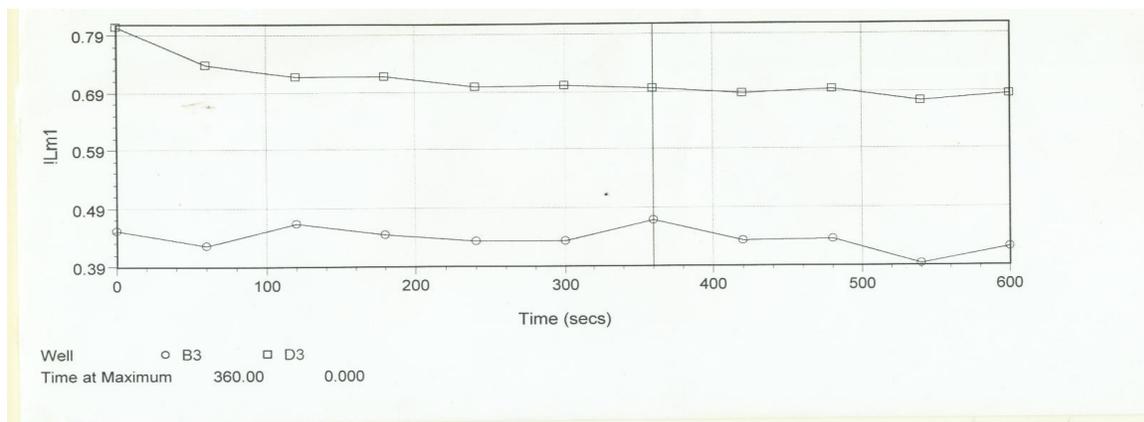


Figure.4a Cytochrome oxidase assay in mosquitocidal protein treated larvae

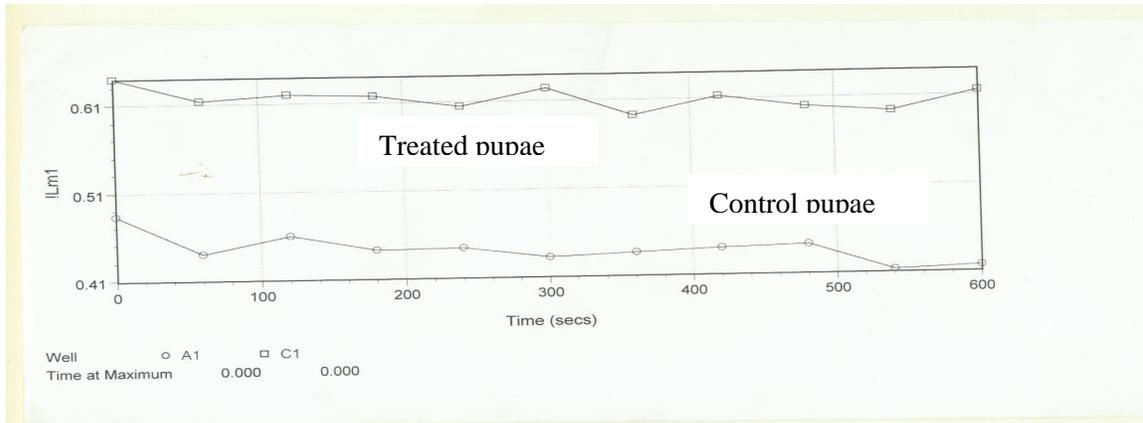
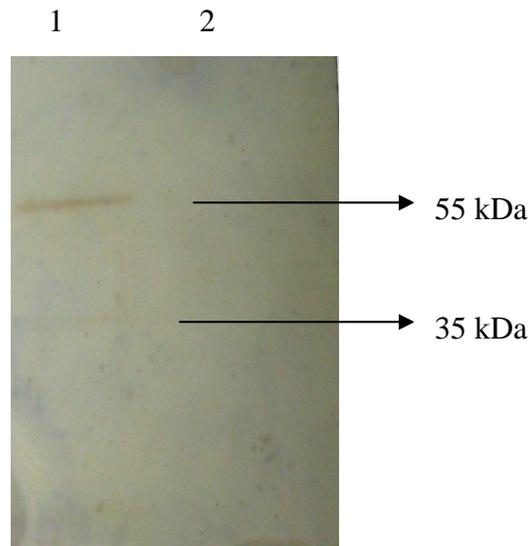
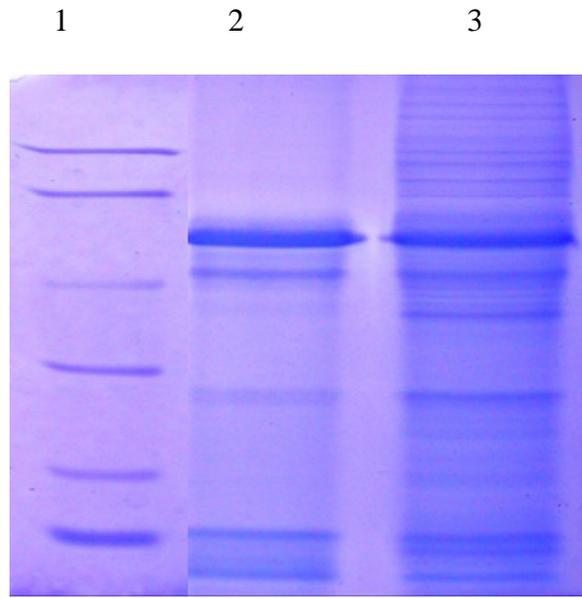


Figure.4b Cytochrome oxidase assay in mosquitocidal protein treated pupae



Lane 1: Mosquitocidal protein treated membrane fractions, Lane 2: Control membrane fractions

Figure.5 Western blot analysis of membrane fractions of *Ae. aegypti* cell lines treated with mosquitocidal proteins



L 1: marker, L 2: treated membrane fractions, L 3: Control membrane fractions

Figure.6 Protein profile of mosquitocidal protein treated membrane fractions of *A. albopictus* cell lines

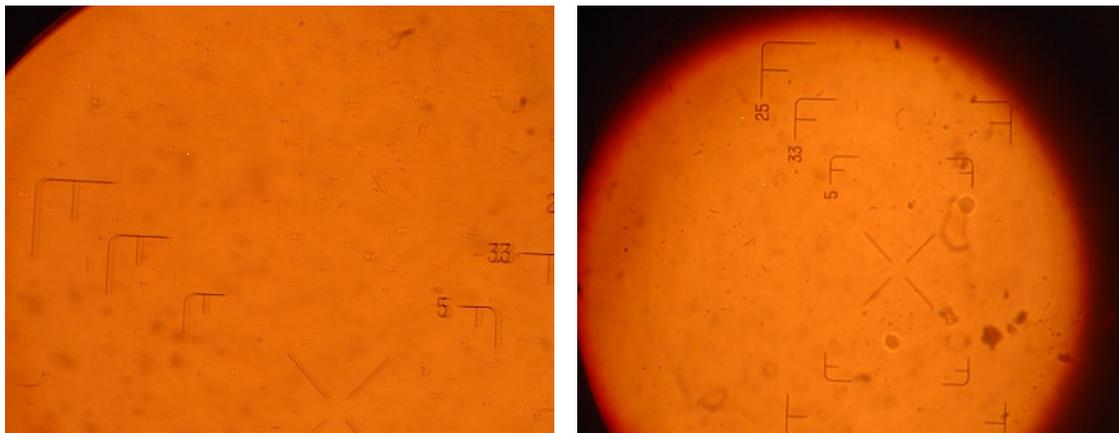
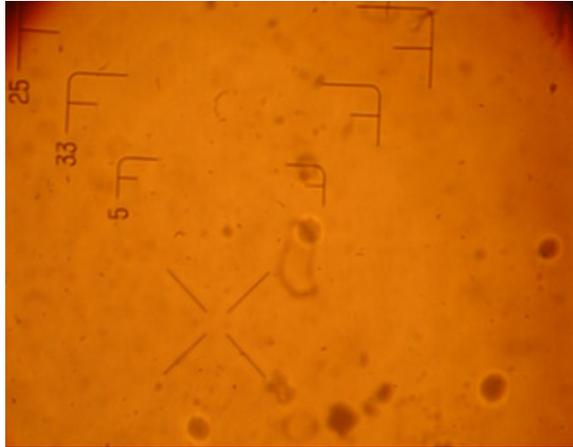


Figure.7 Cytotoxicity assay a) Control cell line b) Initial vacuolation on *Ae. albopictus* C6/36 cell lines after treated with mosquitocidal metabolite



C) Vacoulation on *Ae. albopictus* C6/36 cell lines after treated with mosquitocidal protein

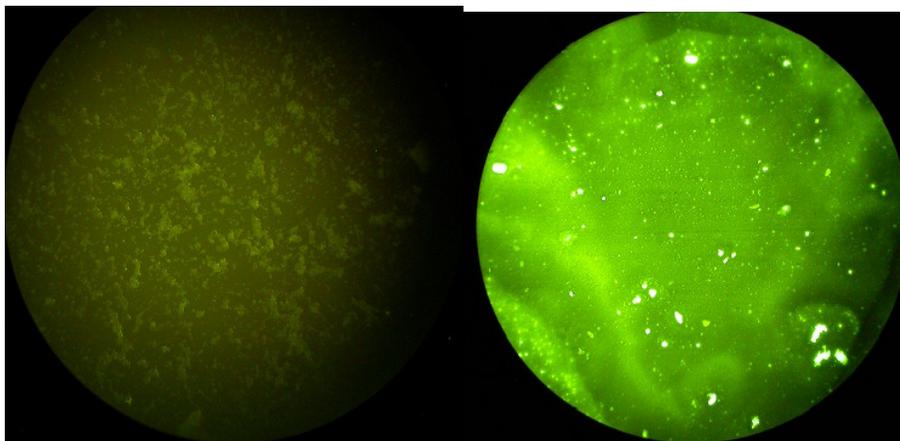
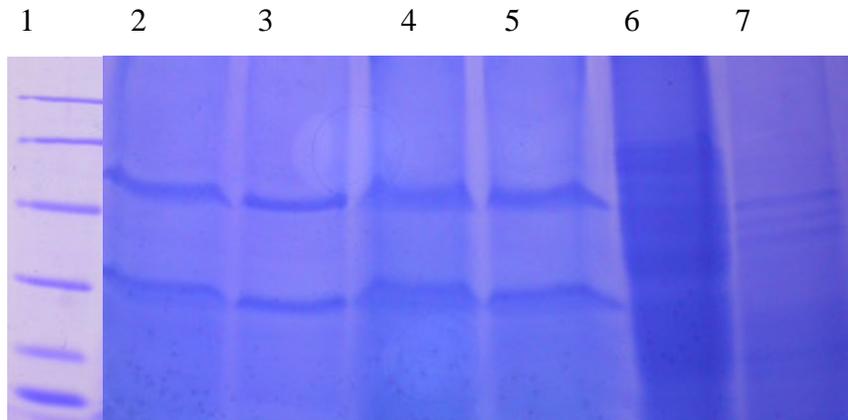


Figure.8 Immunofluorescence toxicity assay (a) Control cell line (b) Cell line treated with FITC Conjugated anti-mouse antibodies



L 1: marker, L 2, 3: protein, L 4: treated pupal gut, L 5: treated larval gut
L 6: larval gut , L 7 : pupal gut

Figure.9 *In vitro* tryptic digestion of midgut extracts of larvae and pupae of *Cx. quinquefasciatus*

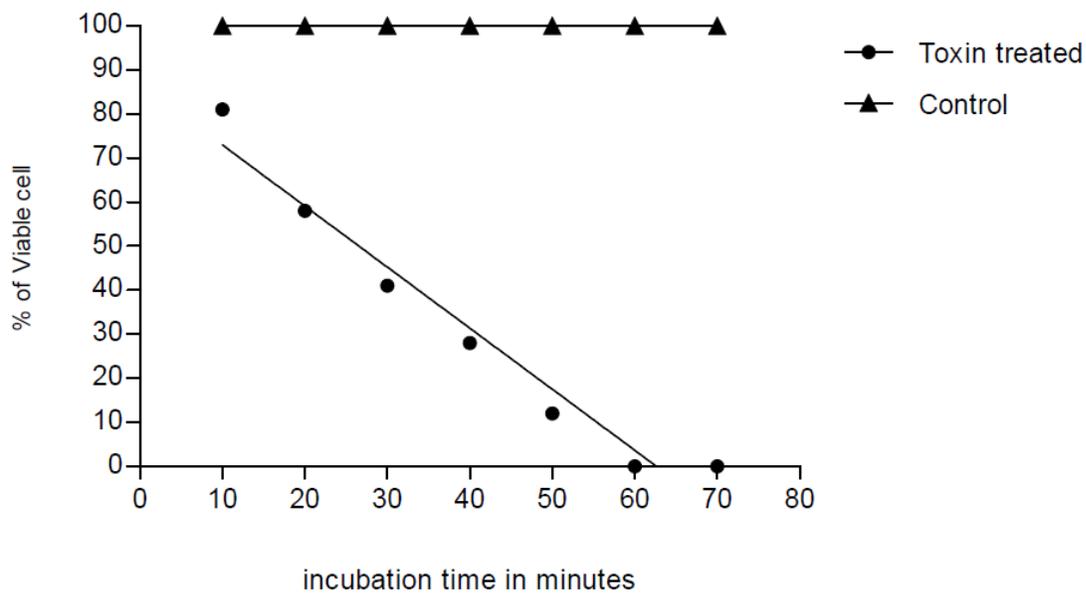


Figure.10 Ex vivo cytotoxicity assay of mosquitocidal protein to isolated midguts of *Cx. quinquefasciatus* larvae

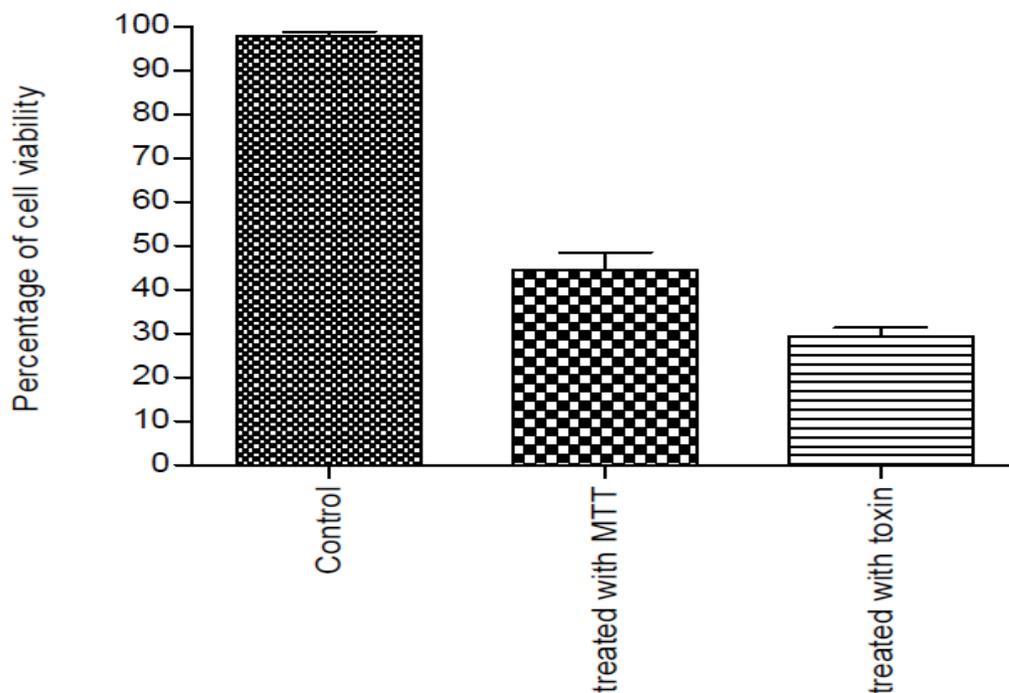


Figure.11 *Ae. albopictus* cell lines C6/36 treated with mosquitocidal protein

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