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## Hypoglycemic Effect of *Semecarpus anacardium* in Streptozotocin Induced Diabetic Rats

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**Abstract:** Diabetes mellitus is one of the major causes of death worldwide, due to the complexity of the diseases associated with it, for which treatment is far from complete. Since synthetic hypoglycemic are often associated with side effects, the study was designed to evaluate the hypoglycemic activity of a herbal drug *Semecarpus anacardium*, as herbal drugs are generally considered nontoxic. Streptozotocin 50 mg kg<sup>-1</sup> b.wt. was administered as intraperitoneal injection to rats. Three days after induction, the animals were divided into 3 groups. One group was left untreated, the other received *Semecarpus anacardium* (SA) nut milk extract at a dose of 300 mg kg<sup>-1</sup> b.wt. and the third group received Metformin at a dose of 500 mg kg<sup>-1</sup> b.wt. Two other groups control and drug control animals were also included for the study. Twenty one days after treatment, the animals were sacrificed. Body weight and blood glucose were determined. Insulin and C-Peptide were assayed. RT-PCR was carried out for O-GlcNAse and GAFT in pancreas. Immunoblot was done to assess the expression of PPAR gamma in skeletal muscle. After treatment with *Semecarpus anacardium*, there was an increase in body weight, insulin and C-peptide, decrease in blood glucose levels and the GAFT. The mRNA levels of O-GlcNAse and the protein levels of PPAR gamma were also increased in the *Semecarpus anacardium* treated group. The results of the present study reveals the hypoglycemic activity of *Semecarpus anacardium* in Streptozotocin induced diabetic rats.

**Key words:** Glucose tolerance test, N-acetyl-β-D glucosaminidase, semecarpoflavonone, insulin, C-peptide, Glutamine: Fructose-6-phosphate amidotransferase

### INTRODUCTION

Diabetes Mellitus is a group of metabolic disorders characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (American Diabetes Association, 2006) in conjugation with gross abnormalities in glucose homeostasis and lipid metabolism, which is affecting several millions of population all over the world (Cohen and Goedert, 2004). World Health Organization (WHO) in the year 2000 estimated the prevalence of Diabetes worldwide to increase from 171 million in 2000 to 376 million in 2030. According to the report of International Diabetes Federation in 2003 the number of people having diabetes was estimated to be about 194 million or 5.1% of adult population which is expected to increase to about 333 million or 6.3% by 2025. The symptoms include polyurea, polydipsia, polyphagia, weight loss, fatigue, cramps, constipation and blurred vision.

Streptozotocin (STZ) was first reported to be diabetogenic in 1963 (Rakieten *et al.*, 1968). It consists of a methyl nitrosourea side chain linked to the C<sub>2</sub> position of D-glucose. Owing to structural resemblance with D-glucose it is up taken by the glucose transporter (GLUT2) which is expressed in the pancreas, liver and kidney of rodents. After administration STZ is rapidly cleared from the bloodstream (serum half-life, 15 min) and beta cell necrosis can be detected by electron microscopy within hours after STZ injection. STZ selectively damages the β-cell of pancreas and the specificity resides mainly on its selective entry into β-cell and weak antioxidant reserve of β cells (Modak *et al.*, 2007). Two hours after STZ injection, hyperglycemia is observed with a concomitant drop in blood insulin. About 6 h later, hypoglycemia occurs with high levels of blood insulin. Finally, hyperglycemia develops and blood insulin levels decrease (Szkudelski, 2001). These changes in blood glucose and insulin concentrations reflect abnormalities in β cell function.

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The use of medicinal plants for the treatment of Diabetes Mellitus dates back from the Ebers papyrus of about 1550 BC. In recent years, traditional and complementary medicine has seen an upsurge in its popularity for the treatment of different diseases, as herbal drugs are generally out of toxic effect (Kameswara Rao *et al.*, 2003). *Semecarpus anacardium* Linn. (family: Anacardiaceae) called as marking nut is used in Indian medicine for the treatment of insanity, fever, asthma, cancer etc. (Kurup *et al.*, 1979). Phytochemical studies revealed the presence of phenolic compounds like Semecarpol in the nuts. The presence of biflavonoids like Jeediflavone, Galluflovone etc. (Murthy, 1992) and sterols have been reported. Bhilawanol, a catechol derivative and monohydroxy phenol called semecarpol, were also isolated from the nuts (Gedam *et al.*, 1974). The TLC and HPLC and HPTLC analysis of the nut and milk extract confirmed the presence of the above compounds (Sahoo *et al.*, 2008; Aravind *et al.*, 2008; Shin *et al.*, 1999; Nair *et al.*, 2009). The presence of vitamins, linoleic, myristic, oleic, palmitic and stearic acids have also been reported (Rai *et al.*, 2000). Vijayalakshmi *et al.* (2000) reported the presence of carbohydrates, phenols and flavonoids in the milk extract and it is non toxic up to a dose of 2000 mg kg<sup>-1</sup> b.wt. when administered in rats. The extract also possess antioxidant, anti-inflammatory, antiarthritic, anticancer (Nair *et al.*, 2009) and hypoglycemic (Arul *et al.*, 2004) activities. Selvam and Jachak (2004) have reported the anti-inflammatory activity and Cyclooxygenase inhibitory property of the nut. Although, a botanical substitute for insulin seems unlikely, compounds that stimulate insulin biosynthesis and secretion or promote peripheral glucose uptake and utilization are realistic possibilities. In the search for new compounds within the exploration of natural resources, the present study was undertaken to evaluate the hypoglycemic effect of *Semecarpus anacardium* in Streptozotocin (STZ) induced diabetic rats.

## MATERIALS AND METHODS

The drug *Semecarpus anacardium* (SA) nut extract contains purified nuts of *Semecarpus anacardium* and cow's milk in the ratio as indicated in the Formulary of Siddha Medicine. Two hundred grams of the nut was boiled with 500 mL of milk, which was repeated thrice. The decoction was stored (Formulary, 1972). The study was conducted from August 22 2006 till May 3 2009 in the Department of Medical Biochemistry, University of Madras, Taramani Campus under the supervision of Dr. P Sachdanandam.

**Animals:** Male Albino rats of Wistar strain weighing 260±10 g were used in this study. The animals were housed in polypropylene cages under a control environment with 12 h light/dark cycles and a temperature between 27 and 37°C and were given a commercial diet with water ad libitum. All experiments involving animals were conducted according to NIH guidelines, after obtaining approval from the Institute's Ethical Committee (02/075/06).

**Experimental design:** Male albino Wistar rats weighing 250-270 g were divided into five groups of six animals each.

**Group I:** Control animals - Normal healthy controls received olive oil (0.5 mL) orally by gastric intubation for 21 days daily

**Group II:** Diabetes induced-(50 mg kg<sup>-1</sup> b.wt.) Streptozotocin dissolved in 0.5 mL of 0.1 M citrate buffer pH 4.5

**Group III:** SA treated - Three days after the induction of diabetes, SA (300 mg kg<sup>-1</sup> body weight dissolved in 0.5 mL olive oil) was administered by gastric intubation for 21 days daily

**Group IV:** Metformin treated-Three days after the induction of diabetes, Metformin (500 mg kg<sup>-1</sup> b.wt. dissolved in 0.5 mL physiological saline) was administered by gastric intubation for 21 days daily

**Group V:** Drug control-Animals received SA at a dose of 300 mg kg<sup>-1</sup> b.wt. in olive oil (0.5 mL) orally by gastric intubation for 21 days daily

**Biochemical analysis:** After the experimental period, the animals were killed by cervical decapitation. The pancreas were excised immediately and immersed in ice-cold physiological saline. Ten percent homogenate was prepared with fresh tissue in 0.01 M Tris-HCl buffer (pH 7.4) and were used for the assays. Blood was collected, serum separated and was also used for the analysis. Skeletal muscle was used for immunoblot analysis.

**Body weight changes and blood glucose measurement:** Body weight changes and the blood glucose level were measured during the initial and the final phase of the study.

**Glucose tolerance test:** After an over night fast a zero-min blood sample was taken from tip of tail vein of the rats. Glucose solution at the dose of 0.5 g kg<sup>-1</sup> b.wt./1 mL

physiological saline was administered intravenously through femoral vein and blood samples were collected at 30th, 60th, 90th and 120th min for the measurement of glucose levels by single touch glucometer after the administration of glucose (Young *et al.*, 1995).

**Estimation of insulin and C peptide:** Insulin measurement was performed using LINCO Research Inc. (St. Charles, MO) radioimmunoassay kit. The values are expressed as pM.

**RT-PCR analysis for OGLcNase and GAFT:** The RT-PCR for mRNA expressions of N-acetyl-β-D glucosaminidase.

(OglcNase) and Glutamine: Fructose-6-Phosphate Amidotransferase (GAFT) were done according to manufacturing guidelines (Qiagen One Step RT-PCR mix). Briefly, the reaction mixture contained 10 μL of 5x Qiagen One step RT-PCR Buffer containing final concentration of 2.5 mM MgCl<sub>2</sub>, 2 μL of dNTP Mix (0.4 mM of each dNTP as final concentration), 5 μL of each sense and antisense primers of OGLCNase and GAFT, 5 μL of sense and antisense primers of housekeeping actin (each of 0.6 μM final concentration), 1.0 μg of template RNA, 2 μL of Qiagen One step RT-PCR enzyme mix and made upto 50 μL with RNase free water. Actin was used as internal control. Generally, the cycling parameters for the PCR reaction were template denaturation and annealing followed by extension. The cycles were followed by incubation at 72°C for 10 min.

To compare the amount of steady state mRNA, 10 μL of each PCR product was resolved onto 2% agarose gel using TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). After electrophoresis, the gels were viewed under UV light and digital images were captured on Hero lab gel documentation system. After initial activation at 50 degrees for 30 min the following procedures were carried out for the respective mRNA's. The base pairs, sequences, product length and the annealing temperature are given in Table 1.

Table 1: The base pairs, product length and the annealing temperature

Name	Primer sequence	Product length (bp)	Tm degrees
GFAT	Sense -5' agc tgt gca aac act cca ga3'	500	55
	Anti-sense - 5' tta cga cca gga ctc taa cc 3'		
O-GlcNac transferase	Sense - 5' gggttagctgagttggcacat 3'	252	56
	Anti-sense - 5' ctgtccagccttcgacactgg 3'		
β Actin	Sense -5' aaccctaaggccaacctgaaaag 3'	226	-
	Anti-sense -5' tcatg ggtagcttcggt c 3'		

Table 2: Body weight and blood glucose of control and experimental animals

Parameters	Group I (Control)	Group II (STZ)	Group III (STZ+SA)	Group IV (STZ+Metformin)	Group V (SA)
Body weight (g)	268.64±28.4	174.11±18.1 <sup>a*</sup>	229.35±20.8 <sup>b*</sup>	193.43±18.76 <sup>b*c*</sup>	264.83±27.32 <sup>NS</sup>
Glucose (mg dL <sup>-1</sup> )	106.35±9.45	277.15±29.14 <sup>a*</sup>	137.68±15.36 <sup>b*</sup>	179.82±17.5 <sup>b*c*</sup>	108.96±9.95 <sup>NS</sup>

Values are expressed as Mean±SD for six animals. Comparisons are made between, <sup>a</sup>Group II vs. Group I, <sup>b</sup>Group III and IV, vs. Group II, <sup>c</sup>Group IV vs. Group III, <sup>d</sup>Group V vs. Group I. The symbol, \*represents the statistical significance at p<0.05, NS: Non significant

**Western blot analysis of PPAR γ in skeletal muscle of control and experimental animals:**

The protein concentration of skeletal muscle was estimated. The samples (equal amount of protein; 50 μg) were boiled with Sample Solubilizing Buffer (SSB) for 5 min and separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was transferred onto a nitrocellulose membrane (Hybond C+, Amersham life sciences) at 30 V for 5 h. Membrane was then washed thrice with PBS and blocking was done with TBST buffer (20 mM Tris, 500 mM NaCl and 0.1% Tween 20, pH 7.5) containing 5% non-fat dry milk. Then, the membrane was incubated with primary antibody (rabbit polyclonal anti PPAR γ) in TBST buffer containing 1% non-fat dry milk and agitated gently at room temperature for 3 h. After incubation with the primary antibody, the blots were washed thrice for 5 min with TBST buffer and incubated for 75 min at room temperature with horseradish peroxidase (HRP) conjugated secondary antibody (1:500 dilutions) in phosphate-free TBST buffer containing 5% non-fat dried milk. The bands were detected using DAB/hydrogen peroxide chromogen system.

**Histopathological studies:** All histopathological studies for pancreas were performed using standard procedures with uniform conditions of fixation and staining of 5 μm sections with Hematoxylin and Eosin (H and E).

**Statistical analysis:** The values are expressed as Mean±SD for six rats in each group. Statistically significant differences between the groups were calculated using one-way Analysis of Variance (ANOVA), followed by student-Newman-Keuls for multiple comparisons using Statistical Package for Social Sciences (SPSS) computer package. Values of p<0.05 were considered to be significant.

## RESULTS AND DISCUSSION

**Effect of SA on body weight and blood glucose level:** The STZ-induced diabetic rats were characterized by hyperglycemia along with hyperphagia, polydipsia and polyurea and decreased body weight. The effects of SA on body weight and blood glucose are shown in Table 2, the body weight in Group II animals decreased by 54.2% when compared to Group I animals which significantly

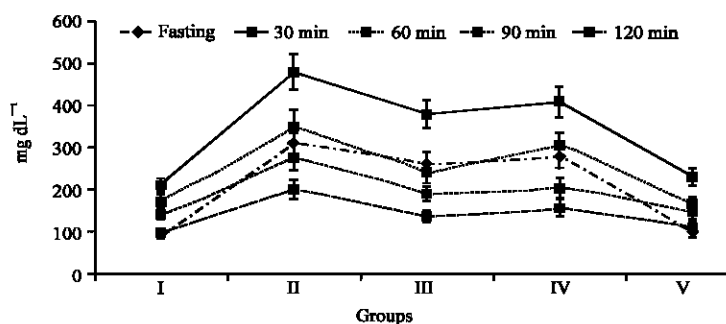


Fig. 1: Glucose tolerance test in control and experimental animals. Values are expressed as Mean±SD for six animals. Comparisons are made between, a- Group II vs. Group I, b- Group III and IV, vs. Group II, c Group IV vs. Group III, d Group V vs. Group I. The symbol, \*represents the statistical significance at  $p < 0.05$ , NS- Non Significant

increased ( $p < 0.05$ ) upon treatment with SA (Group III) and Metformin. (Group IV). The blood glucose increased by 2.6 fold in untreated diabetic animals which decreased by 0.49 fold and 0.35 fold upon administration of SA and Metformin. A significant difference ( $p < 0.05$ ) was found when Group III and Group IV animals were compared. No significance change in body weight and blood glucose was found when the normal (Group I) and the drug control (Group V) groups were compared.

**Effect of SA on glucose tolerance tests:** After an oral glucose load, the glucose peak was seen after one hour which gradually decreased as the time increased and returned to normal level in control (Group I) and drug control animals (Group V) and to near normal levels in the SA treated (Group III) while Metformin treated (Group IV) animals showed a slight increase even after 2 h. When the blood glucose levels between SA treated and Metformin treated groups were compared SA was more effective in decreasing the blood glucose levels than Metformin (Fig. 1).

**Effect of SA in the insulin and C-peptide levels:** Due to the destruction of the pancreatic  $\beta$  cells the values of both insulin and C peptide decreased by 48 and 52 % in Group II animals while it increased by 23.4 and 32.5% in Group III animals and 7.6 and 10.7% in Group IV animals. There was significant difference ( $p < 0.05$ ) when Group IV and Group III were compared while no significance was obtained when Group I and Group V animals were compared (Fig. 2).

**Effect of SA on the mRNA expression of O-GlcNAcase and GAFT:** The decreased mRNA expression of O-GlcNAcase and increased mRNA expression of GAFT in the pancreas of Group II animals and the concomitant increase and decrease in Group III and Group IV animals

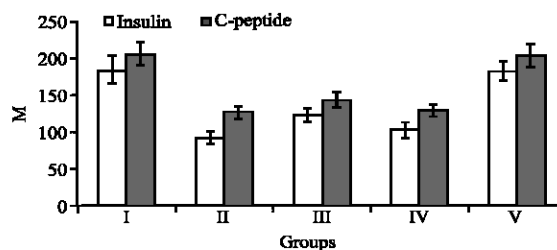


Fig. 2: Insulin and C-Peptide levels in control and experimental animals Values are expressed as Mean±SD for six animals. Comparisons are made between, a- Group II vs. Group I, b- Group III and IV, vs. Group II, c Group IV vs. Group III, d Group V vs. Group I. The symbol, \*represents the statistical significance at  $p < 0.05$ , NS- Non Significant

are shown in Fig. 3a and b. The expression of O-GlcNAcase decreased in the Group II animals due to the administration of STZ. The decreased level significantly increased in Group III animals while there was very minimal increase in Group IV animals. The increased mRNA expression of GAFT in untreated animals (Group II) decreased in the SA (Group III) and Metformin treated groups (Group IV). Group I and Group V animals showed no significant alteration in mRNA expression.

**Effect of SA on the protein expression of PPAR  $\gamma$  in the skeletal muscle of experimental animals:** The increased expression of PPAR  $\gamma$  in the SA treated group is shown in Fig. 4. The decreased expression seen in Group II animals was found to be increased in Group III animals with no increase in Group IV animals. No changes were observed when the control and drug control groups were compared.

**Histopathological observation of the pancreas of control and experimental animals:** The changes that were observed in the H and E stain of the pancreas section are

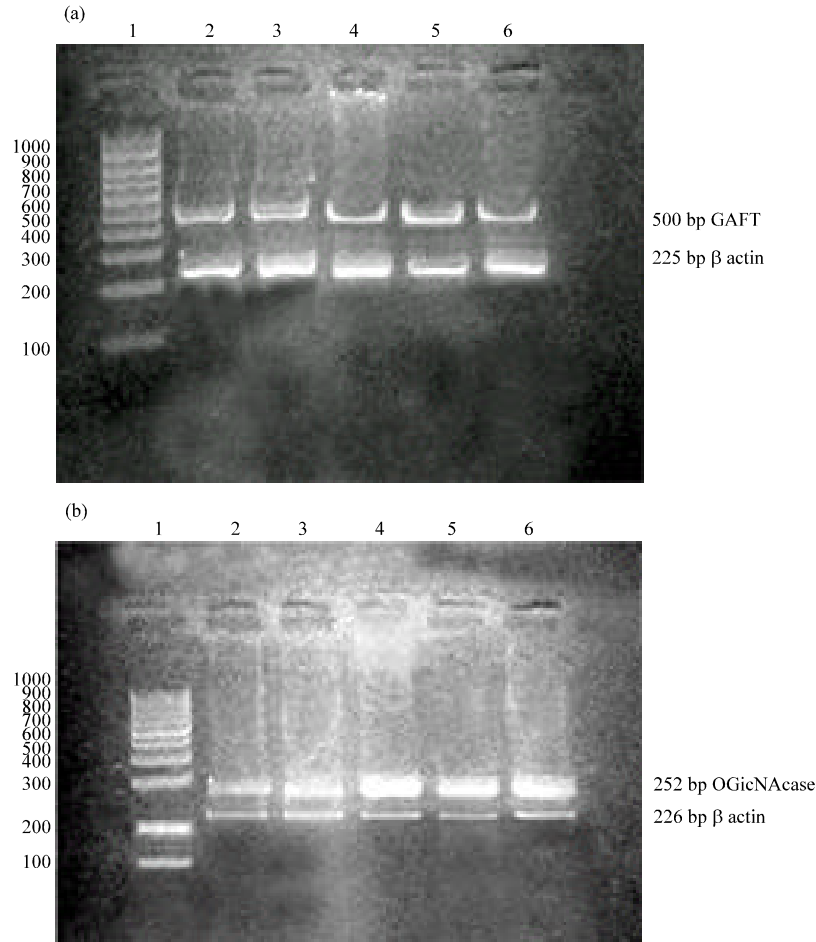


Fig. 3: Effect of SA on the gene expression of (a) O-GlcNAcase and (b) GAFT



Fig. 4: Effect of SA on the protein expression of PPAR  $\gamma$  in the skeletal muscle of experimental animals. Lane 1 control, Lane 2- STZ induced, Lane 3- SA treated, Lane 4- Metformin treated, Lane 5 SA drug control

Table 3: Observations between control and experimental animals

A	Control	The section shows normal histology of pancreas ( $\times 100x$ ).
B	STZ induced	Reduced pancreatic islets with focal fatty change are seen ( $\times 100x$ ).
C	SA treated	The pancreatic islets shows near normal cellularity and normal histology ( $\times 100x$ ).
D	Metformin treated	The pancreatic islets fatty change with near normal histology ( $\times 100x$ ).
E	SA drug control	The section shows normal histology of pancreas ( $\times 100x$ ).

shown in Fig. 5a-e. Table 3 shows the observations that were seen in the control and experimental animals.

In both human and economic terms diabetes is one of the worlds's most costly diseases. Experimental diabetes using various animal models has provided insight into the physiological and biochemical derangement of the diabetic state. Streptozotocin injection resulted in decreased body weight and significant elevation of fasting blood glucose level in the untreated animals compared to control and the diabetic animals were unable to tolerate extra glucose load after given orally. The blood

glucose levels returned to near normal levels after two hours in the SA treated group. The increase in the insulin levels with the concomitant increase in the C-peptide levels shows the hypoglycemic activity of SA which is in accordance with the report of Arul *et al.* (2004). Since insulin and C-peptide are produced in equimolar quantities by the  $\beta$  cells, increase in insulin and C-peptide in Group III animals clearly indicate that the increase in insulin may be due to increased out put from intact residual  $\beta$  cells of the pancreas. C-peptide is a surrogate marker of insulin release which is generally regarded

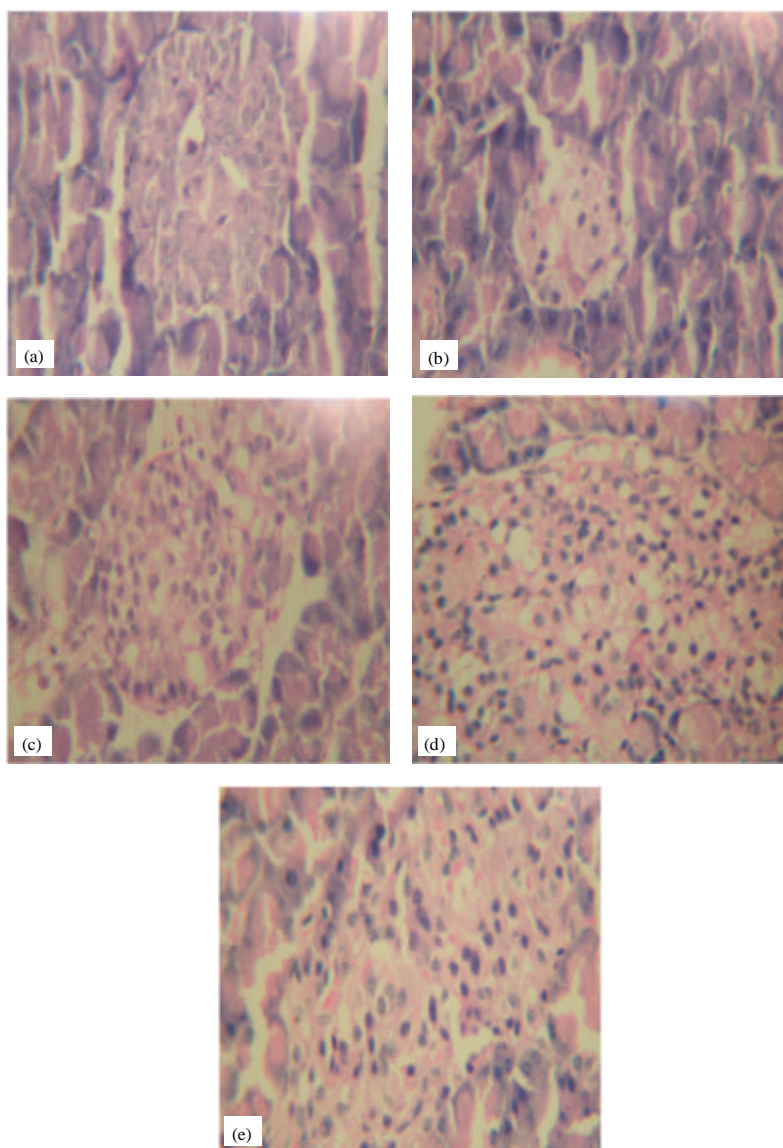


Fig. 5: (a-e) Histopathological observation of pancreas by H and E stain

biologically inert. According to recent reports, apart from the facilitation of proinsulin folding to allow the accurate alignment of the A and B chains of insulin, it plays a protective role in diabetes by ameliorating diabetic complications (Al-Rasheed *et al.*, 2006). C-peptide has been shown to exert beneficial effects on both renal function and morphology in diabetic nephropathy (Samnegård *et al.*, 2005). It has been found to stimulate numerous intracellular signaling pathways in proximal tubular cells such as mitogen-activated protein kinases, phosphatidylinositol 3-kinase (PI3-K)/Akt and protein kinase C (Al-Rasheed *et al.*, 2006). The increase in

C-peptide seen in the drug treated group clearly aligns with the reports that it activates the intracellular pathways.

The ability of pancreatic  $\beta$  cells to sense glucose and secrete appropriate amounts of insulin is critical to glucose homeostasis. Streptozotocin (STZ), displays  $\beta$ -cell-specific toxicity due to the inhibition of O-GlcNAcase, increasing the levels of O-GlcNAc proteins in  $\beta$  cells by the formation of a transition state analog which resides tightly bound in the active site and modifies of the cysteine and sulphhydryl residues present in the active site of the enzyme (Toleman *et al.*, 2006). The

O-linked glycosylation is reversed by O-GlcNAc  $\beta$ -acetylglucosaminidase (O-GlcNAcase) (Kaneto *et al.*, 2001). The source of GlcNAc for the O-GlcNAc-modification is the Hexosamine Biosynthetic Pathway (HBP), with one of the end products being UDP-GlcNAc, the donor sugar nucleotide for O-GlcNAc. Thus, increased glucose flux through the HBP can contribute to increased levels of O-GlcNAc modified proteins. Accumulation of O-GlcNAc-modified proteins is also achieved by inhibition of N-acetyl- $\beta$ -D glucosaminidase (Dong and Hart, 1994). In peripheral tissues such as muscle and fat, the hexosamine biosynthetic pathway serves as a glucose sensor linking the rate of glucose flux to the activity of the glucose transport system and transcription machinery (Luo *et al.*, 2007). Hyperglycemia increases the glucose flux through hexosamine pathway, resulting in increased production of UDP-GlcNAc the donor of O-GlcNAc, which serves as a substrate for O-GlcNAc modification (Farook *et al.*, 2002). A failure to turn over O-GlcNAc on unknown substrates leads to two problems in the  $\beta$ -cells: an acute defect in insulin secretion and eventually  $\beta$ -cell death through apoptosis. Once insulin secretion begins to fail, the glucose excursions become exacerbated and  $\beta$ -cell failure accelerates. Thus, hyperglycemia-induced O-GlcNAc modification can block both activation of Akt kinase activity and transduction of the insulin signal (Vosseller *et al.*, 2002). Flux through the hexosamine biosynthetic pathway has been implicated in the development of insulin resistance and glucose toxicity in peripheral tissues (Daniels *et al.*, 2000). The finding that O-linked GlcNAc transferase and O-GlcNAc residues are at very high levels in  $\beta$ -cells argues that they maintain a high flux through the hexosamine biosynthetic pathway. This is consistent with the hexosamine pathway playing an essential role in glucose metabolism and glucose sensing in the  $\beta$ -cell. The increase in mRNA expression of O-GlcNAcase may be due to the reactivation of the sulphhydryl groups in the active site of the enzyme which were inactivated by STZ. This in turn decreases the accumulation of O-linked proteins and expression of the enzyme OGT, whose product serves as the substrate for the enzyme GAFT. This may be the possible mechanism of the drug by which it decreases the flux through the hexosamine pathway finally leading to the decreased accumulation of the O-GlcNAc modified proteins.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily related to retinoid, steroid and thyroid hormone receptors (Patsouris *et al.*, 2004). The activity of PPARs is regulated by various agents including insulin, fatty acids, fibrates, leukotriene

B<sub>4</sub>, prostaglandin J<sub>2</sub> and synthetic thiazolidinedione drugs (Rieusset *et al.*, 1999). The major role of PPAR  $\gamma$  is to regulate genes involved in carbohydrate and lipid metabolism (Yki-Jarvinen *et al.*, 2004). The PPAR  $\gamma$  agonists have been shown to exert antihyperglycemic and antihyperlipidemic effects in animal and clinical studies (Barroso *et al.*, 1999). In addition to antidiabetic effect, PPAR  $\gamma$  agonists have protective effects against diverse injuries in kidney, heart, brain and peripheral vascular tissues. PPAR's also regulate lipid metabolism (Auwerx *et al.*, 1996). The hypoglycemic action of SA may be due to the presence of flavonoids, since flavonoids could exert an effect on glucose transport, the insulin-receptor function and peroxisome proliferator-activated receptor (PPAR) activation (Yoon *et al.*, 2001).

The difference between the protein expression between group III and IV animals show that SA may bring about the hypoglycemic action by the activation of PPAR  $\gamma$ . The increased expression in Group III animals also indicates that SA may exert hypolipidemic activity by activation of PPAR  $\gamma$ .

The hypoglycemic activity of SA was earlier reported by Kothai *et al.* (2005). The observed hypoglycemic effect of SA may be due to the increased insulin output from the residual intact functioning  $\beta$  cells, reactivation of O-GlcNAcase, decreasing the accumulation of glycosylated proteins which finally leads to decreased flux through the Hexosamine pathway. The observed hypoglycemic activity may be due to the presence of phenolic compounds (Rao *et al.*, 1973) in the nut extract. The major fractions are flavonoids like Semecarpufuranone, Jeediflavanone Gallufuranone, Nallufuranone, Semecarpetin, Anacardiflavanone (Murthy, 1992) which may have the capacity to decrease the blood glucose levels synergistically as in line with other hypoglycemic flavonoids (Jia *et al.*, 2008).

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