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Mosquitocidal activity of thermostable extra cellular glycoprotein of *Pseudomonas fluorescens* Migula

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Abstract
The present study describes the thermostability of glycoprotein of extra cellular mosquitocidal proteins of *Pseudomonas fluorescens* Migula and its involvement during pathogenesis in insects against larvae and pupae of *Culex quinquefasciatus.* Fluorescent glycoprotein detection assay was employed for the detection of glycoprotein. The upper and lower confidential limits of LC 50 and LC 90 values obtained through Probit regression analysis. The amino sugars like D-maltose, D-mannose, D-xylose and sialic acid were checked and determined in purified mosquitocidal protein. Two mosquitocidal proteins corresponding to 55 kDa and 35 kDa mosquitocidal proteins on 10% SDS-PAGE indicated that these proteins could withstand and were stable even at an elevated temperature of 121°C for 15 minutes. The LC50 value of crude and pure protein was 0.3 ug/ml and 0.03 ug/ml for both pre- and post-treatment. P value of liner regression analysis is <0.0001 at 95% confidence intervals, the results of determination of amino sugars D-maltose, D-mannose, D-xylose test is statistically significant. The FT IR spectra of mosquitocidal protein showed amide bonds, Carbohydrates and N-acetyl amino sugars, aliphatic hydrocarbons and hydroxyl groups. Hence, the presence of N-acetyl amino sugar moiety in the mosquitocidal protein confirmed it was glycoprotein. The 0.155 µmol/mg N-acetyl Neuraminic acid was estimated and its presence in the active mosquitocidal protein confirmed it was glycoprotein. The peak at 1.5, 2.4, and 3.3 to 3.5 ppm of 13 C NMR spectrums strongly confirmed the presence of N-acetyl amino sugars. It is concluded that the novel extra cellular mosquitocidal protein of *Pseudomonas fluorescens* Migula is thermostable glycoprotein.

Keywords: *Pseudomonas fluorescens* Migula, *Culex quinquefasciatus*, mosquitocidal, thermostable

Introduction
Mosquitoes are vectors for many diseases around the world like malaria, dengue fever, yellow fever and other types of encephalitis [1]. Mosquito control manages the population of mosquitoes for reducing their damage to human health, economies, and enjoyment. Mosquito control is a vital public-health practice throughout the world and especially in the tropics as mosquitoes spread many diseases, such as malaria. In an integrated Pest management Program, biological control of mosquitoes is an important constituent. Naturally there are several predators of these insect both as larvae and adults. A comprehensive mosquito control program is a carefully planned and executed operation aimed at ensuring effective and continuous control of mosquitoes [2].

There are a number of microbial agents including fungus, protozoa, virus and bacteria which act as mosquitocidal agents. However, among these agents, *Bacillus thuringiensis* var. *israelensis* and *B. sphaericus* are the most potent mosquitocidal agents. *Bacillus thuringiensis* var. *israelensis* and *B. sphaericus* are gram-positive sporulating bacteria producing protoxin crystals during sporulation are highly toxic to susceptible mosquito larvae when ingested. These bacterial agents are environmentally safe due to their host specificity, requiring very low dosage, easy to prepare commercially in large-scale and are in low cost. Field trials with various formulations of *B. sphaericus* and *B. thuringiensis* var. *israelensis* have demonstrated their safety and potential to control mosquitoes. Moreover, cloning and expression of the toxin genes of these organisms in other environment friendly bacteria have also made them important for further investigation. Recent advances with novel types of recombinant microorganisms with new cloning strategies and cloning the toxin genes under strong promoter for over expression together with in vitro gene manipulation and site directed mutagenesis of the active sites for increased toxicity have the potential to provide more effective control of mosquitoes by exploiting these two bacteria. But the toxins of *B. sphaericus* and *B. thuringiensis* var. *israelensis* in particular, do not persist long in nature and require frequent application, a limiting factor for these organisms to be more successful in becoming potent Larvicide. Nevertheless, they are by far the best choice for controlling mosquitoes [3].

A novel natural strain of *P. fluorescens* obtained from a soil sample collected from Pondicherry, India was used in the present study. The active principle present in the culture supernatant of *P. fluorescens* was purified and characterized already [4]. The formulation prepared from the exotoxin of *P. fluorescens* was reported to be toxic to larval as well as pupal stages of mosquitoes [5]. The present investigations aim at Mosquitocidal activity of thermo stable extra cellular

glycoprotein of *Pseudomonas fluorescens* Migula.

Material and Methods

Bacterial culture Assessment of mosquitocidal effect of the culture supernatant

The *P. fluorescens* Migula was grown in the GPS liquid medium containing 1.0% (wt/vol) Glucose, 1.0% (wt/vol) Peptone, 0.1M Potassium dihydrogen Phosphate, pH 7.0. 600 ml of medium was inoculated with 6 ml of seed culture and the activity of extra cellular protein production was checked in the culture supernatant at different stages from 24 hrs to 96 hrs of growth of *P. fluorescens* Migula. The 72 hrs culture was centrifuged at 8,000rpm for 30 minutes at 4°C and the supernatant was collected for the purification of the extra cellular proteins. The proteins present in the culture filtrate of the bacterium *P. fluorescens* Migula (VCRC B426) strain were precipitated with ammonium sulphate, dialyzed and fractionated by gelfiltration using sephacryl S300 columns (Amersham-Pharmacia, Sweden) in an FPLC system. The column is first equilibrated using a50mM PBS buffer containing 0.1M sodium chloride, pH 7.2 at a flow rate of 1 ml per minute. The loop is filled with sample (50ul/200ul) and it was eluted at a flow rate of 1ml per minute. The protein fractions were eluted using PBS (0.05M), containing sodium chloride buffer (0.1M; pH 7.5) and monitored at 280 nm. The protein fractions of 1 ml each collected were assayed for mosquito pupicidal activity against *Cx. Quinquefasciatus* by introducing five pupae of each 1 ml fractions, mixed with 4 ml of tap water as described by Usharani *et al*., 2014 [6]. The mosquitocidal protein concentration was estimated by modification of the Lowry *et al* (1951) [7] method using bovine serum albumin as a standard.

Thermo stability of the mosquitocidal protein

The proteins were autoclaved at 121º C for 15 minutes and dialyzed for 24 h in phosphate buffer (pH 7.2). The dialyzed sample was lyophilized and the protein concentration was estimated by the method of Lowery *et al*. (1951). The autoclaved protein and crude protein were subjected to 10% SDS-PAGE. Laboratory bioassay of the autoclaved protein was performed with pupae of *Cx. quinquefasciatus.* Bioassay with protein without heat treatment served as control. A graph was plotted with the concentration of the protein (µg/ml) Vs the percentage of mortality for the pre- treatment and post-treatment.

Fluorescent Glycoprotein Detection assay

The bacterial proteins were separated by electrophoresis on a 10% SDS-PAGE gel. The gel was stained for glycoproteins with Glycoprofile™ III Fluorescent Glycoprotein Detection Kit [8] and then stained for total protein with EZ Blue Gel Staining Reagent [9] (Sigma Product Code G 1041). Presence of glycoproteins was determined by assessing appearance of bright fluorescent bands.

Glycosylation determination

Determination of glycosylated motifs was performed using an Immuno-Blot System for glycoprotein (Bio-Rad) for detecting glycoproteins acids. The carbohydrate moiety of a glycoprotein was oxidized with a solution of sodium periodate (10mm at room temperature for glycoprotein detection) to form aldehyde groups for reacting with hydrazide. Biotin X hydrazide was used to incorporate the biotin into the oxidized carbohydrate. The biotin was then coupled to streptavidin and this complex was stained with NTB/BCIP solution. The 55 kDa and 35 kDa proteins were transferred onto a nitrocellulose membrane (Sigma Chemicals Co) and the membrane was treated with wheat germ agglutinin lectin (WGA-lectin). Biotinylated proteins from Bio-Rad were used as positive control and native proteins (except for ovalbumin) were used as negative control [10].

Determination of total Carbohydrate in mosquitocidal protein

The protein sample (100 mg) was hydrolysed by keeping it in a boiling water bath for three hours with 5 ml of 2.5 N Hcl and cooled to room temperature. It was neutralised with solid sodium carbonate until the effervescence ceased and the volume was made up to 100 ml and centrifuged. The supernatant was collected and 0.5 and 1 ml aliquots were taken for analysis.

Anthrone Method

Standards were prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard. '0' serves as blank. The volume was made up to 1 ml in all the tubes including the sample tubes by adding distilled water and 4 ml of anthrone reagent. Boiled the tubes for eight minutes in a boiling water bath. Read the tubes at 630 nm after rapid cooling. Standard graph was drawn by plotting concentration of the standard on the X-axis *versus* absorbance on the Y-axis. From the graph, the amount of carbohydrate present in the sample was calculated [11].

Phenol Sulphuric Acid Method

Standards were prepared by pipetting out 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard into a series of test tubes. Pipetted out 0.1 and 0.2 ml of the sample solution in two separate test tubes. The volume was made up to 1 ml in all the tubes including the sample tubes by adding distilled water. The blank was with 1 ml of distilled water and 1 ml of phenol solution was added to each tube. 5 ml of 96% sulphuric acid was added to each tube and after 10 min shaking, the tubes were placed in a water bath at 25–30°C for 20 m. Read the colour at 490 nm. The amount of total carbohydrate present in the sample solution was calculated using the standard graph [12].

Determination of sialic acid (N-acetyl Neuraminic acid)

The lyophilized crude mosquitocidal protein was purified by gel filtration on chromatography using sephacryl column in FPLC system. The protein fractions were eluted using phosphate buffer (pH 7.2) containing sodium chloride and monitored at 280 nm. The active fractions showing the pupicidal activity were pooled and concentrated by lyophilization. The lyophilized protein was passed through the Dowex column and the eluted protein was treated with 0.1 N sulphuric acid as per the method of Warren *et al* (1959) [11]. The amount of sialic acid present in the protein was determined by molecular extinction coefficient of standard N-

acetyl Neuraminic acid.

FT IR Analysis of Mosquitocidal protein

The IR spectroscopy analysis of the active spot was performed using KBr plates (JASCO FT-IR Model-420) with a scanning speed of 2 mm S^{-1}. All solvents and reagents used were of analytical grade and purchased from E. Merck, India. The purified protein (1 mg/ml) was coated on KBr blocky crystals. The crystals were ground and pressed into tablet. The sample tablet was put into the pool of the temperature controller to record the IR spectra *in situ* and then the infrared spectra of all samples were collected Spectrum GX FT-IR system equipped with a DTGS detector with scan ranges from 1000 to 4000 cm^{-1} and resolution of 4 cm^{-1} was employed. The spectra were obtained from the accumulation of a total 20 scans [13].

NMR Analysis of Mosquitocidal protein

The purified protein was prepared to a final concentration of 780 mM (5 mg/ml) in a volume of 0.5 ml 90% H_2O / 10% D_2O. The sample was maintained at pH 5.2 with 10 mM phosphate buffer. All spectra were recorded at 600 and 800 MHz on Bruker Avance 600 and Avance 800 spectrometers, respectively. Temperatures of the spectra were between 5 and 25° C. The 2D experiments included TOCSY experiments using a DIPSI2 spin-lock sequence with mixing times ranging from 40 to 80 ms and NOESY experiments with mixing time of 120 ms. Spectra were routinely acquired with 2K complex data points in F2 and 250 increments in the F1 dimension (in the states-TPPI mode). Solvent suppression was achieved using a Watergate sequence [14]. All data were processed using Xwin Nmr (Bruker). The t2 and t1 dimensions were zero-filled to 4K and 2K data points, respectively, 45 and 908 phase-shifted squared sine bell window functions were applied along t2 and t1, respectively. Chemical shifts were referenced to 2, 2-dimethyl-2-silapentane-5-sulfonate (DSS) at 0.0 ppm [15-16].

Results and Discussion

The thermo stability of the both crude and FPLC fractionated pure protein was studied and the mosquitocidal activity of pre-treatment and post-treatment crude and purified protein was carried out against the pupae of *Cx. quinquefasciatus*. The two mosquitocidal proteins corresponding to 55 kDa and 35 kDa on 10% SDS-PAGE (Figure 1) were intact indicating that these proteins could withstand and stable even at an elevated temperature of 121°C for 15 minutes. The LC50 value of crude protein was 0.3 ug/ml for both pre- and post-treatment. The LC50 value of pure protein was found to be 0.03 ug/ml for both pre- and post-treatment (Figure 2a, 2b). The upper and lower confidential limits of LC 50 and LC 90 values obtained through Probit regression analysis are shown Table 1-4.

A more sensitive fluorescence method was employed for the detection of glycoprotein. The pure mosquitocidal protein, glycoprotein marker and 0.001% of BSA were subjected to 10% SDS-PAGE. Two prominent fluorescent bands (55 kDa and 35 kDa) were observed (Figure 3). They were blotted on nitrocellulose membrane and treated with biotinylated lectin (Wheat germ agglutinin). The glycoprotein marker and purified mosquitocidal protein were visualized as pink colour after the addition of TMB/H_2O_2 substrate (Figure 4).

The amino sugars like D-maltose (Figure 5), D-mannose (Figure 6), D-xylose (Figure 7) and sialic acid were estimated in purified mosquitocidal protein. Since the P value of liner regression analysis is <0.0001 at 95% confidence intervals, the results of determination of amino sugars D-maltose, D-mannose, D-xylose test is statistically significant. The 0.155 µmol/mg N-acetyl Neuraminic acid was estimated and its presence in the active mosquitocidal protein confirmed as glycoprotein.

The IR spectrum shows significant structural information of major constituents present in the mosquitocidal protein. As shown in the figure 8, the strongest peak (O-H) Hydroxyl groups detected at the scan range of 3416.26cm-1. The stronger carbohydrates (C-O) were represented at the peaks ~ 1056 cm-1 and 1114 cm-1. The peak 1732 represents the presence of carbonyl compounds. The structural assignments for the peaks were obtained from 13 C NMR and the spectra were analyzed (Figure 9a, 9b,). One dimensional NMR spectrum (1H NMR) revealed the chemical shifts of the mosquitocidal protein. The chemical constituents of compounds could be identified based on the chemical shifts obtained. The methyl groups appeared between 0.5-1 ppm, the amino acid valine was distinctly seen in the regions of 0.7-0.9 ppm and 5 ppm. The amino acid alanine peak was observed between 3.7-4 ppm. The peak at 1.5, 2.4, and 3.3 to 3.5 ppm represents the presence of N-acetyl amino sugars. The peak at 3.8-4.0 ppm represents the presence of glycine group. At the peak 2.5 ppm, the proline group was observed

The structural assignments for the peaks were obtained from 13 C NMR and the spectra were analyzed. The aliphatic hydrocarbons (C-H) in 13C NMR analysis appeared between 0-55 ppm. The amines, amides and proteins (C-N) groups appeared between 40-55 ppm. Methoxy groups lie in the region between 5560ppm.The anomeric carbons of carbohydrate moieties were determined between 90-105 ppm. IH NMR, 13C NMR revealed the presence of aliphatic hydrocarbons, amides, proteins, methoxy groups and certain functional amino acid groups such as valine, praline and glycine were observed. The presence of N-acetyl amino sugars in the mosquitocidal metabolite conformed the protein as glycoprotein.

The mosquito-active protein crystals produced by *B. thuringiensis subsp. israelensis* contain covalently attached amino sugars which are critical for their larvicidal activity. The 50% lethal concentrations toward *Ae. aegypti* larvae were increased up to 10-fold by mild periodate treatment, up to 40-fold by forming the protein crystals in the presence of tunicamycin, and up to 7-fold by the presence during the mosquito bioassays of N-acetylglucosamine or its trimer, triacetylchitotriose. Periodate-treated crystals and crystals formed in the presence of tunicamycin had greatly reduced binding capacities for wheat germ agglutinin, a Acetylglucosamine-specific lectin. These results suggest that the *B. thuringiensis* subsp. israelensis glycoprotein toxin binds to a lectin like receptor in the larval mosquito gut. Furthermore, the distinct lectin-binding patterns exhibited by diptera active versus lepidoptera-active *B. thuringiensis* crystals suggest that host specificity for the microbial insecticides is determined, in part, by the carbohydrate portion

of their glycoprotein crystals [16] Pfannenstiel *et al*.(1987) [17] purified *B. thuringiensis subsp. israelensis* mosquito larvicidal protein crystals containing roughly 2.7% total carbohydrate, consisting of 1.0% neutral sugars and 1.7% amino sugars (70% glucosamine and 30% galactosamine). The presence of both glucosamine and galactosamine is in qualitative and quantitative agreement with the binding of the fluorescent lectins WGA and SBA by intact crystals. Tyrell, *et al*. (1981) [18] reported the presence of glucose, mannose, fucose, rhamnose, xylose, and galactosamine at a level several fold greater than in crystals from other subspecies of *B. thuringiensis*. As this group has previously reported to have 5.6% carbohydrate in crystals from *B. thuringiensis* subsp. kurstaki, the indicated carbohydrate level in *B. thuringiensis subsp. israelensis* crystals should have been 11.2%. Similarly, Insell *et al*. (1985) [19] reported that *B. thuringiensis subsp. israelensis* crystals contained 6% hexose by dry weight using the anthrone test for neutral sugars. The IR spectrum shows significant structural information of major constituents present in the mosquitocidal protein. Harris *et al*. (1999) [20] had reported amide I absorption occurs in the region 1600–1700 cm-1. Hydrogen bonding and the coupling between transition dipoles are amongst the most important factors governing conformational sensitivity of the amide bands. Amide I bands in the spectral range of 1620–1640 cmy1 with proteins, can be attributed to b-sheet structure. It has been reported that 'β-bands' can occur even below 1620 cmy1 for some proteins [21]. For the homopolypeptide poly-L-lysine, the β sheet band frequency is located at 1610 cm-1. More recently, model calculations by Torii *et al*. (1992) [22] for the protein myoglobin indicated that bands below 1640 cm-1 may also arise from vibrational motions of a-helical structures. In the present study, the strongest peak hydroxyl groups were detected at the scan range of 3416.26 cm-1. The stronger carbohydrates (C-O) were at ~ 1056 cm-1 and 1114 cm-1. The peak 1732 represented the presence of carbonyl compounds. The classical absorptions of N-acetyl amino sugars were observed at the frequencies of 1644.49 cm-1 and 1563 cm-1. The new peak at 1563.3 cm-1 assigned as amide I band (N-C=O) and amide II band of Glycoprotein (N=C-O) at 1644.49 cm-1 appeared very distinctly. The presence of methyl groups was seen clearly at the peak 1400 cm-1. The distinct auto peaks in synchronous spectrum 2859 cm-1, 2927 cm-1, 1463 cm-1 represented the aliphatic hydrocarbons. The trough seen in the scan range of 2000 cm-1 to 3000 cm-1 represented the presence of peptidoglycans. Pelton *et al*. (2000) [23] reported the amide bands I (80% CAO stretch, near 1650 cm21), II (60% N–H bend and 40% C–N stretch, near 1550 cm21), and III (40% C–N stretch, 30% N–H bend, near 1300 cm21) are generally employed to study protein structure. Krishnan *et al*. (1977) [24] reported the 1H NMR spectrum of acetylcholine peaks at 3.25 ppm, due to the-N(CH3)3 group and at 2.18 ppm due to the CH3-co-protons. Henry *et al*. (2007) [25] reported one dimensional NMR spectrum (1H NMR) of the mosquitocidal protein of *P. fluorescens* based on the chemical shifts. In this study, methyl groups appeared between 0.5-1 ppm, amino acid valine was distinctly seen in the region of 0.7-0.9 ppm and 5 ppm. The amino acid alanine peak was observed between 3.7-4 ppm. The peak observed at 1.5, 2.4, and 3.3 to 3.5 ppm represented the presence of N-acetyl amino sugars. The structural assignments for the peaks were obtained from 13 C NMR and the spectra were analyzed. The aliphatic hydrocarbons in 13C NMR analysis appeared between 0-55 ppm. The amines, amides and proteins (C-N) groups appeared between 40-55 ppm. Methoxy groups lie in the region between 55-60 ppm. The anomeric carbon of carbohydrate moieties was determined between 90-105 ppm.

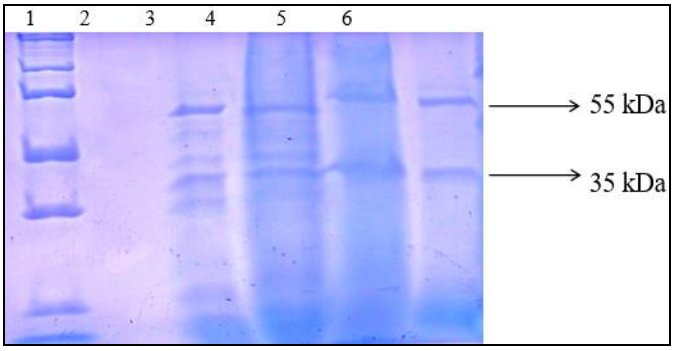

Fig 1: Autoclaved protein on 10% SDS PAGE L-1 protein marker, L-3 Control crude Protein, L-4 Autoclaved crude protein, L-5 Autoclaved pure protein, L-6 Control Pure protein.

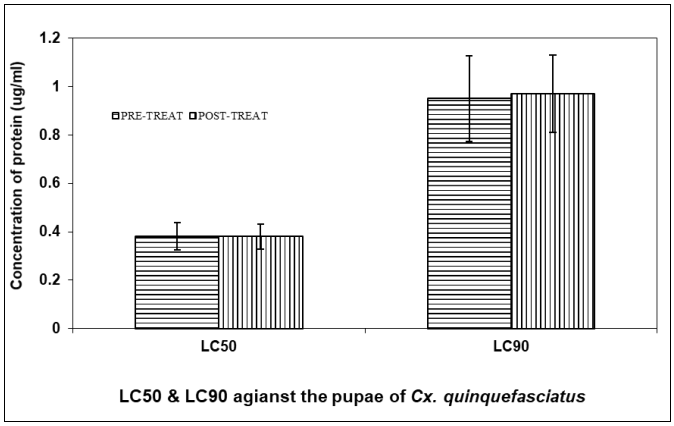

Fig 2a: Stability of bioactivity of crude mosquitocidal protein of *P. fluorescens* after Autoclaving to 121° C for 15 min

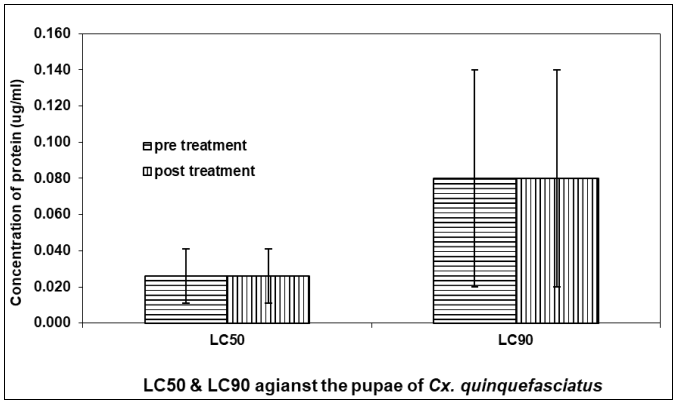

Fig 2b: Stability of bioactivity of pure mosquitocidal protein of *P. fluorescens* after Autoclaving to 121° C for 15 min

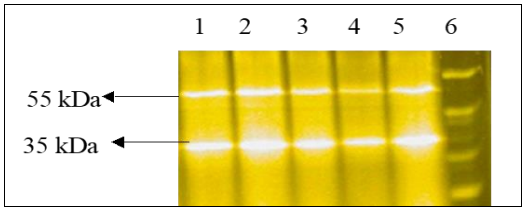

Fig 3: Glycoprotein detection through fluorescent method; 1-5: Pure protein of *P. fluorescens*, 6: Standard glycoprotein marker.

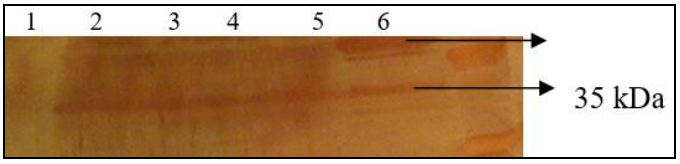

Fig 4: Glycoprotein detection through Western blot method 2-5: Pure protein of *P. fluorescens*, 6: Standard glycoprotein marker.

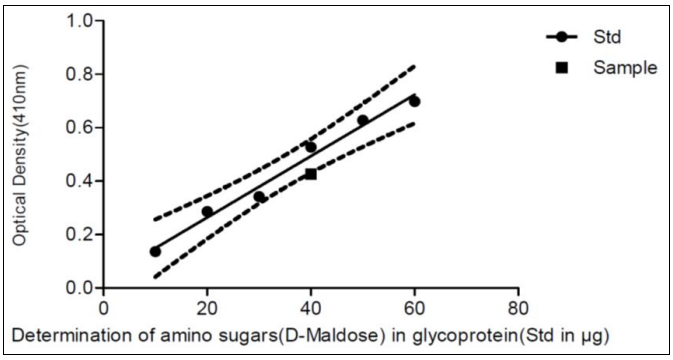

Fig 5: Determination of amino sugar (D-Maltose) in mosquitocidal protein

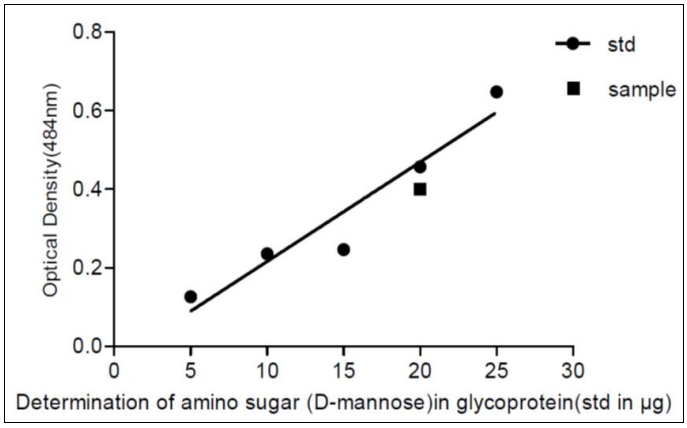

Fig 6: Determination of amino sugar (D-Mannose) in mosquitocidal protein

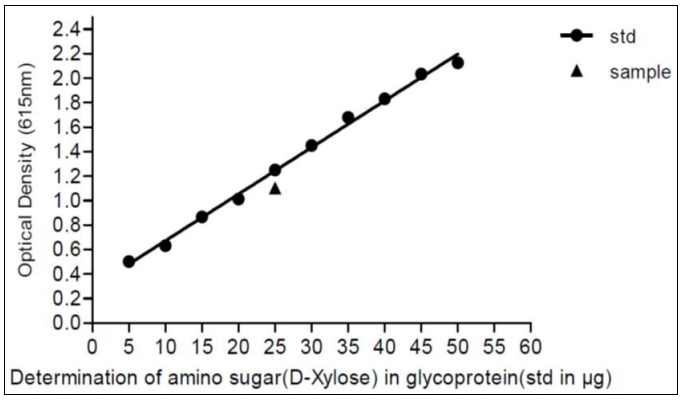

Fig 7: Determination of amino sugar (D-Xylose) in mosquitocidal protein

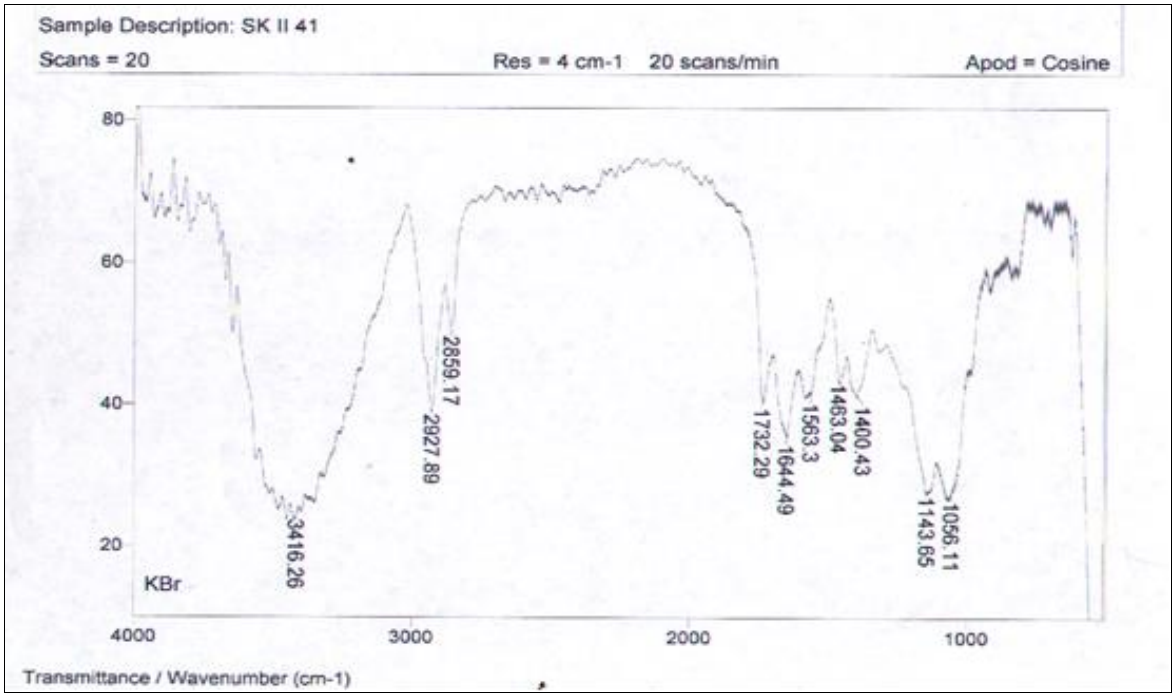

Fig 8

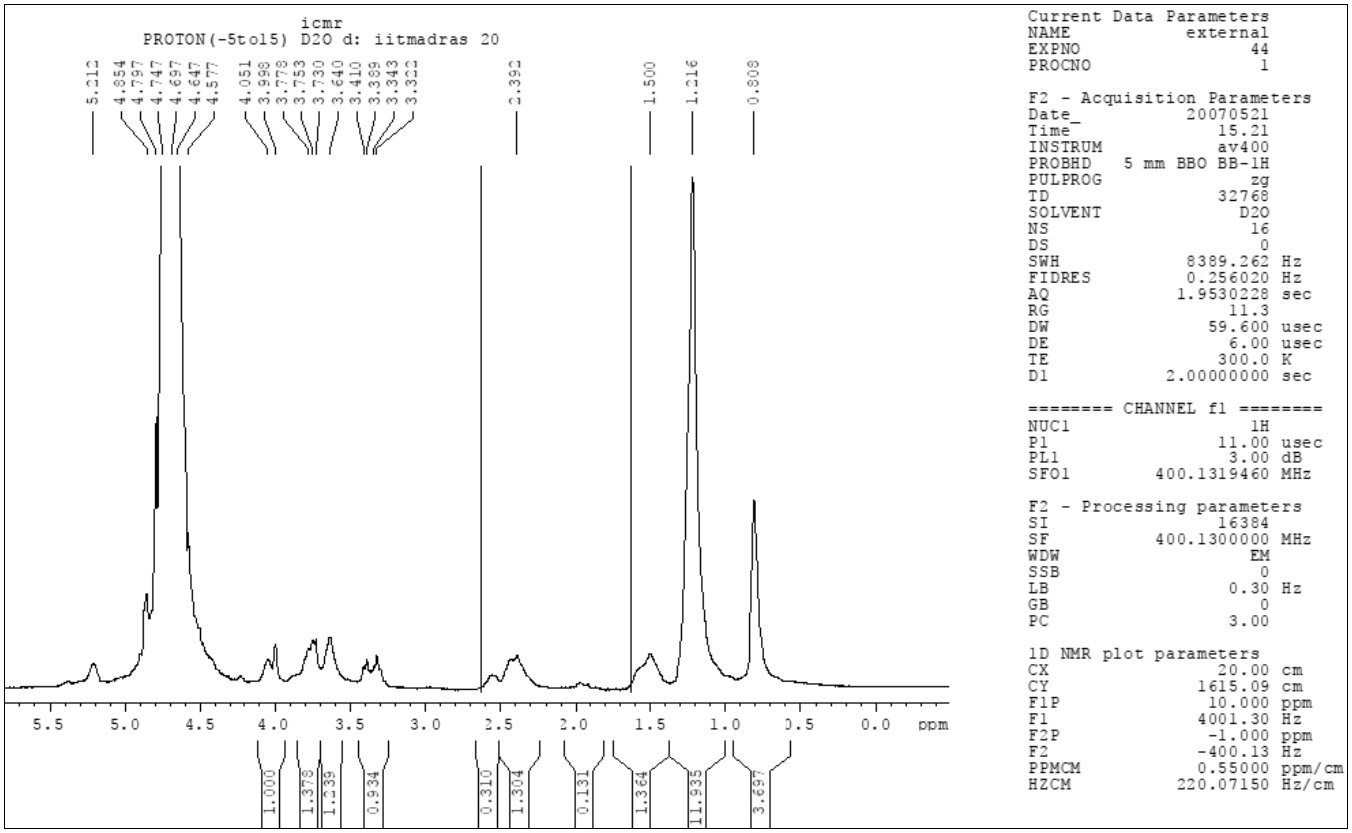

Fig 9a: IH NMR spectrum analysis of mosquitocidal protein

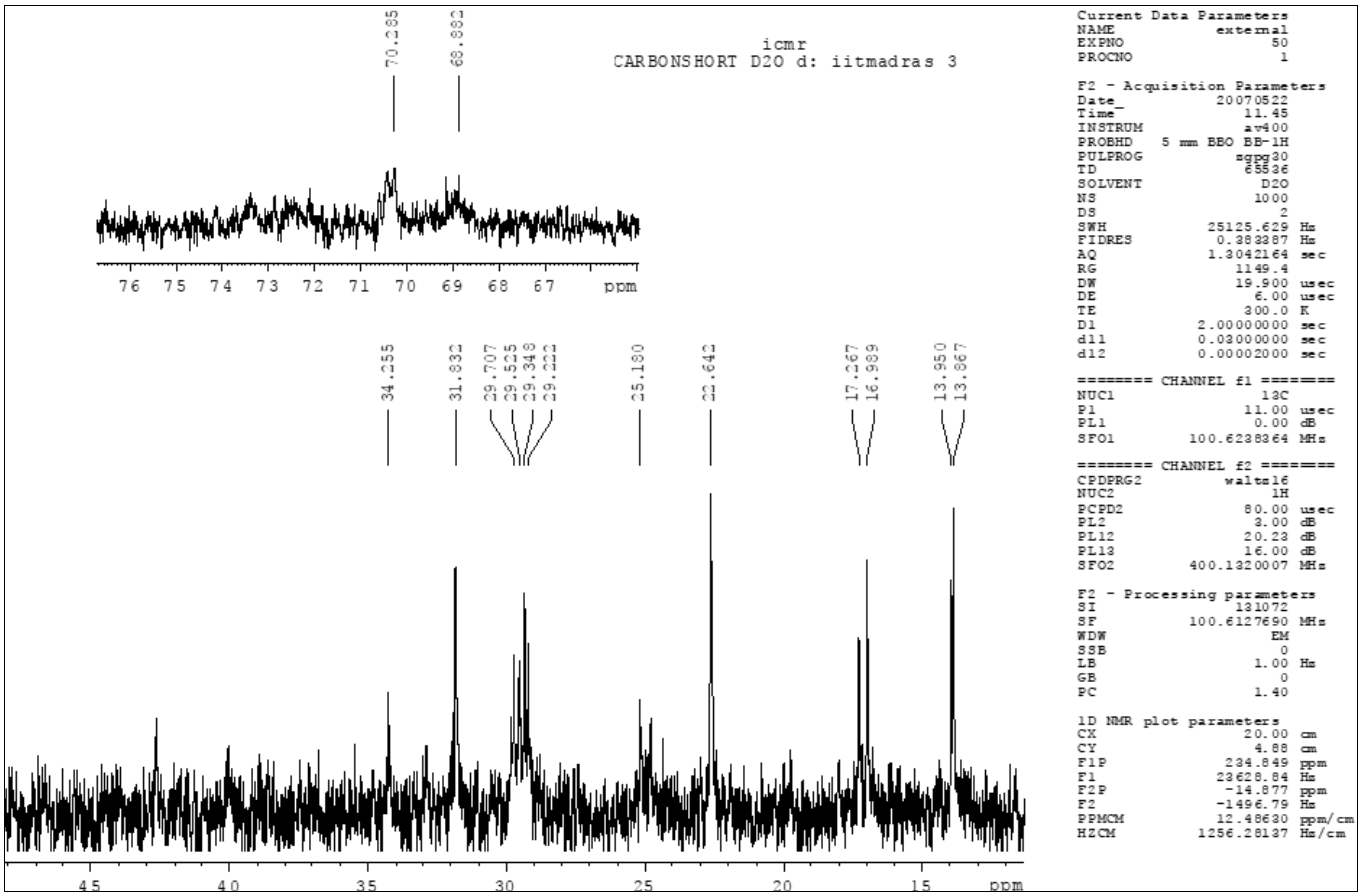

Fig 9b: 13C NMR Spectrum analysis of mosquitocidal protein

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Conflict of interest
The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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