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Protective effect of coded plant (222) leaf extract against streptozotocin-induced diabetes in Wistar albino rats – Part II

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Abstract

In the present study, the antidiabetic effect of the ethanolic leaf extract of coded plant (Code No. 222*) was evaluated against streptozotocin (STZ)-induced diabetic rats. There was a significant increase in blood glucose levels of STZ (50 mg/kg)-induced diabetic rats compared to normal control group of animals. The oral administration of coded plant 222 leaf ethanolic extract (125 mg/kg) resulted in a significant reduction in blood glucose levels of STZ-induced diabetic rats from the 21st day of the treatment. The body weight of animals belonging to the diabetic control group was found to be drastically decreased upon the induction of diabetes. Coded plant 222 leaf ethanolic extract and glibenclamide treated animals were found to exhibit significant gaining in body weight compared to the diabetic control group. The extract treatment also resulted in a significant reduction of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), serum bilirubin (SB), cholesterol and triglyceride levels compared to the toxin control group of animals indicating its anti-hyperlipidemic activity. Glibenclamide (5 mg/kg) was used as the standard drug for comparing the protective effect of the extract administered group. Furthermore, there was a significant increase in the hepatic antioxidant enzyme status of 222 extract (125 mg/kg) treated animals compared to standard control group against the oxidative stress generated by streptozotocin. The liver glycogen was also estimated in streptozotocin induced diabetic study. 47.95 mg/g glycogen was estimated in 222 extract (125 mg/kg) treated group. The histopathological observation also supported the findings of biochemical estimation of serum parameters of the extract treated animals. The results of the present study revealed the anti-diabetic potential of coded plant (222) leaf extract against streptozotocin induced hyperglycaemia and it provided effective protection against other metabolic aberrations induced by diabetes.

Keywords: Diabetes mellitus, Beta cells, Insulin, Streptozotocin, Liver glycogen

* Name of the medicinal plant species will be disclosed only after obtaining the Patent

Introduction

In continuation of our earlier communication on pre-clinical studies of a medicinal plant on traditional knowledge with special reference to Access and Benefit Sharing - Part I (Krishnakumar *et al.,* 2016), in this paper the antidiabetic effect of the ethanolic leaf extract of same coded plant (Code No. 222**) has been evaluated against streptozotocin (STZ)-induced diabetic rats. *Diabetes mellitus* is a chronic metabolic disorder resulting from either insulin insufficiency or insulin dysfunction. It is estimated that the global

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prevalence of diabetes will increase from 4% in 1995 to 5.4% by the year 2025 and WHO has predicted that the major burden will occur in developing countries. It is estimated that there are approximately 33 million adults with diabetes in India and this number is likely to increase to 57.2 million by the year 2025 (Ramachandran *et al*., 2002). Type-I diabetes is insulin dependent and caused due to insulin insufficiency because of lack of functional pancreatic beta cells. Type-2 diabetes is insulin independent caused due to the inability of beta cells to respond to insulin and can be treated with dietary changes, exercise and medication. Diabetes is represented hyperglycaemia, hyperlipidemia and oxidative stress leading to long term complications to the patients such as retinopathy, nephropathy, atherosclerosis and microangiopathy (Glugliano *et al*., 1996).

Although several therapies such as oral hypoglycaemic agents are in use for the treatment of diabetes, there are certain limitations to these synthetic drugs due to high cost and adverse side effects such as development of hypoglycaemia, weight gain, gastro-intestinal disturbances, liver toxicity etc. (Dey *et al*., 2002). There are many herbal remedies suggested for diabetes and diabetic complications and medicinal plants form the main ingredients of these formulations (Dixit *et al*., 2006). Many conventional drugs have been derived from prototypic molecules in medicinal plants. Hence, the studies are being conducted for finding therapeutically more efficient, safer and less expensive drugs from medicinal plants.

The present scientific study was carried out based on Traditional Knowledge related to a coded medicinal plant (Code No. 222) disclosed by a traditional healer and the claim of the traditional healer was that he is using the particular medicinal plant species for treating diabetes and related complications. On verification, no pharmacological studies of the coded plant 222 were so far conducted and the therapeutic usage was kept as trade secret by the healer. The present ethnopharmacological study revealed the antidiabetic potential of the coded plant 222 leaf ethanolic extract on streptozotocin-induced diabetic rats.

Materials and Methods

1. Preparation of the coded drug extracts

100 g of the coded drug 222 extracted with 1000 mL of ethanol overnight, at room temperature with constant stirring. The extract was filtered, concentrated and the solvent evaporated completely on a rotary evaporator and dried in a desiccator and they were reconstituted in 0.5 % Tween-80 to required concentrations and used for the experiments.

2. Animals

Wistar albino rats, male and female (200–250 g) obtained from the Institute's Animal House were used for the present study. They were housed in poly-acrylic cages with three animals per cage and maintained under standard laboratory conditions (temperature 24-28 ± 1°C, relative humidity 60 ± 5% and 12 h light/dark cycles), fed commercial rat feed (Lipton India Ltd., Mumbai, India) and boiled water *ad libitum*. All experiments involving animals were carried out according to National Institute of Health (NIH) guidelines, after getting the approval of the Institute's Animal Ethics Committee.

3. Streptozotocin-induced diabetic study

The antihyperglycemic study of the ethanolic leaf extract of coded drug 222 was

carried out in streptozotocin-induced diabetic rats. Diabetes was induced in rats by tail vein injection of streptozotocin (50 mg/kg, intravenously). 48 h after streptozotocin administration, blood samples were drawn and glucose levels determined to confirm diabetes. The diabetic rats exhibiting blood glucose levels in the range 375-475 mg/dL were selected for the study. These rats were subdivided into normal control group (without diabetes), diabetic control, standard control and groups treated with 125 mg/kg dose of the extract. The treatment was continued for 21 days. Blood was collected from tail vein on days 1, 7, 14 and 21 for glucose estimation. After 21 days, the animals were sacrificed by carbon dioxide inhalation; blood was collected for biochemical estimation of serum parameters. Liver tissue was sliced out to estimate the antioxidant enzymes. Liver, pancreas and kidney tissue samples were sliced out for histopathological studies (Babu *et al*., 2003).

3.1. Biochemical Estimation

The collected blood allowed to coagulate for 1h at room temperature and centrifuged at 1500 rpm for 15 min at 37ºC to separate the serum. . The serum then used for the assay of marker enzymes, namely Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Serum Bilirubin (SB) and Serum Alkaline phosphatase (ALP) according to the standard methods. Other serum parameters such as serum cholesterol, triglycerides and total protein levels were estimated and compared to the standard modern drug, glibenclamide.

3.2. Histopathological studies

Liver, pancreas and kidney specimens obtained from the control and the treated groups of the experiment were trimmed to small pieces and preserved in formalin (10% solution) for 24 h, which is subjected to dehydration with acetone of strength 70, 80 & 100% respectively, each for 1 h. The infiltration and impregnation done by the treatment with paraffin wax, twice each time for 1 h. Specimens cut into sections of 3-7 µm thickness and stained with haematoxylin and eosin. Mounting of the specimens was done by the use of Distrene Phthalate Xylene (DPX).

3.3. Estimation of antioxidant enzymes in liver tissue homogenate

0.5 g of the rat liver tissues of all the groups were sliced and homogenized with 10 mL of 150 mM KCl-Tris-HCl buffer (pH 7.2) to prepare the tissue homogenate. Superoxide dismutase (SOD) was estimated by using the method of Misra and Fridovich (1972), catalase by Aebi (1974) and glutathione peroxidase (GPx) was estimated by using the method of Rotruck *et al.* (1973).

3.4. Estimation of liver glycogen (*in vivo*) in streptozotocin-induced diabetic study

The glycogen content in liver tissue was determined by the anthrone method. Briefly, 3 mL KOH 30 % (w/v in distilled water) was added to 1 g of liver tissue and heated at 100^0 C for 30 minutes. After dilution with distilled water to 1:50, 20 µL of this mixture was transferred to 2 mL anthrone reagent (2 mg/mL in sulphuric acid) and the resultant mixture boiled for 10 minutes. The samples were cooled to room temperature and absorbance was determined at 620 nm (Fong *et al*., 1953).

4. Statistical analysis

All the analyses carried out in triplicate. The

statistical significance between the samples were determined by the analysis of variance (ANOVA) and the data were recorded as mean ± Standard Deviation (SD) , $p \leq 0.05$ was considered to be statistically significant. Significant differences between means were determined by Duncan's Multiple Range tests (Raghava, 1987). The computer software employed for the statistical analysis was IBM SPSS Statistics, version 20 (USA).

Results and Discussion

Streptozotocin (STZ) induced diabetes has been described as a useful experimental model to study the effect of antidiabetic agents with or without insulin (Ledoux *et al.*, 1986). It generally causes the destruction of β-cells after three days and reaches its peak at three to four weeks in rats (Adeghate and Ponery, 2002). It is a potent DNA methylating agent and acts as a nitric oxide donor in pancreatic islet cells and beta cells are particularly sensitive to damage by nitric oxide and free radicals because of their low levels of free radical scavenging enzymes (Spinas, 1999). The diabetes induced animals exhibited reduced response to insulin in hepatic and peripheral tissue. The results show that STZ injection effectively induced diabetes in normal fasted rats as reflected by the blood glucose level, body weight and other serum parameters.

The body weight of animals of diabetic control group was found to be drastically decreased upon the induction of diabetes. The extract 222 (125 mg/kg) and glibenclamide (5 mg/kg) administered groups were found to gain body weight significantly ($p \leq 0.05$) when compared to the diabetic control group as shown in Table 1.

The level of blood glucose was significantly increased in diabetic rats when compared to normal rats. Oral administration of coded drug extract 222 (125 mg/kg) and glibenclamide (5 mg/kg) to diabetic rats significantly ($p \leq 0.05$) decreased the blood glucose level. In 222 (125 mg/kg) treated group, the significant anti-hyperglycemic effect was evident from 21st day onwards. This was almost equal to the standard drug, glibenclamide in its efficiency in lowering blood glucose level in STZ -induced diabetic model (Table 2). The sulfonylureas such as glibenclamide have been used for many years to

Table 1: Streptozotocin-induced diabetic study of coded plant 222 leaf ethanolic extract: Influence on body weight (in grams)

Groups	Day-1	Day-7	Day-14	Day-21
Normal control	150.00 ± 0.00	155.00 ± 0.00	165.00 ± 0.00	170.00 ± 0.00
Diabetic control Streptozotocin (50 mg/kg)	150.00 ± 0.00	125.00 ± 0.00	120.00 ± 0.00	100.00 ± 0.00
Coded plant 222 (125 mg/kg)	150.00 ± 0.00	125.00 ± 0.00	125.00 ± 0.00	130.00 ± 0.00**
Standard control Glibenclamide (5 mg/kg)	150.00 ± 0.00	120.00 ± 0.00	135.00 ± 0.00	135.00 ± 0.00**

Values are the mean ± SD, n=6 in each group, analysis of variance (ANOVA) followed by Duncan's multiple range test. **Significance p≤0.05 compared to the diabetic control.

Table 2: The effect of coded plant 222 leaf ethanolic extract on rat serum glucose levels (mg/dL) in streptozotocin-induced diabetic rats

Groups	DAY-1	DAY-7	DAY-14	DAY-21
Normal control	92.00 ± 0.89	93.00 ± 0.89	92.00 ±0.89	90.00 ± 0.89
Diabetic control Streptozotocin (50 mg/kg)	480.00 ± 0.89	424.00 ± 1.05	430.00 ±0.89	486.00 ± 0.78
Coded plant 222 (125 mg/kg)	463.00 ± 0.89	349.00 ± 0.89	326.00 ± 0.54	287.04 ± 0.57
Glibenclamide (5 mg/kg)	435.01 ± 0.89	318.00 ± 0.89	301.00 ±0.41	223.01 ± 0.58

Values are the mean ± SD, n=6 in each group, analysis of variance (ANOVA) followed by Duncan's multiple range test. **Significance p≤0.05 compared to the diabetic control.

treat diabetes, to stimulate insulin secretion from pancreatic β-cells principally by inhibiting ATP-sensitive K_{ATP} channels in the plasma membrane. The oral administration of glibenclamide to the STZ-induced diabetic rats decreased the blood glucose level and also could be considered as a standard anti-diabetic drug to compare the efficacy of hypoglycaemic compounds (Courtois *et al*., 2003).

In diabetic rats, the serum levels of liver enzymes like ALT, AST and ALP were significantly increased when compared to normal control rats. The ethanolic extract of 222 at 125 mg/kg dose significantly ($p \leq 0.05$) reduced ALT and bilirubin level. The total protein level was significantly ($p \leq 0.05$) increased in 222 (125 mg/kg) and glibenclamide (5 mg/kg) treated groups compared to the diabetic control group (Table 3).

Table 3: The effect of the coded plant 222 leaf ethanolic extract on rat serum parameters in streptozotocin-induced diabetic rats

Groups	ALT (IU/L)	AST (IU/L)	ALP (KAunits/ 100 mL)	Bilirubin (mg/dL)	Total protein (g/dL)
Normal Control	89.92±0.89	55.00±0.89	96.07±0.91	0.12±0.02	5.07±0.99
Diabetic control Streptozotocin (50 mg/kg)	251.43±0.89	219.17±1.47	320.14±0.96	0.48±0.01	3.30±0.16
Coded plant 222 (125 mg/kg)	104.45±1.02**	129.13±0.95	201.01±0.89	0.12±0.01**	4.61±0.19**
Glibenclamide (5 mg/kg)	104.76±0.89**	96.15±0.97	154.04±0.90	0.13±0.01**	4 .14±0.05**

Values are the mean ± SD, n=6 in each group, analysis of variance (ANOVA) followed by Duncan's multiple range test. **Significance p≤0.05 compared to the standard control.

Table 4: The effect of coded plant 222 leaf ethanolic extract on rat serum parameters in streptozotocin-induced diabetic rats

Groups	Glucose (mg/dL)	Cholesterol (mg/dL)	Triglycerides (mg/dL)
Normal Control	92.99 ± 0.10	7.87 ± 1.52	152.25 ± 0.61
Diabetic control Streptozotocin (50 mg/kg)	465.66 ± 9.02	28.24 ± 1.52	329.22 ± 4.92
Coded plant 222 (125 mg/kg)	285.65 ± 0.89**	18.21 ±1.72**	208.62 ± 11.01**
Glibenclamide (5 mg/kg)	225.09 ± 0.88	12.50 ± 0.50	157.87 ± 0.62

Values are the mean ± SD, n=6 in each group, analysis of variance (ANOVA) followed by Duncan's multiple range test. **Significance p≤0.05 compared to the standard control.

Table 5: The effect of coded plant 222 leaf ethanolic extract on rat serum parameters in streptozotocin-induced diabetic rats

Groups	Albumin (g/dL)	Urea (mg/dL)	Uric acid (mg/dL)	Creatinine (mg/dL)
Normal Control	3.91± 0.30	11.22 ± 1.91	0.20 ± 0.07	0.31 ± 0.01
Diabetic control Streptozotocin (50 mg/kg)	7.73±0.51	34.21 ± 6.25	1.27 ± 0.25	2.04 ± 0.05
Coded plant 222 (125 mg/kg)	6.29± 0.09	12.58 ± 0.08**	0.61 ± 0.23**	1.26 ±0.03**
Glibenclamide (5 mg/kg)	4.79± 0.56	13.91 ± 0.85**	0.29 ± 0.11	0.73 ± 0.03

Values are the mean ± SD, n=6 in each group, analysis of variance (ANOVA) followed by Duncan's multiple range test. **Significance p≤0.05 compared to the standard control.

Lipids play a vital role in pathogenesis of diabetes mellitus. Abnormalities in lipid profile are one of the most common complications in diabetes mellitus found in 40% of diabetic cases. The most common lipid abnormalities in diabetes are hyper-triglyceridemia and hyper-cholesterolemia. In the present study, elevated levels of serum lipids such as cholesterol and triglycerides were noticed in diabetic rats, while as it was found to be significantly ($p \leq 0.05$) lowered in 222 (125 mg/kg) treated group (Table 4). Diabetes induced hyperlipidemia is attributable to excess mobilization of fat from adipose tissue due to underutilization of glucose. STZ-induced diabetic rats were found to have significantly elevated serum creatinine and urea

Table 6: Estimation of liver glycogen in streptozotocin-induced diabetic rats treated with coded plant 222 leaf ethanolic extract

Groups	O. D. Value	Liver Glycogen (mg/g)
Normal control	0.243	62.31 ± 0.02
Diabetic control Streptozotocin (50 mg/kg)	0.170	43.59 ± 0.78
Coded plant 222 (125 mg/kg)	0.187	47.95 ± 0.94**
Glibenclamide (5 mg/kg)	0.208	53.33 ± 0.58**

Values are the mean ± SD, n=6 in each group, analysis of variance (ANOVA) followed by Duncan's multiple range test. **Significance p≤0.05 compared to the standard control.

Table 7: Effect of coded plant 222 leaf ethanolic extract on oxidative stress of streptozotocin induced diabetic rats

Groups	Catalase (U/mg protein)	SOD (U/mg protein)	Glutathione peroxidise (U/mg protein)
Normal Control	83.91± 0.30	11.22 ± 1.91	12.20 ± 0.07
Diabetic control Streptozotocin (50 mg/kg)	37.73±0.51	3.21 ± 6.25	1.24 ± 0.25
Coded plant 222 (125 mg/kg)	66.29± 0.09**	8.58 ± 0.08**	9.61 ± 0.23**
Glibenclamide (5 mg/kg)	74.79± 0.56**	9.91 ± 0.85**	10.29 ± 0.11

Values are the mean ± SD, n=6 in each group, analysis of variance (ANOVA) followed by Duncan's multiple range test. **Significance p≤0.05 compared to the standard control.

levels as compared to non-diabetic control rats. This is because STZ-induced diabetic rats have diminished ability to filter urea and creatinine from blood and excrete them in urine. This is another characteristic change in diabetes. After treatment with 222 extract at 125 mg/kg dose, the levels of serum urea, uric acid and creatinine were significantly ($p \leq 0.05$) reduced (Table 5).

The liver glycogen was also estimated in STZ-induced diabetic study. 47.95 mg/g glycogen was estimated in the animal group treated with 222 (125 mg/kg) dose compared to the normal control group (Table 6). In diabetes, glucose 6-phosphatase increases in liver facilitating glucose release into the blood. The opposing enzyme, which phosphorylates glucose, is glucokinase, which decreases in diabetes. As a result, the liver continues to produce glucose even with severe hyperglycaemia. This results in glycogen degradation and inhibition of glucose utilization. The elevation of depressed glycogen stored by the drug in STZ-treated rats may be attributed to either an inhibition of hepatic glucose output by improvement in plasma insulin levels or by stimulating the enzyme glycogen synthase responsible for the incorporation of glucose moieties into pre-existing glycogen chain.

The antioxidant enzymes such as SOD,

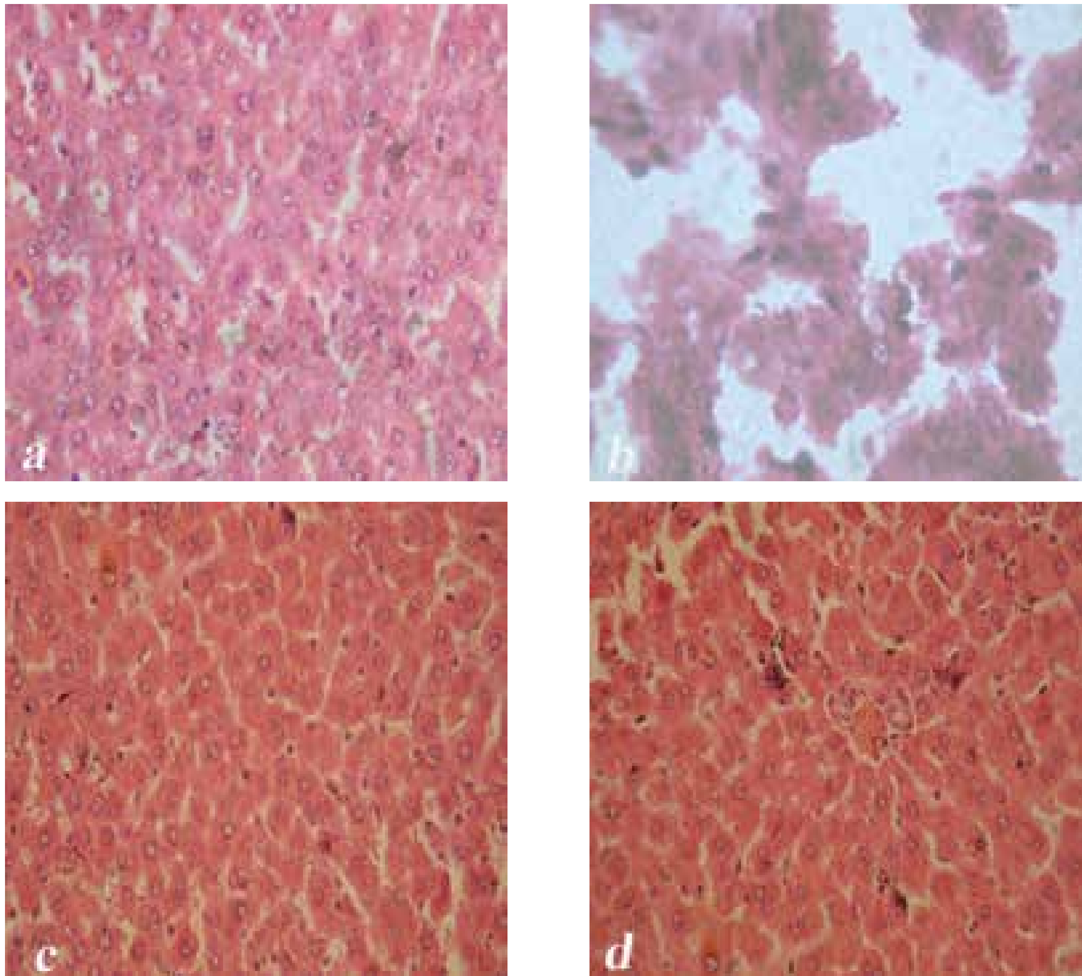

Fig - 1 Histological evidence of the protective effect of the ethanolic extract of coded drug 222/glibenclamide on liver of streptozotocin induced diabetic Wistar rats. a) Normal control rat liver showing normal hepatocytes with well brought out nuclei and cytoplasm. B) Liver of diabetic rat showing degenerated parenchymatous cells with severe necrosis and dialation of sinusoids. c) Liver of diabetic rat after treatment with 222 extract at 125 mg/kg, showing hepatocytes with near normal appearance and minimal necrosis. d) Liver of diabetic rat after treatment with glibenclamide showing normalcy of hepatocyte arrangement. (*Magnification: x* 400)

catalase and GPx are responsible for the detoxification of deleterious free radicals. SOD is one of the most important enzymes in the antioxidant enzymatic system which catalyses the disputation of superoxide radicals to produce H_2O_2 and molecular oxygen, hence diminishing the toxic effects caused by the radicals. The enzyme catalase catalyses the reduction of H_2O_2 and protects the tissues from highly reactive hydroxyl radicals (Baynes, 1995). GPx minimizes the oxidative damage by detoxifying H_2O_2 in low concentration. Table 7 shows the estimated values of catalase, SOD and GPx in the liver of normal and diabetic rats treated with the coded plant 222 leaf ethanolic extract and standard drug glibenclamide. The levels of these antioxidant enzymes were found to be lowered in STZ-induced diabetic rats. However, treatment with the coded plant 222 leaf ethanolic extract (125 mg/kg) and glibenclamide (5 mg/

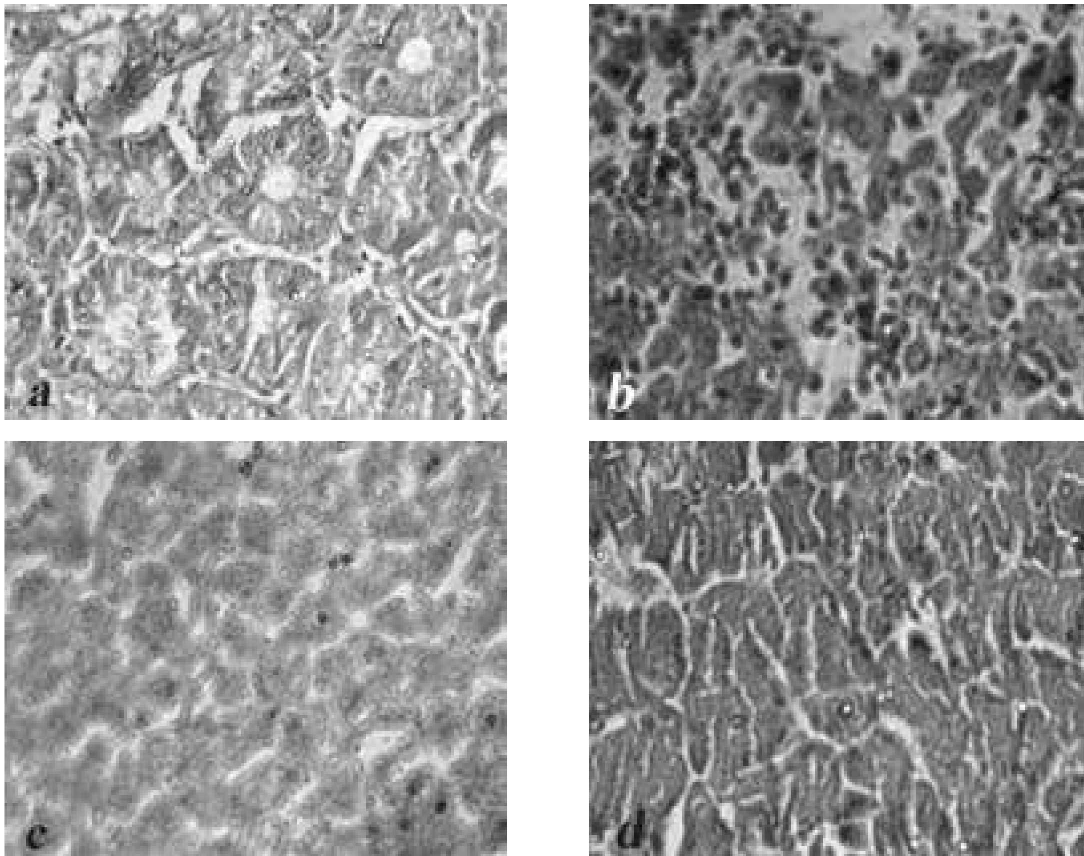

Fig - 2 Histopathological evidence of the protective effect of the ethanolic extract of 222/glibenclamide on pancreas of streptozotocin-induced diabetic rats. a) Normal control rat pancreas showing normal pancreatic cells (x 400). b) Pancreas of diabetic rat showing damaged pancreatic β-cells (x 400). c) Pancreas of diabetic rats after the treatment with 222 ethanolic extract (125 mg/kg) showing pancreatic β- cells almost similar to normal control (x 400). e) Pancreas of diabetic rat after treatment with glibenclamide, showing normalcy of pancreatic β - cells (x 400).

kg) resulted in the elevation of these enzymes compared to diabetic control group.

Histopathological examination confirmed the protective effect of 222 extract (125 mg/kg) (Fig. 1-3). The section of pancreas from diabetic control showed atrophy of β-cells and vascular degenerative changes in islets. The coded plant 222 leaf ethanolic extract (125 mg/kg) and glibenclamide (5 mg/kg) treated rats showed increased number of islets in the pancreas. The possible mechanism by which the coded plant 222 leaf ethanolic extract brings about decrease in blood glucose level may be by stimulation of surviving β-cells of islets of langerhans to release more insulin. The present study also showed an increased activity of antioxidant enzyme system of the liver indicating an adaptive mechanism in response to oxidative stress generated by STZ.

The results of the present study indicate that the coded plant 222 extract lead to the regeneration or proliferation of the pancreatic β-cells. Since pancreas contains quiescent and stable β-cells which have the capacity, thus the surviving cells proliferate by replication to

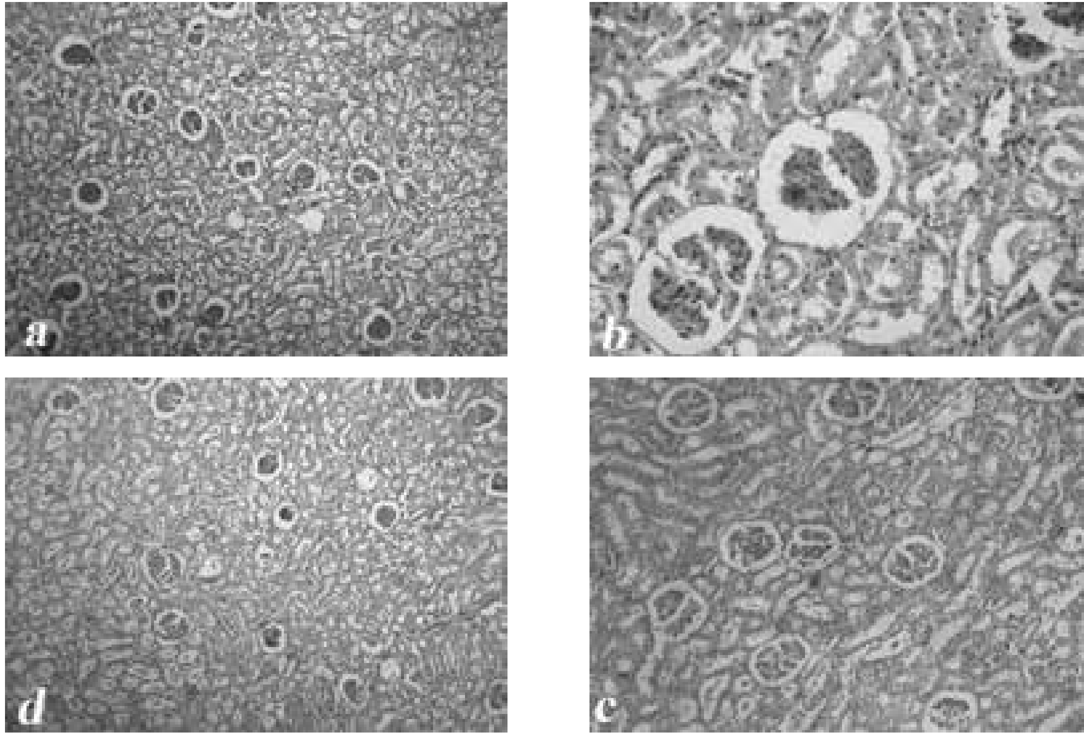

Fig - 3 Histological evidence of the protective effect of the ethanolic extract of 222/glibenclamide on kidney of streptozotocin-induced diabetic rats. a) Normal control rat kidney showing normal architecture of kidney (x 400). b) Kidney of diabetic rat showing degenerative changes and expanded glomerulus and thickening of the walls of renal tubules (x 400). c) Kidney of diabetic rat after the treatment with 222 ethanolic extract (125 mg/kg) showing glomerulus with reduction in thickening of walls of renal tubules (x 400). d) Kidney of diabetic rat after treatment with glibenclamide showing normalcy of Bowman's capsule, almost similar to normal rat kidney (x 400).

supplicate the lost cells. New pancreatic cells can be formed by neogenesis or by replication of pre-existing differentiated cells. Hence it is assumed that the extract is also responsible for the proliferation of β-cells, as there are already reports showing extracts of other medicinal plants have β-cell regenerative potential (Yadav *et al*., 2014)

Conclusion

In conclusion, the coded plant 222 leaf ethanolic extract exhibited anti-hyperglycaemic effect by significantly reducing the blood glucose levels in diabetic rats and it also reduced the lipid profile parameters in diabetic animals. The extract prevents the free radical formation or it may scavenge the reactive oxygen species through various antioxidant systems. The histopathological investigation along with the biochemical evaluation suggests the possibility of the regeneration of islets of diabetic pancreas by the extract treatment.

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