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Ameliorative effects of *Costus speciosus* on biochemical and histopathological changes in alloxan-induced diabetic mice

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Abstract
The present study evaluated the ameliorative effects of *Costus speciosus* (CS) on biochemical and histopathological changes in alloxanized mice. Diabetes was induced in mice by administration of alloxan monohydrate (0.65mg/100g BW). Root extract of CS (10, 20 and 50 mg/100g BW) and metformin (19.5 mg/100g BW) as the standard drug were administered orally using a gavage to alloxanized mice daily for 14 days.Our study showed that oral administration of CS significantly decreased the blood glucose and total serum cholesterol levels. Moreover, CS restored the altered plasma enzyme (AST, ALT, LDH, ALP and ACP) levels. The reversal in liver and pancreatic histopathology further supports the protective effect of the CS extract towards diabetic damage. Extract of CS is effective in controlling blood glucose in diabetes and protecting liver and pancreatic tissues from diabetic damage. However, further studies are indeed required to prove the safety and efficacy of CS extracts as a potential anti-diabetic agent in clinical practices.
Keywords Alloxan, biochemical changes, *Costus speciosus*, *Diabetes mellitus*, histopathology, mice.

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Introduction

Diabetes mellitus (DM) is one of the most common metabolic disorders worldwide, affects the life quality of patients by bringing huge pressure to society and public health [1]. The global prevalence of DM has shown an upward trend over the past few decades. Nearly 2.2% of the total deaths in the world are caused by DM [2]. Every ten seconds a person dies from DM related causes. DM, therefore, has become a very serious public health problem with a heavy socioeconomic burden to most of the South Asian countries including Indonesia. Current numbers of DM patients are approximately 150 million to 300 million, which is predicted to be double by the year 2025[2]. Persistent hyperglycemia in DM leads to the development of secondary complications including neuropathy, nephropathy, and retinopathy [3, 4]. Increased production of free radicals and reactive oxygen species (ROS) have been observed during DM leading to increased lipid peroxidation and degradation of DNA and proteins. Autoimmune reactions and inflammatory cytokines initiate ROS production in type 1 diabetes, which cause β-cell dysfunction [5]. While in type 2 diabetes, β-cell apoptotic pathways impair insulin synthesis and are activated by ROS leading to insulin resistance [3, 6].

Despite the great strides made in the understanding and management of DM, related complications are increasing vigorously unabatedly [7]. Currently, there are no therapeutic regimens available which can fully cure DM, although most of them can reduce blood glucose and fat levels in blood circulation [1]. Traditional treatment aims to restore blood glucose levels and pancreatic islet function only. Additionally, some oral antihyperglycemic agents display various adverse effects such as indigestion, constipation, insulin resistance, hypoglycemia and edema. Metformin, a biguanide antihyperglycaemic agent, is widely used to lower blood glucose in type 2 DM and do not cause hypoglycaemia [8].However, many researchers reported unsatisfactory therapeutic results for current medicines. The basic reasons for limiting compliance for therapeutic regimen are lack of confidence, patient education/ belief, side effects of medicine and medicine taste. These issues have led to the selection of alternative therapeutic regimen for the treatment of DM. Searching for alternative treatment of DM and related complications are highly demanded.

Plants are considered an important source for novel drugs due to the potent efficacy with few side effects. Plants with anti-diabetic activities could provide useful ingredients for the management and treatment of DM [9]. Active compounds found in plants are being evaluated and used for the treatment of various diseases, including high blood pressure, cancer, heart problems and gastrointestinal disturbances [10]. Recently, antidiabetic agents of plant origin, such as *Costus speciosus* (CS) have

gained the attention of scientist due to unwanted side effects with other pharmacological antidiabetic drugs. CS (Family: *Costaceae*) is a valuable medicinal plant which is being used for the treatment of various health issues in humans [11, 12]. Diosgenin is an important phytochemical of CS and being used as herbal medicine for the control of DM in Indonesia. CS has gained value as antidiabetic plant and being cultivated on a large scale [10]. Herbal medicines possessing a polymodal protective action are under the intensive research in this context [11, 12]. However, the data on the mechanisms of action of these drugs are insufficient. Keeping this in view, the present study was designed to study the antidiabetic and antilipidemic effects of CS along with ameliorative effects on pancreas and liver histopathology in alloxan induced diabetic mice.

Materials and Methods

Plant material

The plant material for the present investigation was collected from the field areas of Bandar Lampung, Lampung Indonesia in June 2014. Plant material was verified by a botanist and a voucher specimen deposited at Department of Botany, Lampung University Indonesia.

Preparation of the extract

Soxhlet apparatus is a method to extract a soluble fraction from a solid medium. This apparatus consists of a condenser, an extraction unit and a round bottom flask. It has a standard paper round glass joint. The extraction unit and control unit plays a vital role in this apparatus. The sun-dried coarsely ground rhizome of CS (100g) was weighed and placed inside the extraction unit. The flask was filled with solvent100% ethanol (250ml) and apparatus was switched on. After a few hours (approximately four cycles), it was switched off and the extract was collected from the flask. The evaporated final content was used for the phytochemical work and animal treatment [6].

Animals and diabetes induction

All animal experiments were approved by the University Ethics Committee on the use of laboratory animals and experiments were performed according to the Committee guidelines. Thirty male mice (*Mus musculus*) were purchased from the Veterinary Investigation Center (BPPV), Lampung, Indonesia (age 4 months and weight 30-40 g). Mice were conditioned in polyurethane boxes, placed in an air-conditioned environment at 25°C with controlled lighting and exhaust, and received standard laboratory diet (protein, 16.04%; fat, 3.63%; fiber, 4.1%; and metabolic energy, 0.012 MJ) and water *ad libitum*. Prior to the inoculation of alloxan monohydrate, no feed was offered to mice for 12 hours. Type 2 DM was induced using intravenous alloxan (Sigma-Aldrich 3050 Spruce St., St. Louis, MO 63103, USA) at a dose of 0.65mg/100g of body weight into tail veins. Mice with a fasting plasma glucose range >200 mg/dl were considered diabetic and used for the investigation. The control animals were administered with phosphate buffer saline (PBS; pH 7.0).Diabetic animals that died during the post-induction period or at follow-up were replaced to avoid compromising the final number of mice in the sample. Animals were randomly assigned to 6 experimental treatments as shown in Table 1.Hyperglycemia mice were treated using an ethanol extract of CS dissolved in Tween 40, through oral administration daily upto 14 days.

Table 1 Experimental design.

Treatments
Control negative
Control positive (un treated diabetic)
DM+ CS (10mg/100g BW)
DM+ CS (20mg/100g BW)
DM+ CS (50mg/100g BW)
DM + metformin (19.5mg /100 g BW)

BW= body weight; CS= *Costus speciosus*; DM= *Diabetes mellitus*

Collection of blood sample

Mice were euthanized using pentobarbital sodium after 14 days of treatment. Blood samples were taken and harvested sera stored at -20°C for biochemical analysis.

Biochemical analysis

Blood samples were examined to determine plasma glucose insulin, C-peptide level and total cholesterol concentration by using radioimmunoassay kits (Perkin Elmer, Massachusetts, USA). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and acid phosphatase (ACP) activities were measured using Bayer Express plus Clinical Chemistry Auto-analyzer (Bayer® Germany).

Histopathological investigations

Tissue specimens (liver, pancreas) were dissected and preserved in a 10% solution of formaldehyde. The tissues were dehydrated because the reagents used at a later stage were immiscible with water. Varying concentrations of isopropyl alcohol (70%, 80%, 90%, 96%, and 100%) were used for the

dehydration. The minimum time for dehydration between two different concentrations was 1 h. The fixed tissues were then processed for routine histological examination. The sections (5 μm) from each of the tissues were examined using a light microscope (×40) after staining with hematoxylin and eosin dye [13].

Statistical analysis

Biochemical and histological lesion data were compared using one-way analysis of variance (ANOVA) followed by Post Hoc Dunnett's test(SPSS 13.0 for Windows program; SPSS Inc., Chicago, Illinois, USA). The *P* value of <0.05 was considered statistically significant and values were expressed as the mean ±standard deviation of the mean (SDM).

Results

Biochemical analysis

The anti-hyperglycemic effect of the C Son the fasting serum glucose levels in diabetic mice was determined. Diabetic mice showed significantly higher levels of glucose than mice in the negative control group. Our data revealed a drop in serum glucose levels with CS in a dose dependent manner (Table 2). It was revealed that CS can significantly decrease (P<0.05) serum glucose levels at a dose rate of 50mg/100 g BW/day compared with the diabetic mice. Similarly, mice treated with metformin also showed a significant reduction (P<0.05) in glucose level than mice in the negative control group. Moreover, a significant reduction in (P<0.05) insulin and C-peptide levels in alloxanized mice was observed. After treatment with CS, the level of insulin and C-peptide was increased in a dose dependent manner. At the end of the study, values of insulin and C-peptide were similar to that of negative control mice in CS (50 mg/100 g BW/day) and metformin treated mice. In addition, a significant increase (P<0.05) in the levels of total cholesterol in diabetic mice than in normal mice was observed. Administration of CS brought back the levels of serum lipids to near normal, especially at higher doses (Table 2).The activities of plasma enzymes AST, ALT, LDH, ALP and ACP were significantly increased in diabetic mice compared to normal controls. However, oral administration of CS (50mg/100g BW) for 14 days significantly (P<0.05) decreased the levels of these enzymes in CS and metformin treated mice. However, a decrease in the level of these enzymes was more prominent in those mice treated with higher doses of CS (Table 3).

Histopathologic observations

Non diabetic control mice showed normal liver parenchyma with general structures preserved, including hepatic lobules with normal hepatocytes surrounded by sinusoids and distributed radially towards the centrilobular veins, and containing kupffer and red blood cells in the capillary lumen. Portal spaces were also normal, with no observed inflammatory infiltration, fatty degeneration or abnormal distribution of fibroblasts or collagen in mice sacrificed at 14 days of follow-up (Fig. 1A). More prominent pathological lesions were observed commonly in the liver and pancreas due to toxicity of alloxan. Severe micro-vesicular fatty degeneration was observed in the livers of diabetic untreated mice associated with the presence of dilated sinusoids and a progressive loss of general organ structure (Fig. 1B). Inflammatory changes consistent with steatohepatitis, which were represented by mononuclear inflammatory infiltrates of moderate intensity of analyzed liver histological sections. CS nullified the toxic effects of alloxan on liver tissue in a dose dependent manner. Interestingly, metformin showed better ameliorative effects than CS at dose rate of 10 and 20 mg/100g BW, but less than 50 mg/kg BW CS (Fig.1C and 1D).

The sections of the pancreas from untreated diabetic mice showed an extensive destruction of islet cells as compared with that of healthy control mice (Fig. 2Aand2B). Further, there was a definite reduction in the number of islets in diabetic mice than in the healthy mice. However, hemorrhages were not observed and acinar cells were intact in the pancreatic tissues of alloxan induced diabetic control mice. Further, severe inflammatory cell infiltrations in islets were also observed in diabetic control mice. The best amelioration was seen with CS 50mg/100g BW in the islets of Langerhans compared to CS 10 and 20 mg/100g BW (Fig. 2C and 2D). The pancreas sections from CS treated diabetic mice revealed a statistically significant regeneration of islet cells with some hyperplastic islets (P< 0.05). Further, the CS extract produced a significant increase in the mean profile diameter in large islets compared to the alloxan induced diabetic control mice. Severity of histopathologic lesions was significantly reduced in CS treated group (50mg/100g BW) (Table 4).

Discussion

Antidiabetic activities of CS on mice have been discussed in this article. The blood glucose level of diabetics can be effectively controlled by utilizing

Table 2 Effect of oral administration of *Costus speciosus* on blood glucose, total cholesterol, plasma insulin and C-peptide levels (mean + SD) in normal and alloxan-induced diabetic male mice.

Treatments	Glucose (mg/dL)	Cholesterol (mg/dL)	Plasma Insulin (µU/ml)	C-Peptide (ng/ml)
Control negative	153.50± 52.00^{c}	115.00 ± 42.04^{c}	18.15±0.33^{a}	6.38±0.11^{a}
Control positive (DM)	309.75±194.76^{a}	210.50 ± 25.94^{a}	6.9±0.1.2^{d}	3.44±0.29^{d}
DM+ CS (10mg/100g BW)	172.20±30.80^{b}	141.60 ± 24.94^{b}	9.33±0.6^{c}	3.75±0.66^{c}
DM+ CS (20mg/100 g BW)	166.25±35.83^{b}	139.25 ± 42.92^{b}	10.31±0.73^{c}	3.89±0.42^{c}
DM+ CS (50mg/100 g BW)	158.00±36.31^{c}	129.75 ± 32.99^{b}	14.47±1.2^{b}	5.35±0.15^{b}
DM + Metformin (19.5mg /100 g BW)	170.25±4.84^{b}	133.25 ± 29.35^{b}	11.28±1.4^{b}	4.53±0.48^{b}

$^{a-d}$Mean values in the same column that do not share a common letter differ significantly ($P < 0.05$)

Table 3 Effect of oral administration of *Costus speciosus* on plasma AST, ALT, LDH, ALP and ACP levels in normal and alloxan induced diabetic male mice.

Treatments	AST (U/dl)	ALT (U/dl)	LDH (U/dl)	ALP (U/dl)	ACP (U/dl)
Control negative	32.6 ± 1.7^{d}	53.0± 1.2^{c}	1089.2 ± 12.3^{d}	47.3± 4.7^{c}	13.8± 0.2^{c}
Control positive (DM)	66.4± 3.6 ^{a}	97.3± 2.6^{a}	1727.2 ± 22.2^{a}	86.2 ±3.9^{a}	27.0± 6.3^{a}
DM+ CS (10mg/100 g BW)	56.7 ±1.2^{b}	71.8± 6.3^{b}	1356.2 ± 25.9^{b}	67.8± 3.7^{b}	18.9± 1.7^{b}
DM+ CS (20mg/100 g BW)	47.8 ±3.7^{b}	69.5± 3.9^{b}	1266.4 ± 23.5^{c}	64.5± 3.2^{c}	17.2± 2.7^{b}
DM+ CS (50mg/100 g BW)	42.2 ±6.5^{c}	68.2± 6.7^{b}	1203.2 ± 12.3^{c}	61.4± 7.5^{c}	17.44± 1.4^{b}
DM + Metformin (19.5mg /100 g BW)	49.9 ±2.2^{b}	65.8± 5.7^{b}	1262.2 ± 17.6^{c}	67.6± 2.0^{b}	18.67± 2.2^{b}

CS: *Costus spiosus*, DM: Diabetes mellitus, AST: Aspartate aminotransferase, ALT: Alanine aminotrasferase, LDH: Lactate dehydrogenase, ALP: Alkaline phosphatase, ACP: Acid phosphatase. $^{a-d}$Mean values in the same column that do not share a common letter differ significantly ($P < 0.05$)

Table 4: Severity of histopathological lesions in liver and pancreas in normal and alloxan induced diabetic male mice.

Treatments	Fatty degeneration		Necrosis		Hemorrhages		Cellular infiltration		Fibrosis	
	Liver	Pancreas	Liver	Pancreas	Liver	Pancreas	Liver	Pancreas	Liver	Pancreas
Control negative	-	-	-	-	-	-	-	-	-	-
Control positive (DM)	+++	+++	++	+	++	+++	++	++++	++	+++
DM+ CS (10 mg/100 g BW)	+++	++	++	+	++	+++	+	++	+	++
DM+ CS (20 mg/100 g BW)	++	+	+	+	++	++	+	++	+	++
DM+ CS (50 mg/100 g BW)	+	+	+	+	-	-+	-	+	+	+
DM + metformin (19.5mg /100 g BW)	++	+	+	-	+	++	+	++	+	+

- normal ; + very mild ; ++ mild; +++ moderate; ++++ severe

currently available antidiabetic agents. The FDA approved anti-diabetic agents are used for the therapy to control blood glucose level [14, 15]. Incorporation of natural antidiabetic preparation is given the privilege to make use of it, as it holds less toxicity and negligible side effects. Almost all the flavonoids have potential for antidiabetic activity, but are limited in usage due to lack of studies on their antidiabetic activities.

In the present study, low dose of alloxan monohydrate (0.65 mg/100g BW) induced type II DM by partial destruction of pancreatic beta cells leading to insufficient insulin production [16]. Dose dependent decrease in the glucose level was observed during the present study in CS treated diabetic mice. This decrease in the level of glucose might be due to insulin like effects of CS or its ingredients which lead either to increase in glucose uptake mechanism possibly by inhibiting the process of gluconeogenesis [17].Moreover, the CS might also enhance the regeneration process of β-cells in pancreas [18-21]. Further, CS might inhibit the expression of nitric oxide synthase leading to increase insulin secretion [22].

It has been reported that hypoglycemic activity of herbal medicines might be due to improvement in the effect of insulin on the target cells, stimulating glucose dependent insulin secretion or by enhancing the recovery process for the damaged β-cells of islets of Langerhans in the pancreas of alloxan-induced diabetic rats [24].Rapid decrease in the enzyme levels in CS treated diabetic mice indicated that CS has the ability to nullify the toxic effects of alloxan. Moreover, an increase in the level of AST, ALT, LDH, ALP and ACP might be due to necrosis of liver tissue. Similar findings have been reported in past indicating liver dysfunction in streptozotocin induced diabetic rats [27, 28]. Therefore, an increase in the activities of these enzymes might be due to the

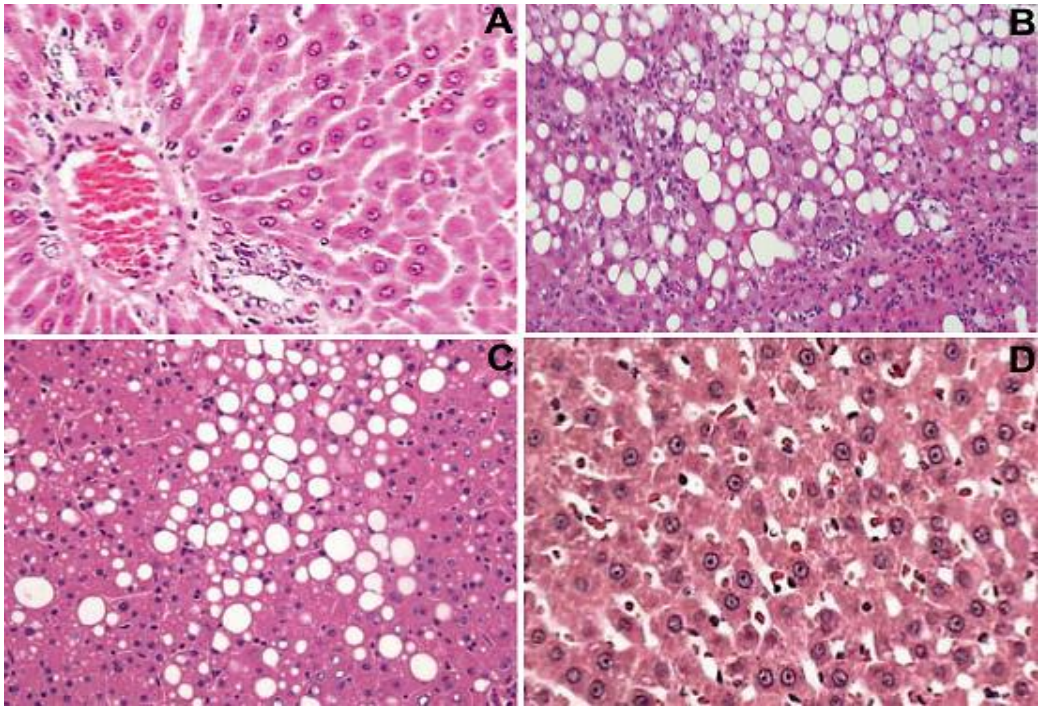

Fig 1 Histological pattern of liver from nondiabetic control mice showing normal appearing of hepatocytes, portal space, sinusoids, and Kuppfer cells (**A**); Histological pattern of liver in untreated diabetic showing severe fatty degeneration, sinusoidal enlargement and interlobular mononuclear inflammatory infiltrate (**B**);liver of metformin-treated diabetic group showing mild fatty degeneration and mononuclear inflammatory infiltrates (**C**); liver of CS (50mg/100g BW) treated diabetic group showed normal hepatic architecture which was almost similar to that of the control group (**D**), (×400).

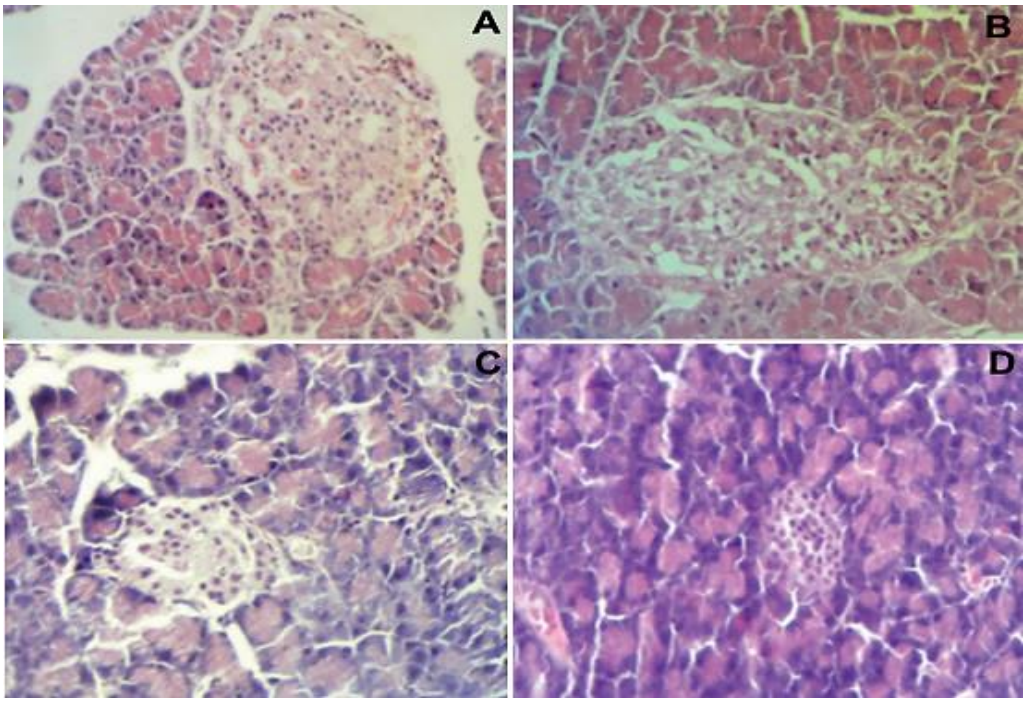

Fig 2 Photomicrographs of histopathology of the pancreatic tissues stained with hematoxylin and eosin.Healthy control mice, islets of Langerhans with normal islet cell population(**A**);diabetic control mice, an islet with few preserved islet cells, fibrosis, and infiltration by inflammatory cells (**B**);*Costus speciosus* (50mg/100g BW) treated diabetic mice, restoration of pancreatic islet cells with prominent islets (**C**);metformin treated (19.5mg/100g BW) diabetic mice with mild restoration of islet cells (**D**).

leakage of these enzymes from the damaged liver tissue to blood circulation indicating the hepatotoxic effect of alloxan. However, we observed that treatment of the diabetic mice with CS resulted in alleviation in liver damage and ultimately leading to decrease in the activity of these enzymes. These findings are in agreement with those obtained by El-Demerdash et al. [29] in rats.Moreover, CS treated mice showed increased levels of insulin and C-peptide, indicating the effect of CS on the regeneration of pancreatic tissue. C-peptides have been reported to have insulin-mimetic effects and can stimulate insulin receptors; leading to increased glycogen synthesis from blood glucose [30-31].Phytochemical compounds in plants may regulate the enzymes of glycolysis and gluconeogenesis [25]. However, we have not studied the mode of action of these phytochemical agents in the present study; therefore our analysis is based on assumptions.

Abnormalities in lipid profile are considered one of the leading complications in most of the DM cases [26].Low levels of insulin during DM can result in increased lipase secretion leading to enhanced mobilization of fatty acids [27]. Fatty degeneration was seen during histopathological examination of liver that might be the result of increase fatty acid mobilization in diabetic mice [32, 33]. Regeneration of pancreatic and liver tissue with minimal lesions was observed in CS treated mice. Similarly, the regeneration of pancreatic tissues was observed by Dhanavathy [34] in streptozotocin induced diabetic mice after treatment with plant extract.

In conclusion, oral administration of ethanolic extract of CS to alloxan-induced diabetic mice improved their glucose tolerance, an important finding in the control of diabetes. This suggested that CS is useful in the protection and amelioration of diabetic complications through the enhancement of regeneration of β-cells of the islets of Langerhans. Moreover, the present results showed that CS consumption reversed most of the histological changes in the liver and pancreas of the diabetic rats in a dose dependent manner. In addition, the plant extract exerts anti-hyperlipidemic activities in diabetic mice. So, we can say that CS had a significant hepatoprotective role in diabetic rats and offers promising perspectives deserve further investigation. Further studies are needed to define the active agents present and their mode of activity.

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