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RESEARCH ARTICLE

STUDY OF TOXICITY OF BACILLUS THURINGIENSIS CRY PROTEIN ISOLATED FROM 1953 STRAIN OF BACILLUS THURINGIENSIS ON THE RAT HEPATIC AND RENAL TISSUES IN A 90 DAY FEED DESIGN

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ABSTRACT

Bacillus thuringiensis is a spore forming gram positive bacteria that have a number of different chemical compounds which are used for different types of insecticidal purposes. Though other various species of Bacillus are used for insecticidal purpose the effect on mammalian system has not been elaborated till now. In our study we observed some adverse effects of the cry toxins on the mammalian tissues i.e. specifically in the renal and hepatic tissues. The renal tissues and the hepatic tissues are the markers of toxin excretion and accumulation in physiological systems. Some results show no significant differences at all while some showed potent differences. Our study in brief reflects the different biochemical and histological changes that occurred by Bt Cry proteins in daily diet to the group of animals for observing the toxicological effects of the said insecticidal protein.]

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INTRODUCTION

Bt is an aerobic or anaerobic facultative and sporulatingbacterium. It can remain latent in the environment even in adverse conditions for its development. *Bt* can be found in soil, insects and their habitats, stored products, plants, forest, and aquatic environments. The insecticidal bacterium Bt is a Gram-positive bacterium that produces proteinaceous inclusions during sporulation [Apaydin *et al.,* 2004]. These inclusions can be distinguished as distinctively shaped crystals by phase-contrast microscopy. The inclusions are composed of proteins known as crystal proteins, Cry proteins, or δendotoxins, which are highly toxic to a wide variety of important agricultural and health-related insect pests as well as other invertebrates. Because of their high specificity, crystal proteins are a valuable alternative to chemical pesticides for control of insect pests in agriculture and forestry and in the home. It has been proposed that the rational use of Bt toxins will provide a variety of alternatives for insect control and for overcoming the problem of insect resistance to pesticides. Bt cotton refers to transgenic cotton which contains endotoxin protein inducing gene from soil bacterium Bacillus thuringiensis (Bernhard, 1997). It has been reported that in pigs and calves, Cry protein fragments are detectable but are progressively reduced in size as they travel down the GI tract. None were detected in the liver, spleen, or lymph nodes (Bradford, 1976) indicating they were too large to be systemically absorbed from the GI tract. It has been suggested that transgenic nucleic acids and proteins from GM crops are handled in the gut like their conventional counterparts, with no evidence for systemic absorption of intact proteins or genes (Cannon, 1996). Bt cry protein that has non-insecticidal effect which is the strong crytocidal activity against various cell with a markedly divergent target specificity to the normal hepatic tissue & in renal tissue. It has been reported to form liver & kidney cancer cells. Bt toxin can induce hepatotoxicity & renal toxicity. Hence in our work we coined the observations after the treatment of the protein in case of the toxicologicaly important organs i.e liver & kidney.

MATERIALS AND METHODS

Purification of the crystal proteins: After attaining the autolysis phase of the bacterium (Strain-1953, *Bacillus thuringiensis*) at 110 hour of incubation,the crystal proteins from all the strains were isolated after the specified time. It was then subjected to ammonium sulphate $[(NH_4)_2SO_4]$ precipitation followed by filtration through dialysis membranes

of various pore sizes for purification and concentration of the samples. Male albino rat models were selected for the study. 90 days feeding study was performed on them. With the crystal proteins of Bt 1953, the animals were sacrificed after the experiment and the tissue samples were collected for histopathological studies.

Procurement of materials:

- **Chemicals:** Analytical grade chemicals required for the preparation of various reagents and solutions were procured.
- **Media:** Media were required for the culture of the bacteria and for performing various biochemical tests.
- **Reagents and Solutions:** Reagents and different solutions were made to perfom different biochemical and staining experiments.
- **Test Organisms:** Bacillus thuringiensis

(Bt) Strains: Vegetative growth of the strains were obtained by adding them into sterilized nutrient broth media and incubating at 28°C for 24h.

Rat: Male albino rats were were bought which weighed 180-200gm and were 6-8 weeks old at the time of procurement. The rats were allowed to acclimatize to the housing conditions for one week. The rats were needed for histopathological and histological experiments.

METHODS

Bacterial culture : The Bt strain were cultured to produce vegetative cells by incubating for 24hrs at 28°C in sterilized Nutrient Broth and then were spread on Nutrient Agar plates to obtain the pure culture of the respective strains.

Isolation and purification of crystal proteins: The Bt strain was inoculated in 50 ml sterile conical flask containing 20ml Nutrient Broth for 110hrs to rach complete autolysis phase. Crude Protein was extracted by centrifugation at 10,000 rpm at 4°C for 15 minutes. Supernatant was discarded and the pellet was suspended in 2ml sterile distilled water. The suspension was crude protein obtained from the stran. The protein quantification has been done using Bradford method (1976). The rats were divided into two groups in which one group contained five treated rats where as other contained one control albino rat respectively. The 1953 strain treated rats were named as T1, T2, T3, T4 and T5 and the untreated was named as C or control.

Histological & Histochemical test: Histological & histochemical test of laboratory prepared tissue section is effective diagnosis of tissue abnormalities & cancerous condition.

Haematoxylin –Eosin stain: This protocol describes H&E staining of tissue and cell sections (13).

Bromophenol blue stain: It has recently come into general use of histochemical reagent for the recognition of protein in tissue.bromophenol blue is a acid phenopthelin dye, commonly used as a pH indicator. This method is employed for the localization of total proteins for the following advantages Mercuric bromophenol blue method (www.cicr.org.in).

Biochemical test:

Total protein: Protein can be estimated by different method as Lowry method.the blue color developed by the biurul reaction of the protein with the alkaline copper tartrate are measured in the Lowry method.

Billirubin test: The billirubin estimation in tissue is performed by Diazo method. The main principle is conversion of billirubin to the purple colored azobillirubin when coupled with diazolised sulphanilic acid. The water soluble billirubin reacts fast with the diazo reagent.

SGOT (serum glutamic oxaloacetic transaminase): Aminotransferares are a group of enzyme that catalyse the transfer of an amino group from an α amino acid.this specific enzymes are diagnosis significance & arise from different tissue like liver & kidney etc are reach sources of AST(Aspartate aminotransferase) (Zhang, 2017).

SGPT (Serum Glutamic Pyruvic Transaminase): Aminotransferares are a group of enzyme that catalyse the transfer of an amino group from an α amino acid.this specific enzymes are diagnosis significance & arise from different tissue like liver & kidney etc are reach sources of AST (Aspartate aminotransferase) (Zhang, 2017).

Catalase test:

Inhibition (%) = $(A_{\text{Blank control 1}} - A_{\text{Standard or Sample}})/(A_{\text{Blank control 1}} - A_{\text{Blank control 2}})$ x 100%.

The standards were used with known Catalase activities and their inhibitions to establish a standard curve. Then, the curve was used to calculate the Catalase activities of samples by their inhibitions (21).

Alkaline phosphatase test: Were done using the OD values observed from the test samples.

RESULTS

The diversity of B. thuringiensis strains facilitates isolation of new types of cry and vip which is known as vegetative insecticidal protein genes (15). For better pest control, the cry genes have been transferred to plants. Stacking of more than one insecticidal gene is required for resistance management in transgenic crops.

Modification of these proteins through protein engineering for increasing the toxicity and the insecticidal spectrum is also a promising approach, but requires detailed understanding of the structure and function of these proteins (17).

Total Protein content of Liver & Kidney: The total protein content in case of liver has increased significantly. After feeding the protein for 90 days the transverse section of rat's liver showed some of the morphological differences of the cells of the liver. The total protein content increased about ±30.211 from the control group respectively (Fig 1). Elevated total protein may indicate, inflammation or infections, such as viral hepatitis B or C, bone marrow disorders, such as multiple myeloma.

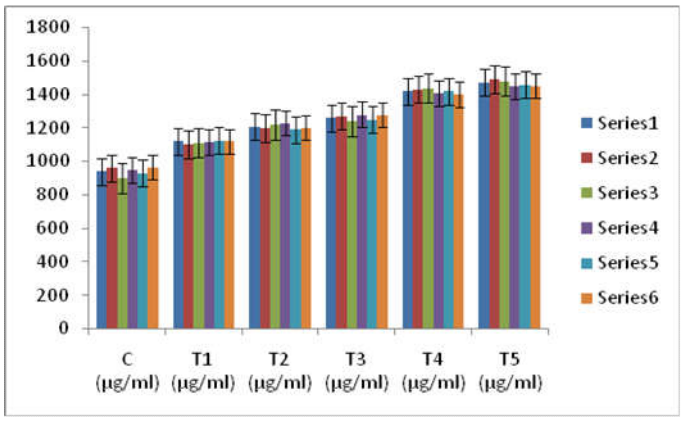

Fig 1. Graphical representation of changes of total protein in liver

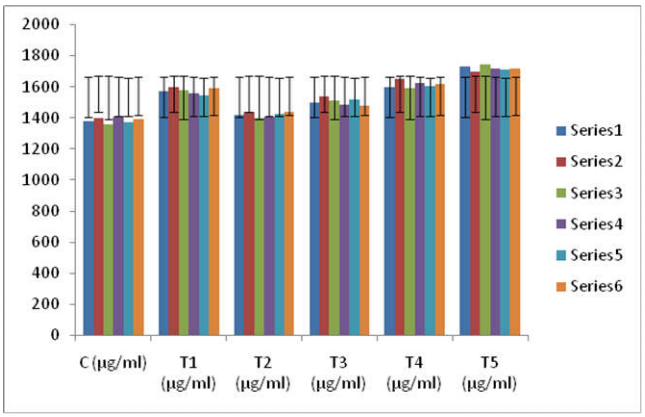

Fig 2. Graphical representation of changes of total protein in kidney

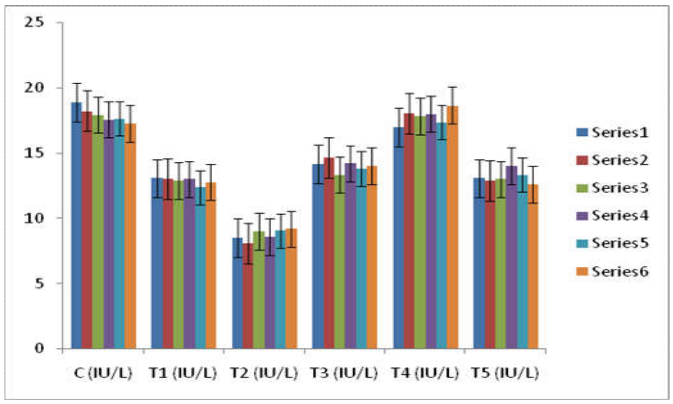

Fig. 3. Graphical representation of changes of SGOT in liver

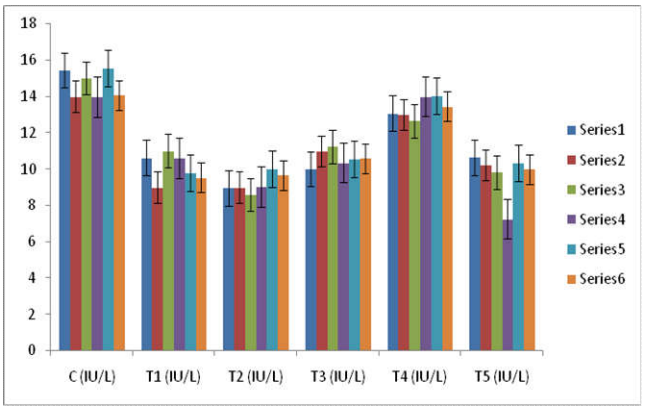

Fig. 4. Graphical representation of changes of SGOT in kidney

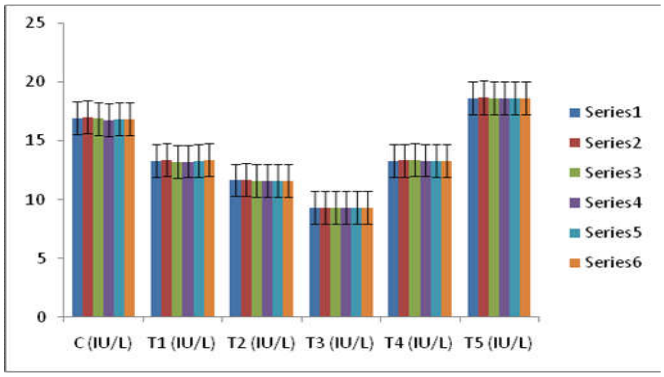

Fig. 5. Graphical representation of changes of SGPT in liver

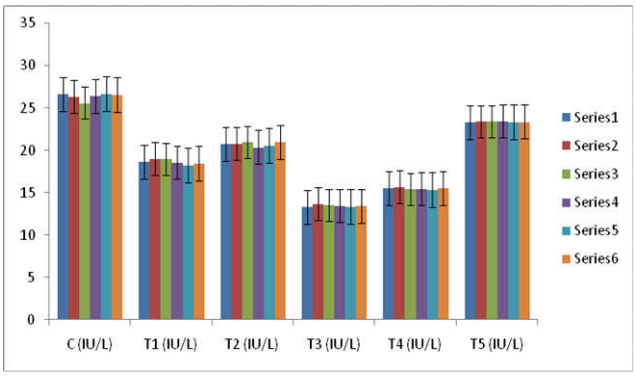

Fig. 6. Graphical representation of changes of SGPT in kidney

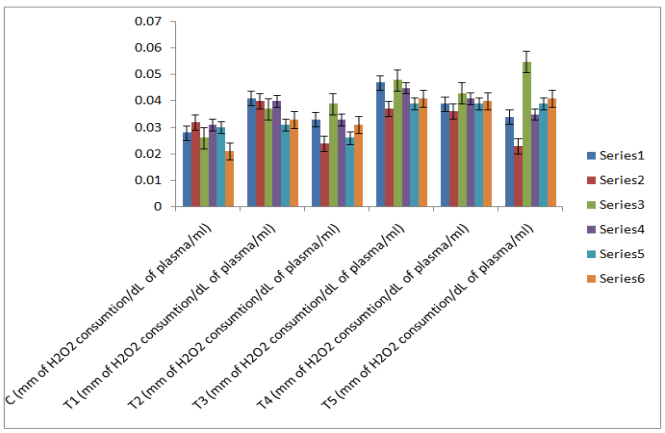

Fig 7. Graphical representation of changes of catalase activity in liver

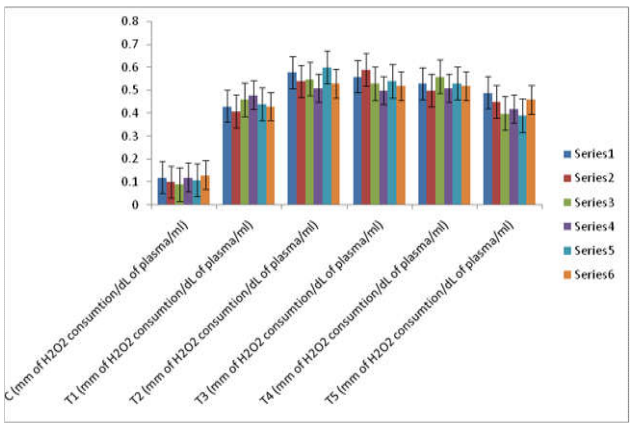

Fig 8. Graphical representation of changes of catalase activity in kidney

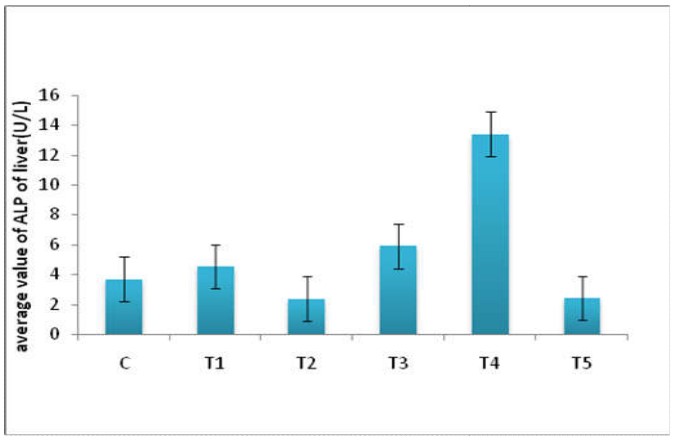

Fig 9: Graphical representation of changes alkaline phosphatise in liver

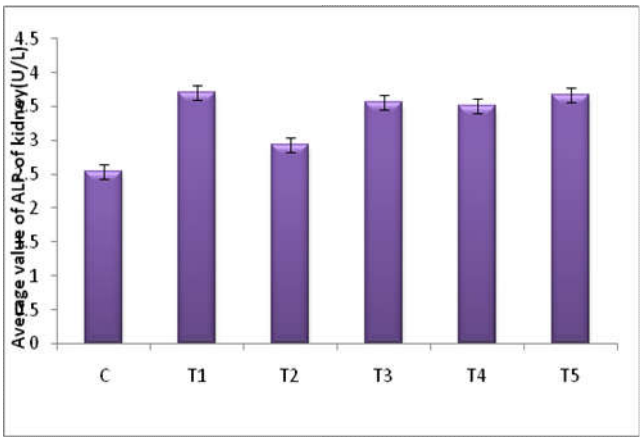

Fig 10. Graphical representation of changes of alkaline phosphatise level in kidney

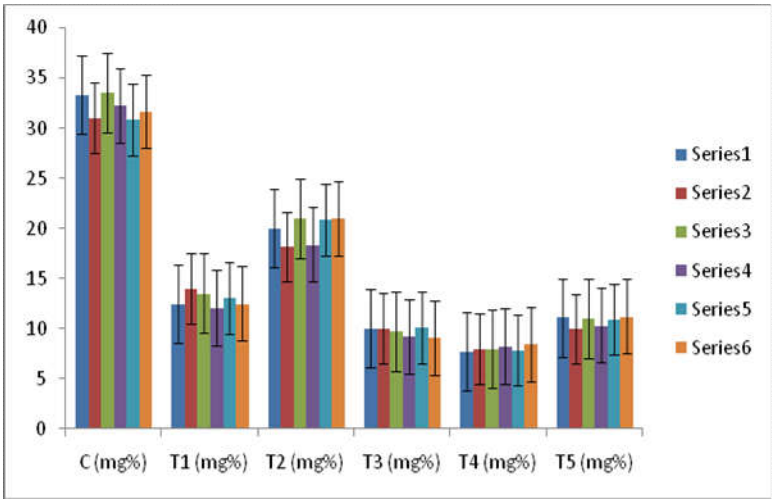

Fig 11. Graphical representation of changes bilirubinlevel in liver

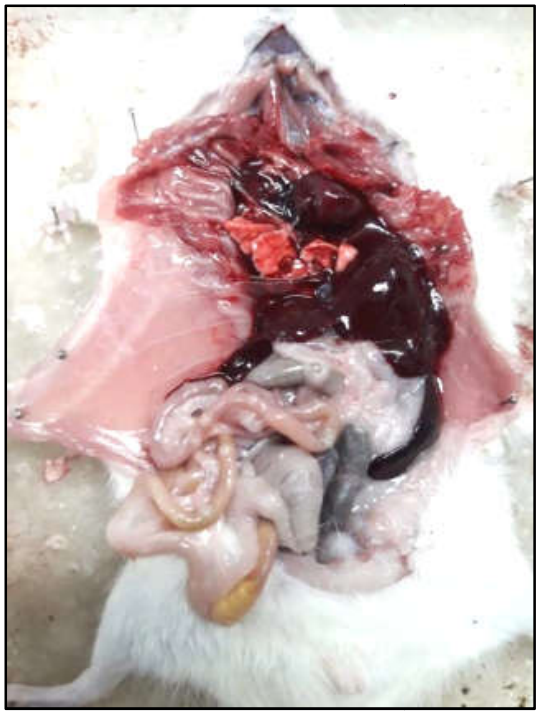

Fig12:Macrophotography of sacrificed rat after 3months feeding of bt cry protein

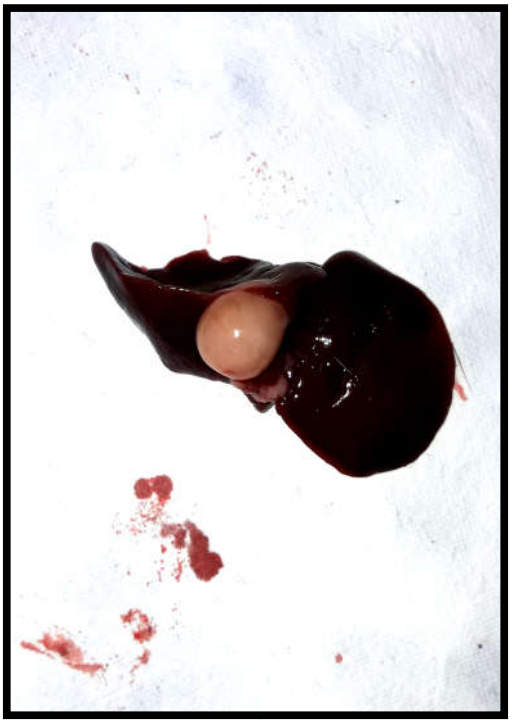

Fig. 13 : Observation of cystic appearances in liver tissue after sacrifie of the treated rats.

The total protein level differed about ±19.59843, which differs significantly from the control animals (Fig :2).

SGOT level of liver and kidney: Extremely high levels of AST indicate conditions that cause severe liver damage including the decrease in enzyme levels. The SGOT level differed about ±1.409016 from the control respectively (Fig 3). Extreme high levels of SGOT in kidney may degrade the renal reabsorptions in DCT and PCT. The SGOT level of kidney differed about ±0.361185 from the control animals respectively (Fig 4).

SGPT level of liver & kidney: Extremely high levels of AST indicate conditions that cause severe liver damage including the decrease in enzyme levels. The SGPT level differed about ±0.527127 from the control respectively (Fig :5). High or low levels of SGPT or AST denotes less kidney functioning from the normal. In this context the SGPT level of kidney differed about ±0.746713 from the control animals respectively (Fig 6).

Catalse activity of liver & kidney: The catalase activity of the liver tissues showed drastic difference. As from the diagram it can be seen the protein exerted crucial effects on the reactive oxygen and nitrogen species generation.

The catalase level differed about ±0.025944 from the control respectively (Fig 7). Renal antioxidant enzymes can be down-regulated, as well, by certain biological stimuli. Renal antioxidant enzymes undergo postnatal development. Oberley et al demonstrated immunohistochemical localization of catalase in the developing hamster kidney [6]. Here in this context, the catalase activity differed about ±0.001232 from the control animals (Fig 8).

Alkaline phosphatise levels in liver & kidney: The highest concentrations of ALP are present in the cells that comprise bone and the liver. Elevated levels of ALP in the blood are most commonly caused by liver disease or bone disorders. The ALP level differed about ±1.43398, which differs significantly from the control animals. Abnormal levels of ALP in kidney indicate malnutrition, kidney cancer tumors, intestinal issues, a pancreas problem, or a serious infection. Here the level in ALP differed about ±0.10623 from the control animals respectively.

6. Bilirubin content of liver: Adults with jaundice generally have bilirubin levels greater than 2.5 milligrams per deciliter (mg/dL). In an otherwise healthy newborn, bilirubin levels greater than 20 to 25 mg/dL may cause problems.

Representing Histological & Histochemical sections of Liver tissues

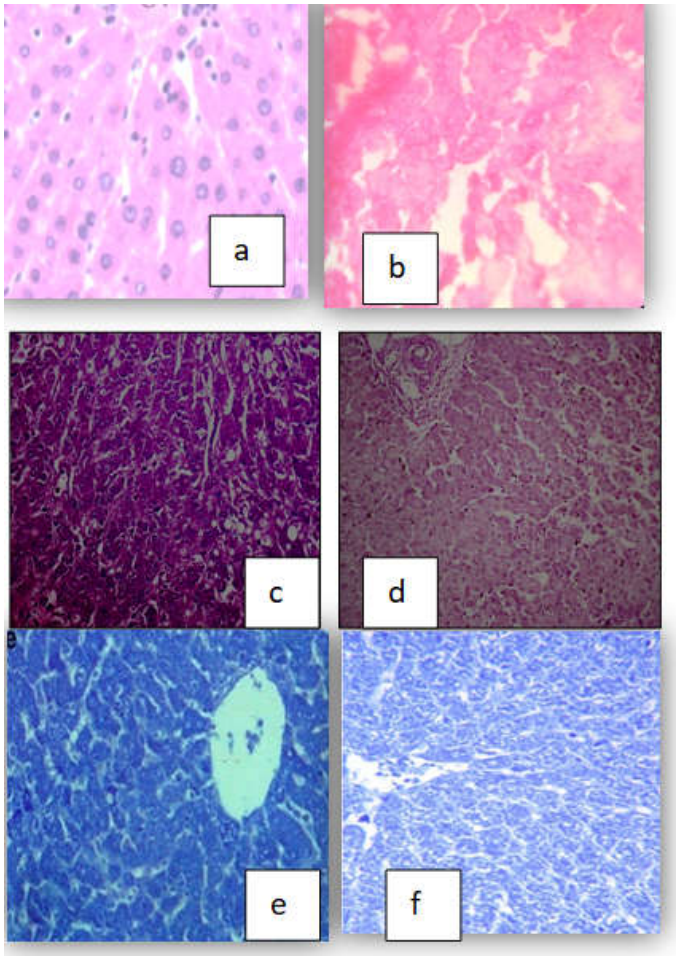

Fig 14. Representing the hematoxyline eosin(a,b) , PAS(c,d) , Bromophenol(e,f) stained histological & histochemicall slides of normal and treated albino rats .“a” , “c” , “e” denotes the normal histology and histochemistry of liver from the control rat whereas “b” denotes the treated rat’s liver histology which shows degenerated cell morphologies of the respective hepatocytes, whereas “d” and “f” denotes the diffrenece in protein and glycogen content respectively of the treated rats

Representing Histological & Histochemical sections of Kidney tissues

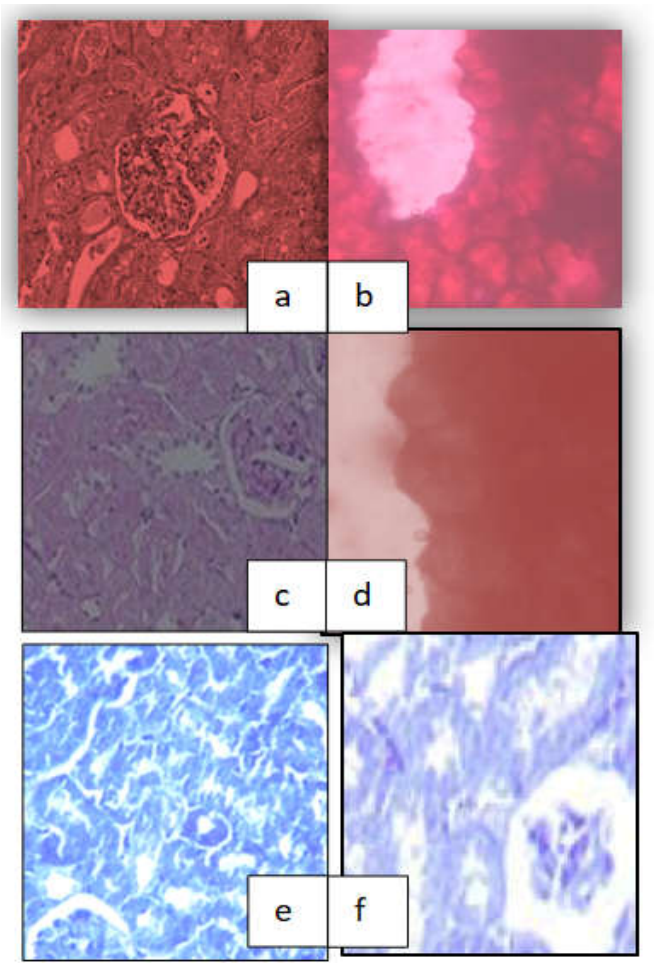

Fig 15. Representing the hematoxyline eosin(a,b) , PAS(c,d) , Bromophenol(e,f) stained histological & histochemicall slides of normal and treated albino rats .“a” , “c”,”e” shows the normal histology and histochemistry of kidney of the control rat whereas “b” denotes the treated rat’s kidney histology which shows degenerated cell morphologies.

The Bilirubin level differed about ±1.409016, which differs significantly from the control animals.

DISCUSSION

In the histological study the transverse section of both the organs liver and kidney were observed. In case of hematoxylene eosin staining of liver the sections containing cysts were observed under 400X(40*10) magnification. The liver hepatocyte cells observed after the treatment of Bt protein in the H/E staining showed some structural deformities. The kidney sections observed under microscope for Bromophenol blue and PAS staining didn't show any significant difference.

Total protein content was estimated from tissue homogenate mainly for liver & kidney, isolated from control & Bt cry protein treated rat by lowry method, and it was observed increased level of total protein in liver & kidney tissue in Bt fed rat as compared to the control rat. In this study elevated levels of protein content in case liver tissue observed in treated animal as compared to control rat & showing the P value is less than 0.05($P<0.05$),indicating there was a significant difference. Further we measured the amount of bilirubin is formed in case of liver tissue. The breakdown of old red blood (RBCs) in the body produces bilirubin, which thus travels to the liver is stored in the bile duct.RBCs have a lifespan of around 120 days & renew continuouslly. In my case Bt cry protein treated rats showing gradual decrease in bilirubin content in case of liver compaired to control group & it gives the P value less than the 0.05,thus it can be concluded that there was a significant difference affecting the normal functioning of liver. The fluctuation observed in values of treated animal may be due to variation of waste product excretion by treated animals.

Poulsen *et al.* observed that the level of SGOT & SGPT is decreased in case Bt cry protein treated rat compared to the control,so they concluded that Bt cry protein does effect SGOT & SGPT level in case of renal & hepatic tissue. In this study a decreased level of SGOT/AST & SGPT/ALT levels in case of treated rats both liver & kidney represents Bt cry protein does affect the hepatic & renal tissue but causes less damage to it.

It was observed that ALP& catalase level in liver does not increase significantly. In my study the statistical analysis of catalase, for both the hepatic & renal tissue, showed the P value less then 0.05.Thus there is a significant difference between the control & treated rats. This catalase activity increased to protect hepatic & renal cells from oxidative stress induced by Bt cry protein ingestion for consecutive 90 days. Thus it can be concluded that Bt cry protein shows some detrimental effects in term of oxidative stress, where as enzyme (ALP) level in hepatic tissue showed insignificant effects.

Conclusion

It can be concluded that, total protein content level increased significantly both in hepatic and renal tissue in Bt cry protein treated rat. But Bt cry protein diet does not affect bilirubin content of hepatic tissue. In addition to this, it was observed that the SGOT & SGPT levels are decreased significantly for both renal and hepatic tissues, and the catalase activity increased significantly showing no damage to the tissues thus protecting the cells from oxidative stress induced by Bt toxin. The ALP level gradually increases in case of renal tissue whereas the ALP levels does not show any significant result for hepatic tissue. Hence it can be said that the Bt Cry proteins treated here showed some detrimental effects and further work is required to establish its negative effects.

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