



## Effects of Crystal Derived from *Stevia rebaudiana* Leaves on Alloxan Induced Type-1 Diabetic Mice

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### Authors' contributions

This work was carried out in collaboration between all authors. Authors SRD and KR designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript.

Authors ASMEI, MKHB and PH managed the analyses of the study. Author UH managed the literature searches. All authors read and approved the final manuscript.

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### ABSTRACT

The study was undertaken to investigate the antidiabetic effects of crystals derived from *Stevia rebaudiana* leaves in normal rats and alloxan induced diabetic mice. Normal rats were used to evaluate whether the stevia crystal have any adverse effects on body weight and blood glucose of healthy rats. The rats were divided into two groups (n=5); healthy control rats and stevia treated healthy rats (1 g/kg/day orally). To evaluate the antidiabetic effects of stevia crystal, mice were divided into five equal (n=5) groups; healthy control, diabetic control, stevia crystal @ 250 and 500 mg/kg bwt, and Amaryl<sup>®</sup> @ 800 µg/kg daily to compare the efficacy. Alloxan monohydrate was injected in mice at a dose rate of 120 mg/kg intraperitoneally for diabetic induction. Result of the present study showed that stevia crystal at a high daily dose did not have any significant effect on body weight but it reduced blood glucose level non-significantly in healthy animal. The antidiabetic effects of stevia crystal was evaluated based on glucose lowering capacity, improvement of body weight loss, changes in lipid profile, and renal and pancreas protective capacity. Treatment with stevia crystal @500 mg/kg improved body weight loss and reduced blood glucose level significantly

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( $P < 0.01$ ) in diabetic animal. Stevia @500 mg/kg decreased cholesterol level non-significantly whereas it reduce the triglyceride level significantly ( $P < 0.01$ ). In histopathology study, stevia crystal showed renal and pancreas protective effect with slightly restoration of structural damage in both organs. Based on present studies data it may conclude that crystalline compounds derived from *Stevia rebaudiana* leaves may have antidiabetic properties that need further characterization.

**Keywords:** *Stevia crystal; diabetic nephropathy; pancreatic injury; diabetic mice.*

## 1. INTRODUCTION

Diabetes is generally acknowledged to be due to insulin deficiency (Type I) which results from pathologic changes in pancreatic  $\beta$ -cells, or due to insulin insensitivity (Type II). Elevation of blood glucose level and increased risk of vital organs malfunction are common symptoms in diabetic patients [1]. As the number of people with diabetes multiplies worldwide, the disease takes an ever-increasing proportion of national and international health care budgets. According to World Health Organization, the diabetic population is likely to increase to 300 million or more by the year 2025 [2]. As the prevalence of this disease increases, there is a need to look for more efficient drugs with fewer side effects. However, currently available drugs may lead to obesity and hyperandrogenemia while reducing blood glucose. Traditional medicinal plants are used throughout the world for the treatment of Diabetes mellitus, because the plants are considered to be less toxic, low cost and free from side effects than the synthetic medicines [3]. Regarding its treatment, a suitable drug is yet not to be available which can permanently cure this disease. Since the discovery of insulin in 1922, it has been used successfully in insulin-dependent diabetes mellitus (IDDM). But it cannot be given orally, daily intake through injection is obviously troublesome and hypoglycemic reactions as an adverse effect may occur in any diabetic patient treated with insulin. Again insulin resistance, a state of relative tissue insensitivity to the action of insulin, is another drawback for patients taking insulin for a long period [4]. On the other hand, oral hypoglycemic agents such as glimepiride, glibenclamide etc also have some adverse effects such as vomiting, epigastric discomfort, jaundice, headache etc. [5].

*Stevia rebaudiana* is a small perennial shrub that belongs to the family aster or chrysanthemum family. It grows primarily in the Amambay mountain range of Paraguay [6]. The constituents responsible for stevia's sweetness were documented in 1931, when eight novel plant chemicals called glycosides were

discovered. Of these eight glycosides, one called stevioside is considered the sweetest one and it has been tested to be approximately 300 times sweeter than sugar [7]. The leaves of the stevia shrub contain specific substances (glycosides), which produce a sweet taste but have no caloric value, apart from protein, fibers, carbohydrates, phosphorus, iron, calcium, potassium, sodium, magnesium, rutin (flavonoid), zinc, vitamin C and vitamin A [8]. The dry extract from the leaves of *S. rebaudiana* also contains flavonoids, alkaloids, water-soluble chlorophylls and xanthophylls, hydroxycinnamic acids, neutral water-soluble oligosaccharides, free sugars, amino acids, lipids and essential oils. Stevia sweetener extractives have been suggested to exert beneficial effects on human health [9].

So far there has been no study reported on the antidiabetic activities of the crystalline compound derived from *S. rebaudiana* leaves available in Bangladesh. Therefore, the present study was undertaken to explore the antidiabetic activities of *S. rebaudiana* in relation to its folklore medicinal properties with special attention on diabetic nephropathy and pancreatic cell injury.

## 2. MATERIALS AND METHODS

The proposed experiment was conducted in the Department of Pharmacology, Bangladesh Agricultural University (BAU), Mymensingh. Experimental protocols and animal care were performed according to the guidelines for the care and use of animals established by Bangladesh Agricultural University. The experiments were approved by the Animal Experimentation Ethics Committee at Faculty of Veterinary Science, Bangladesh Agricultural University.

### 2.1 Animal

The experiment was carried out on 10 male Long Evans (*Ratus norvegicus*) rats and 25 male swiss albino (*Mus musculus*) mice. The rats and mice were collected from International Centre for Diarrhoeal Diseases Research and

Rehabilitation, Bangladesh (ICDDR,B). During the experimental period, the animals were fed standard rat pellet food and tap water *ad libitum*. The animals were maintained in this condition for a period of one week to acclimatize them prior to experimental use.

## 2.2 Plant Material

Stevia plants were grown in the field of Physics Department, Bangladesh Agricultural University, Mymensingh. Stevia leaves were collected from the field. Stevia crystal preparation was done in the Department of Physics, Bangladesh Agricultural University, Mymensingh as follows.

Stevia leaves were sun-dried and thereafter they were dried in an oven at below 70°C. Dried leaves were grinded to get fine natural colored leaf-powder. The dried powders were mixed with hot water (about 65°C) for water extraction. The crude extract containing stevioside was filtered by Whatman No. 3. This filtrate was purified by addition of 5% Ca(OH)<sub>2</sub>. The filtrates were collected passing through ion exchange column (packed with Amberlite IR-4B Resin) to remove the undesirable color at rate of 1 mL/sec at 25°C. The clear and colorless solution containing stevioside was collected. Stevioside containing sample were then heated at 45-50°C to get light yellowish powder. Then methanol was added to ground leaves powder at ratio 4:1 (v/w) and remained for 7 hours, then filtered through Whatman No.3 filter paper. The filtrate containing solvent was evaporated to dryness by using rotary evaporator at 45°C. The residue was washed with ether and extracted with butanol (three times). The organic phase was evaporated and the residue was recrystallized at 4°C overnight and purity during extraction and purification steps were determined by determination of pigments.

## 2.3 Induction of Diabetes

Alloxan monohydrate (SIGMA-ALDRICH Company, UK) was dissolved in 0.9% v/v cold normal saline solution. To induce diabetic condition in mice a dose of 120 mg/kg body weight were injected intraperitoneal as done previously. Since alloxan is capable of producing fatal hypoglycemia as a result of massive pancreatic insulin release, the mice were then kept for the next 24 hours on 5% glucose solution to prevent hypoglycemia. The diabetes was assessed in alloxan induced mice by determining the blood glucose concentration 72

hours after injection of alloxan monohydrate. Blood samples were collected for blood glucose measurements at alternate weeks.

## 2.4 Experimental Design

**Study-1:** Normal rats were used to evaluate whether the stevia crystal have any adverse effects on body weight and blood glucose of healthy rats. The rats were divided into two groups; healthy control rats and stevia treated healthy rats (1 g/kg/day orally).

**Study-2:** To evaluate the antidiabetic effects of stevia crystal, mice were divided into five equal groups (n=5 in each group) and treated as follows: group-A as healthy control. After 2 weeks of Alloxan injection diabetic mice were divided into four groups (groups B, C, D, and E). Group-B; diabetic control. Group-C; Diabetic + Amaryl<sup>®</sup> at a dose rate of 800 µg/kg, Group-D; Diabetic + stevia crystal at a dose rate of 250 mg/kg orally, Group-E; Diabetic + stevia crystal at a dose rate of 500 mg/kg orally. The drugs were administered orally once a day. During the second phase of the experiment we use mice instead of rat due to unavailability of animals.

## 2.5 Sample Collection

At the end of the treatment, food was withdrawn from the mice and they were fasted overnight but the animals had free access to water. They were then anesthetized under sodium pentobarbital (65 mg/kg, i.p.) anesthesia and sacrificed to collect blood samples, kidney and pancreas for further analysis. The abdominal cavity and thoracic cavity were opened surgically and then blood was collected directly from the heart with the sterile syringe and needle. About 1mL of blood from the syringe was taken in the test tube containing anticoagulant (3.8% Na citrate solution) for hematological studies. Plasma was then separated by centrifugation at 4500 rpm for 10 minutes and finally stored at -20°C temperature for further analysis. After perfusion with normal chill saline kidney and pancreas were surgically removed and were immediately blotted using filter paper to remove traces of blood and then weighed with an analytical balance. Thereafter, the tissues were suspended in 10% formal saline for fixation preparatory to histological processing.

## 2.6 Histopathology

For histopathology, the kidney tissues were immediately transferred to 10% formal saline for

paraffin embedding and staining with Hematoxylin and Eosin (H&E). Tissue samples from the pancreas were fixed in 10% buffered neutral formalin, embedded in paraffin, sectioned at 5 µm and stained with H&E. Haematoxylin and Eosin stains were used to stain the pancreas to demonstrate the pancreatic islets. Photomicrographs were obtained using a microscope eye piece attached to a computer monitor and observations made.

### 2.7 Statistical Analysis

All data were expressed as Mean±SEM. Differences among the groups of study-1 were compared using Student's t-test. In study-2 comparison were done by using one-way ANOVA with post-hoc Bonferroni test. Statistical significance was set at  $P < 0.05$ . Statistical analysis was performed using SPSS software version 20 (SPSS Inc., Chicago, IL, USA).

## 3. RESULTS

### 3.1 Effect of Stevia Crystal on Healthy Rat

At post treatment day 0 and 7, average body weights of healthy control rats were 152.2±2.78 g and 149±1.87 g respectively and healthy rats treated with stevia crystal showed 142.4±9.96 g and 139.2±9.53 g, respectively. This data indicates that stevia crystal at a high daily dose for 7 days did not have any significant effects on body weight of normal healthy rats (Fig. 1a).

Average blood glucose of healthy rats were 4.88±0.29 mmol/L and 5.14±0.27 mmol/L from the beginning to end and stevia treated healthy rats showed 5.08±0.15 mmol/L and 4.00±0.07 mmol/L at day 0 and 7, respectively. Suggesting that stevia crystal non-significantly lowers the blood glucose level in normal healthy rats even at a high daily dose for seven days (Fig. 1b).

### 3.2 Effect of Stevia Crystal in Alloxan Induced Diabetic Mice

From the beginning to end, the average body weight of normal healthy control and diabetic control mice were 29.67±1.60 g to 46.17±1.14 g and 29.33±1.80 g to 31.33±1.49 g, respectively. At week 0 and week 8 of treatment, Amaryl® treated group showed 29.67±1.43 g and 34.33±1.31 g body weight, respectively. On the other hand, treatment with stevia crystal 250 mg/kg and 500 mg/kg showed 29.5±1.47 g to 31.17±1.22 g and 29.5±2.22 g to 32.67±1.11 g body weight, respectively (Fig. 2).

Average fasting blood glucose level of normal healthy control and diabetic control mice was 7.88±0.10, 7.7±0.09 mmol/L and 13.83±1.17, 16.45±0.85 mmol/L on week 2 and 8 respectively. The blood glucose level of Amaryl® treated group was 13.93±0.82 mmol/L and 10±0.47 mmol/L. Treatment with stevia crystal 250 mg/kg and 500 mg/kg showed 13.93±0.79 mmol/L, 14.2±0.55 mmol/L and 14.00±0.76 mmol/L, 11.98±0.74 mmol/L, respectively (Fig. 3).

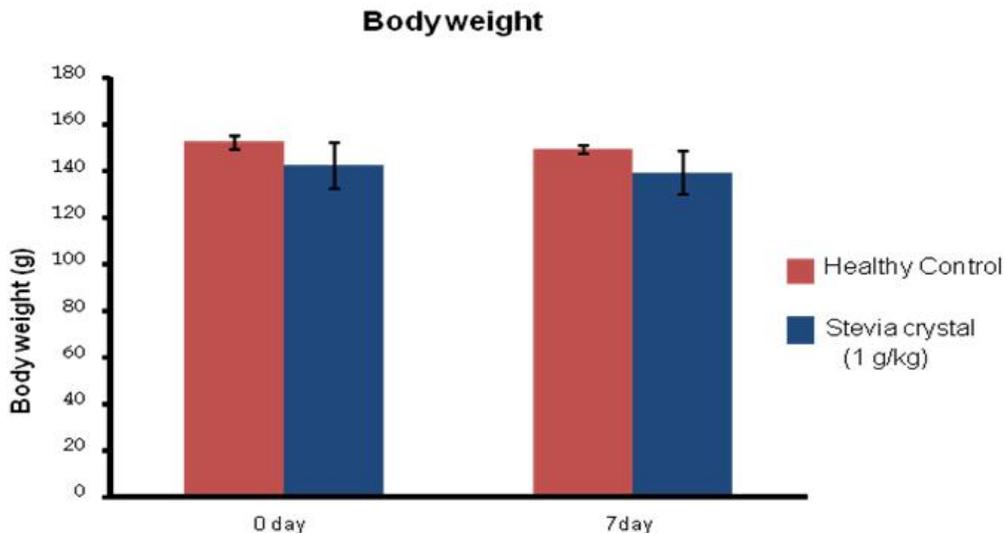
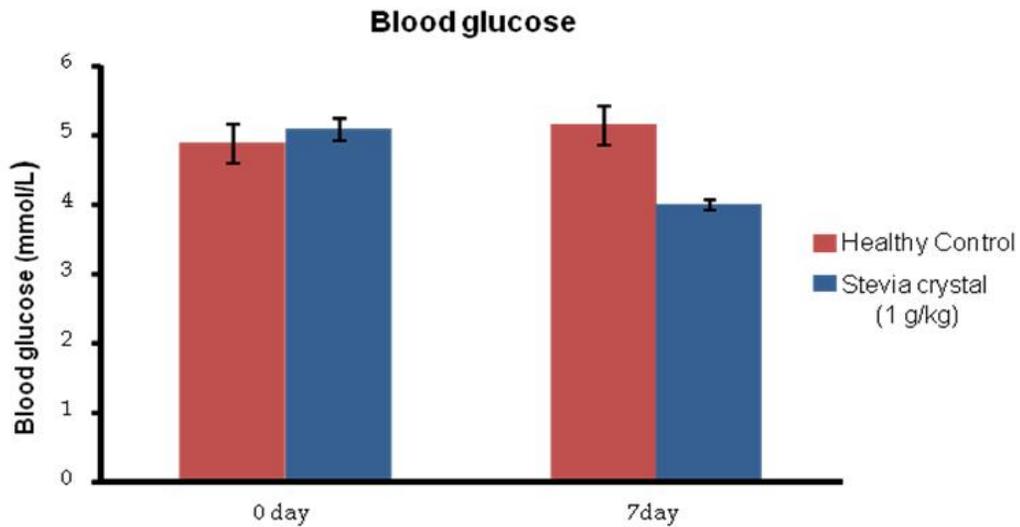
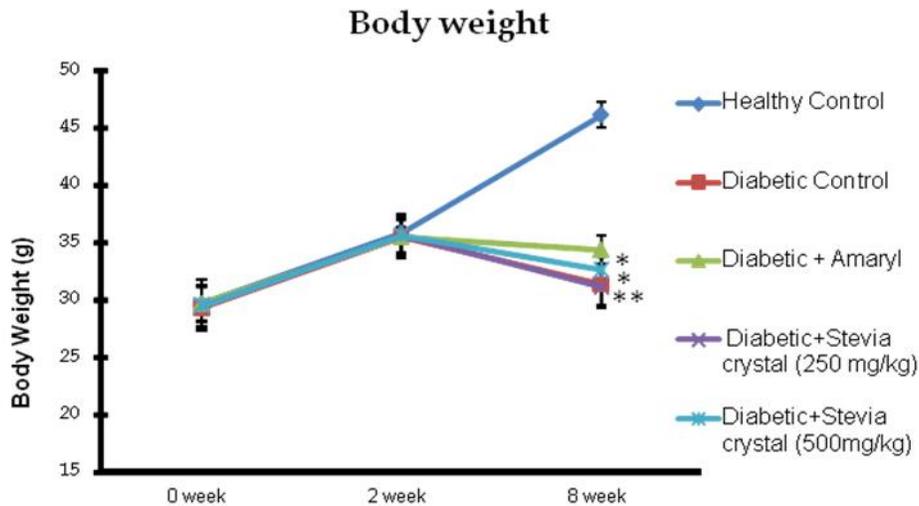


Fig. 1a. Effect of stevia crystal on body weight of healthy rat. Stevia crystal no significant effects on body weight of health rats



**Fig. 1b. Effect of stevia crystal on whole blood glucose level of healthy rat. Stevia crystal non significantly lowered blood glucose level in health rats**



**Fig. 2. Effect of stevia crystal on body weight in alloxan induced diabetic mice. Alloxan induced body weight lowering effects was prevented by stevia crystal @ 500 mg/kg/day**  
\* $P < 0.05$ , \*\* $P < 0.01$

At the end of the experiment, total cholesterol level was raised ( $122.62 \pm 2.70$ ) significantly ( $P < 0.01$ ) in diabetic mice compared to control group ( $100.03 \pm 0.52$ ). There was no significant changes in total cholesterol values after treating them with Amaryl® or Stevia crystal (500 mg/kg) but stevia treatment prevent the further increase in cholesterol level in alloxan induced diabetic animal.

After treatment with stevia crystal @500 mg/kg bwt, the triglyceride values were slightly lowered

the value compared to Amaryl® treated group ( $168.00 \pm 6.38$ ) which was not statistically significant. Stevia treatment also decreased the plasma creatinine levels.

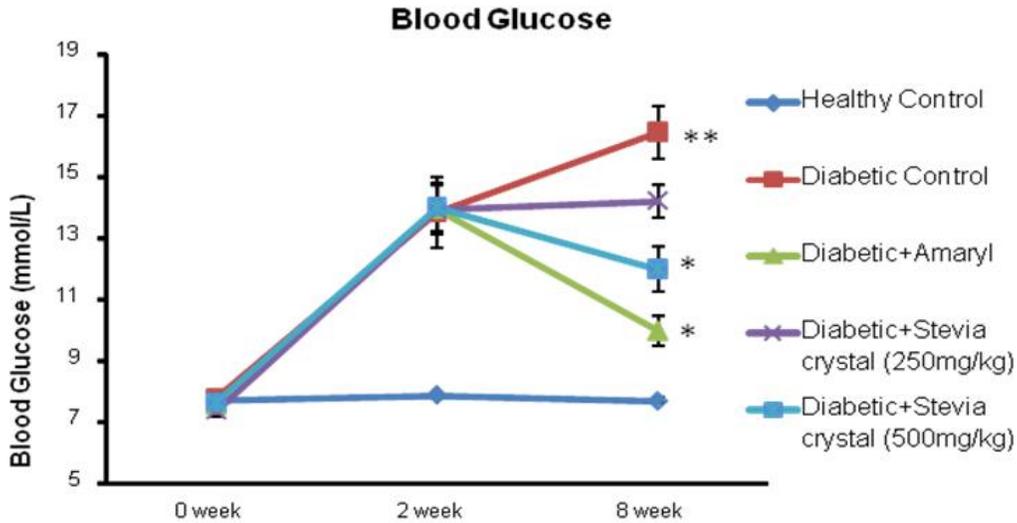
### 3.3 Effects of Stevia Crystal on Diabetic Nephropathy and Pancreatic Injury

Kidney section from control mice exhibited normal renal capsules, glomerulus and tubules (proximal and distal). On the other hand, diabetic control mice showed enlarged renal corpuscles

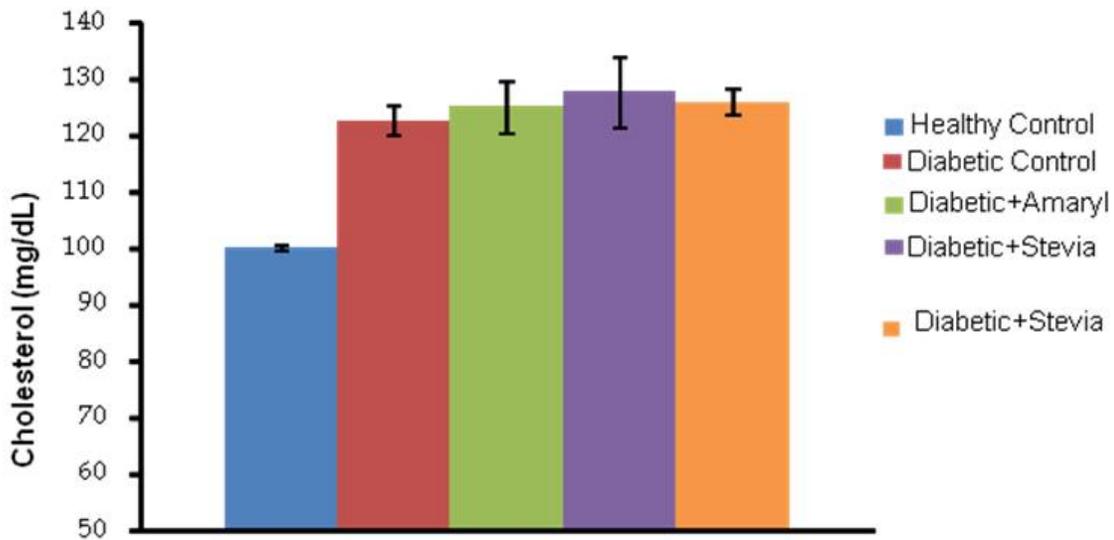
with glomerular sclerosis, lipid deposition in tubules leading to tubular damage with interstitial fibrosis and glomerular hypertrophy. Stevia treated groups showed slightly improved renal damage and lower incidence of tubular lipid deposition.

Alloxan monohydrate induced diabetes in experimental animal causing destruction of

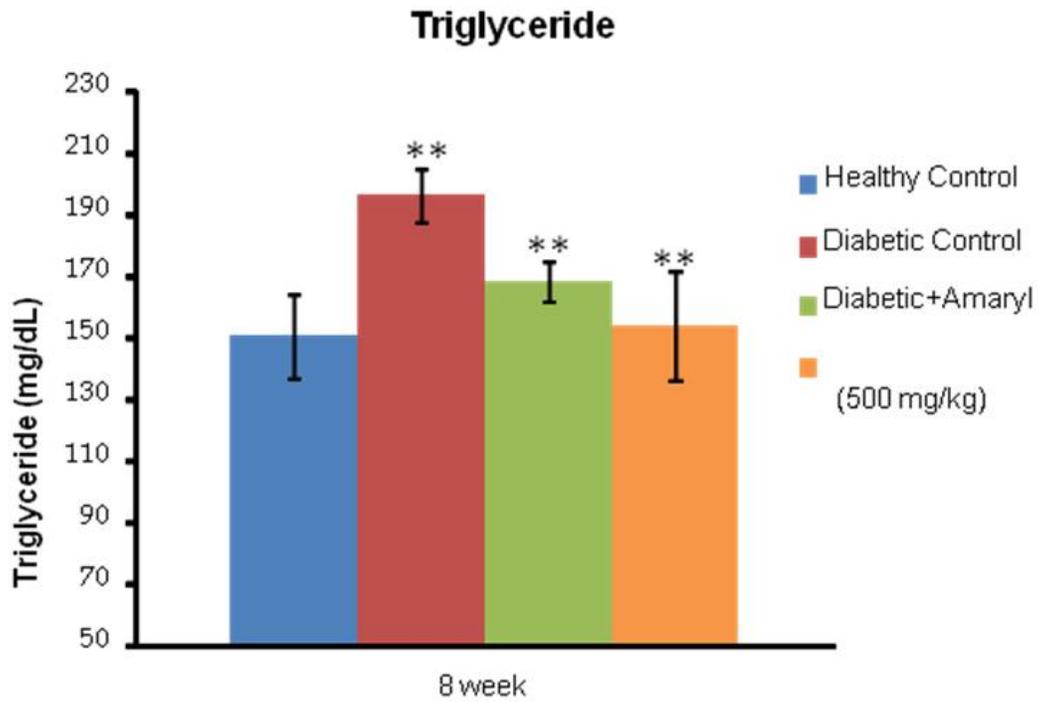
pancreatic  $\beta$ -cells. Pancreatic section from the control mice exhibited normal endocrine portion with islets of Langerhans cell. The  $\beta$ -cells of islets of Langerhans were degenerated, vacuolated in diabetic mice and the number of islets significantly decreased. Partial regeneration of islets was observed in groups treated with Stevia (500 mg/kg bwt orally).



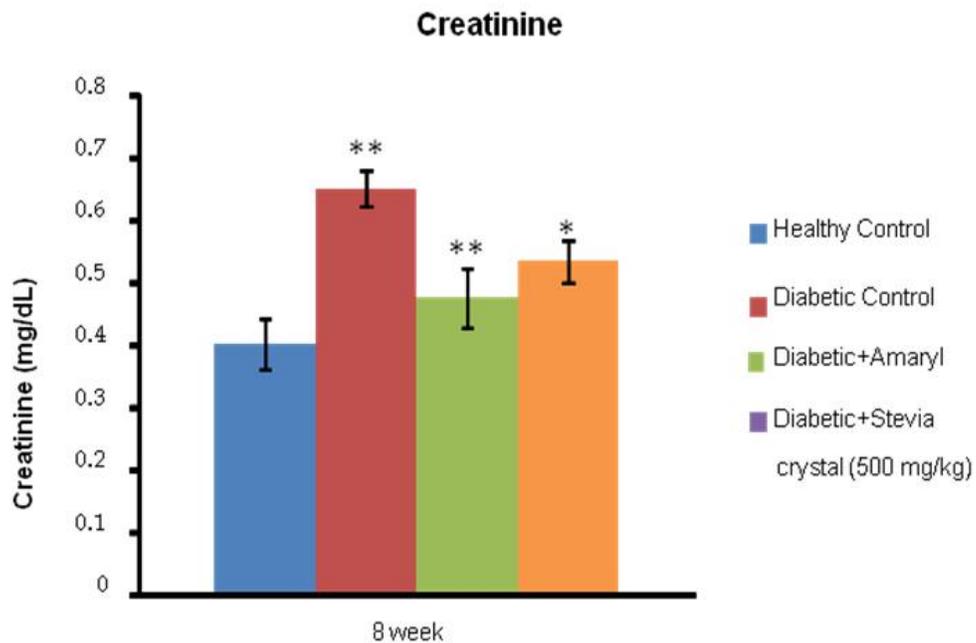
**Fig. 3. Effect of stevia crystal on whole blood glucose level in alloxan induced diabetic mice. Alloxan induced hyperglycemia was lowered by stevia crystal @ 500 mg/kg/day.**  
\* $P < 0.05$ , \*\* $P < 0.01$



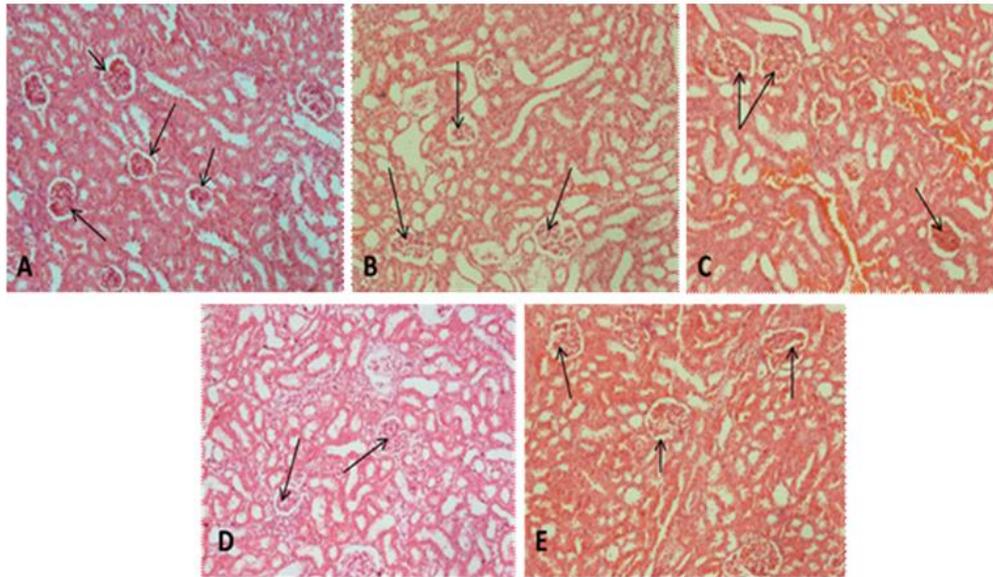
**Fig. 4a. Effect of stevia crystal on plasma lipid profile in alloxan induced diabetic mice. Alloxan induced elevated total cholesterol was unchanged by stevia crystal @ 500 mg/kg/day**



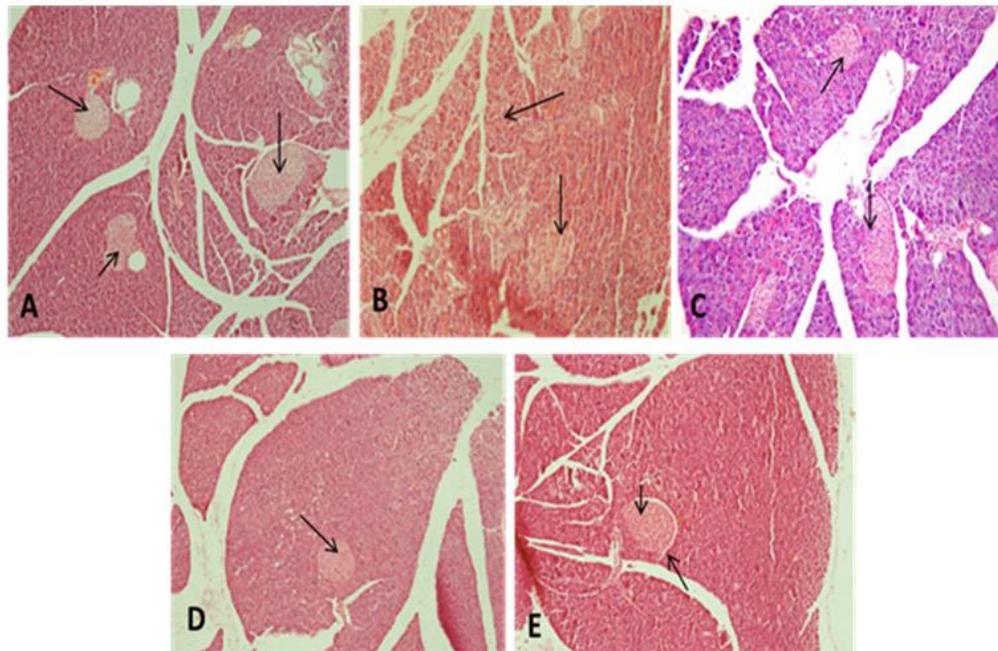
**Fig. 4b. Effect of stevia crystal on plasma lipid profile in alloxan induced diabetic mice. Alloxan induced increased triglyceride level was lowered by stevia crystal @ 500 mg/kg/day**  
**\*\*P<0.01**



**Fig. 5a. Effect of stevia crystal on plasma creatinine in alloxan induced diabetic mice. Alloxan induced increased plasma creatinine level was lowered by stevia crystal @ 500 mg/kg/day**  
**\*P<0.05, \*\*P<0.01**



**Fig. 5b.** Photomicrographs of histopathological studies of Kidney sections of normal and experimental diabetic mice. Alloxan induced diabetic nephropathy was partially attenuated by stevia crystal @ 500mg/kg/day. Sections of renal cortex were stained with hematoxylin and eosin (H&E). Representative light micrographs (10 X) from each mice groups are shown



**Fig. 6.** Photomicrographs of histopathological studies of pancreas of normal and experimental diabetic mice. Alloxan induced pancreatic tissue injury was partially attenuated by stevia crystal @ 500mg/kg/day. Representative histopathological profiles on  $\beta$ -cells (arrow show one islet) Sections of pancreatic tissue were stained with hematoxylin and eosin (H&E). Representative light micrographs (10 X) from each mice groups are shown

#### 4. DISCUSSION

Diabetes of long duration is associated with several complications such as atherosclerosis, myocardial infarction, neuropathy and nephropathy. These complications have long been assumed to be related to chronically elevated glucose levels and subsequent oxidative stress. Stevia treatment at a high daily dose did not have any adverse effect on normal animal. Hazali et al. [10] found that stevia does not raise blood glucose significantly when consumed in short period. Stevia is effective to be used by healthy people to maintain blood glucose even when consumed in short length of time. In the present study, higher doses (500 mg/kg) of crystals derived from *S. rebaudiana* leaves could produce a significant fall in blood glucose levels in diabetic mice without any significant changes in body weights. Similar findings were observed by other researchers [10]. Treatment with Amaryl<sup>®</sup> and Stevia crystal @500 mg/kg improves the body weight loss significantly in diabetic animal. Stevia @250 mg/kg have no significant effect on body weight of mice. This data partially supported the findings of Shukla et al. [11] as they use ethanolic extract of *Stevia rebaudiana* leaves and found a comparable change in body weight in extracts treated diabetic rats with diabetic control and normal animals.

Alloxan induces "chemical diabetes" in a wide variety of animal species by damaging the insulin secreting pancreatic  $\beta$  -cell, resulting in a decrease in endogenous insulin release [12]. In the present study, higher doses (500 mg/kg) of crystals derived from *S. rebaudiana* leaves could produce a significant fall in blood glucose levels in diabetic mice without any significant changes in body weights. Similar findings were observed by other researchers [13]. Shivanna et al. [13] also found the glucose lowering capacity of *Stevia rebaudiana* leaves. Rafiq et al. [14] also found that stevia has a positive effect on reducing the blood glucose level.

Alloxan induced diabetic mice showed elevated total cholesterol level ( $P<0.001$ ) compared to control group, this result are in agreement with pervious study [15]. The levels of glucose, cholesterol, triglycerides and creatinine in serum were found to be significantly increased in alloxan-induced diabetic group when compared to normal control group [16]. Following the administration of stevia crystal at a dose rate of 500 mg/kg the plasma total cholesterol level was

reduced non-significantly and it prevents the further increase in cholesterol level of diabetic animal. But there was no significant change between the effects of stevia at a dose rate 250 mg/kg and 500 mg/kg with Amaryl<sup>®</sup> treated group. It also decreased the triglyceride values significantly ( $P<0.05$ ). This result is partially supported by the findings of Park et al. [17], as they found that total cholesterol and triglyceride concentrations in serum were significantly higher in the high fat diet group than in the Stevia treated group.

Plasma creatinine level was increased significantly ( $P<0.01$ ) in diabetic control group compared to control group. Yang et al. [18] described that Diabetic control rats exhibited higher serum creatinine, urinary creatinine and serum urea levels compared to those of normal rats. In this study, after treating with stevia @500 mg/kg, the plasma creatinine level was decreased non-significantly. Plasma creatinine was not detected in stevia @250 mg/kg treated group as it has no significant effects on lipid profiles, may be due to low dose.

The kidney are an important target organ in diabetes, and kidney failure often leads to death in diabetes. Diabetes causes glomerular lesions, atherosclerosis of renal veins, pyelonephritis, and nephropathy [19]. In this study, a normal histological structure of the kidney section was observed in the control group. However, diabetic control group exhibited enlarged renal corpuscles with glomerular sclerosis, lipid deposition in tubules, interstitial fibrosis and glomerular hypertrophy. Pathological changes were lowered by stevia treatment partially. Stevia @500 mg/kg produce better renal protective effect than @250 mg/kg dose. This result partially support the findings of Ozbayer et al. [20], as they use *Stevia rebaudiana* (Bertoni) leaves extract to determine its effects on renal function in streptozotocin-nicotinamide (STZ-NA)-induced diabetic rats.

Alloxan selectively destroy the pancreatic  $\beta$ -cells via production of reactive oxygen species. Alloxan induces type-1 diabetes mellitus in experimental animal [21]. In this experiment, histopathological examination of control mice shows a normal histological structure of pancreas with distinct islets but in diabetic mice, the Islets of Langerhans were degenerated and the number of islets significantly decreased. Treatment with stevia causes partial regeneration of islets in diabetic animal. This result supports the findings of Assaei et al. [22]. They showed

that aqueous extract of stevia leaves in STZ induced rats exhibit pancreatic protective effects. However, there was no report using stevia crystal in alloxan induced diabetic nephropathy and pancreatic injury. To the best of our knowledge, this is the first time we evaluate the reno-pancreatic effects of stevia crystal in alloxan induced mice. Therefore, due to lack of previous report we did not compare our result with previously published data. We speculate that this reno-pancrease protective effects of stevia crystal is may be due to improvement of oxidative stress.

## 5. CONCLUSIONS

These present study findings express that crystalline compounds derived from locally available *Stevia rebaudiana* leaves possess antidiabetic properties characterized by the capacity of lowering blood glucose level with partially protective effects on kidney and pancreas in alloxan induced type-1 diabetic mice may be through reducing oxidative stress. Still to draw a definite conclusion in this regards it demands details further study.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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