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ANTIHYPERGLYCEMIC EFFECT OF CHITOSAN OF *PODOPHTHALMUS VIGIL* IN STREPTOZOTOCIN INDUCED DIABETIC RATS

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ABSTRACT

The present study was designed to investigate the antihyperglycemic activity of chitosan of *Podophthalmus vigil* on normal and streptozotocin (STZ) diabetic rats. Diabetes was induced into male albino wistar rats by intraperitonial administration of STZ. Chitosan was administered orally at three different doses to normal and STZ-diabetic rats for 30 days. The diabetic rats showed an increase in levels of blood glucose and glycosylated hemoglobin (HbA1c) and a decrease in the levels of insulin and hemoglobin addition, diabetic rats showed a significant reduction in the activity of glucokinase and an elevation in the activities of gluconeogenic enzymes such as glucose-6-phosphatase and fructose-1, 6-bisphosphatase. Treatment with chitosan significantly decreased plasma glucose and HbA1c, and increased the insulin and Hb. Chitosan administration to diabetic rats reversed these enzyme activities in a significant manner. Thus, the results show that chitosan possesses an antihyperglycemic activity in the control of diabetes. The 300 mg dose of the extract produced a better effect than 100 and 200 mg doses.

INTRODUCTION: Diabetes mellitus (DM) is a group of metabolic disorder characterized by elevated blood glucose level resulting from the defects in insulin secretion, insulin action or both [1]. The world prevalence of diabetes among adults is expected to be 6.4%, affecting 285 million adults, in 2010, and will increase to 7.7% i.e. about 439 million adults by 2030. Between 2010 and 2030, there will be a 69% increase in numbers of adults with diabetes in developing countries and 20% increase in developed countries [2]. Currently available oral hypoglycemic drugs for the treatment of DM have characteristic profile of adverse effects. Hence, research is focused to screen the natural source that are used traditionally for the treatment of DM to find a newer lead drug molecule with more potential and lesser side effects than the existing hypoglycemic agents [3].

Chitosan, a biopolymer of glucosamine derived from chitin that is chemically similar to that of cellulose, is not digestible by mammalian digestive enzymes and acts as a dietary fiber in gastrointestinal tract [4]. It is well known for its cholesterol-lowering effect. However, relative less information is available about the effect of chitosan on plasma lipids and glucose control in diabetic subjects.

Previous study has reported that chitosan reduced the concentration of plasma cholesterol in animals [5, 6, 7] and type II diabetes patients in combination with hypercholesterolemia [8]. Increased fecal cholesterol accompanied with or without bile acid excretion by interfering intestinal micelle formation was proposed to be the mechanisms responsible for the hypocholesterolemic properties [4, 6, 7].

One of the recently reports demonstrated that chitosan has a hypoglycemic effect in STZ-induced diabetic animals [9]. Other studies also found that low molecular weight chitosan (average MW about 2.0 X10^4 Da) [10, 11] as well as chitosan oligosaccharides [12], can reduce plasma glucose level in diabetic animals. The aim of the present study was to investigate the antihyperglycemic effect of the chitosan in STZ-diabetic rats.

MATERIALS AND METHODS:

Raw materials preparation: Shell materials were obtained from the wastes of *Podophthalmus vigil* were collected from Mudasalodai landing centre, south east coast of India. Then packed in plastic bags and stored at -20°C before and during transportation to the laboratory. Shells were scraped free of loose tissue from the crab wastes in laboratory, washed in double distilled water and dried at 60°C. Standard chitin and chitosan obtained from Himedia, Mumbai. All other reagents were of analytical grade.

Isolation of Chitin by Takiguchi, (1991a) [13]:

A. **Demineralization:** 20 gm of sample powder was demineralised with 300ml of 2N Hcl for 24 hours with constant stirring and thus filtered. The filterate was again washed with distilled water and filtered till the liquid showed neutral PH. The filterate was then dried in a vacuum dryer and weighed.

B. **Deprotenization:** The sample was then deprotenized with 300ml of 1N NaOH at 80°C for 24 hour with constant stirring. The NaOH was exchanged intermittently and the sample was washed with distilled water every time before adding fresh NaOH. After 24 hour the sample was filtered. The sample filtrate was washed as before and dried. The weight was noted.

Isolation of Chitosan by Takiguchi, (1991b) [14]: The deprotenized sample was deacetylated with 250 ml of 40% NaOH, treated for 6 hours at 110° C constant stirring. The sample was filtered and the filtrate was washed as before, 200 ml of 10% acetic acid was added to the sample and stored for 12 hours at room temperature with constant stirring. The dissolving sample was reprecipated by adding 40% NaOH to pH 10. The sample was then disalysed by deionised water. It was then centrifuged at 10,000 rpm for 10 minutes and the precipitate was freeze dried.

Animals: Healthy adult male albino Wistar rats, bred and reared in Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College, Annamalai University were used for the experiment. Weight matched animals (160-180 g) were selected and housed in polypropylene cages layered with husk and kept in a semi-natural light/dark condition (12 h light/12 h dark). The animals were allowed free access to water and standard pellet diet (Amrut Laboratory Animal Feed, Pranav Agro Industries Ltd., Bangalore, India). Animal handling and experimental procedures were approved by the Institutional Animal Ethics Committee, Annamalai University (Registration Number: 160/1999/CPCSEA, Proposal no. 858) and animals were cared in accordance with the "Guide for the care and use of laboratory animals" [15].

Source of Chemicals: Streptozotocin was purchased from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals were of analytical grade obtained from S.D. Fine Chemicals, Mumbai or Himedia Laboratories Pvt. Ltd., Mumbai, India.

Experimental induction of Diabetes: The animals were rendered diabetes by a single intraperitonial injection of STZ (40 mg/kg b.w.) in freshly prepared citrate buffer (0.1M, pH 4.5) after an overnight fast. STZ injected animals were given 20% glucose solution for 24 h to prevent initial drug-induced hypoglycemic mortality. STZ injected animals exhibited massive hyperglycemia (determined by glucose oxidase method) and diabetes in STZ rats was confirmed by measuring the fasting blood glucose concentration, 72 hour after injection with STZ. The animals with blood glucose more than 220 mg/dl were considered diabetic and used for the experiment.

Dosage Fixation study: Preliminary study was carried out to assess the effect of chitosan on blood glucose levels in diabetic rats. For each test chitosan preparation (100, 200 and 300 mg kg^{-1} b.w.) 6 animals were used. The chitosan and glibenclamide were administered orally using vehicle solution (1% glacial

acetic acid). After 15 days, the animals were fasted for 12 hours; blood was collected by retro orbital puncture for the estimation of plasma glucose. Administration of chitosan (15 days), at all the three doses, gave significant reduction of plasma glucose in STZ-diabetic rats. Since the chitosan at a 300 mg dose gave significant improvement on the body weight, and decreased blood glucose and thiobarbituric acid reactive substance levels, it was fixed as the optimum dosage for further work.

Experimental study: The animals were randomly divided into five groups of six animals each. Chitosan was suspended in 1% glacial acetic acid and fed by intragastric tube daily for 30 days.

Group I:	S Normal control (1 ml of 1% glacial acetic acid)
Group II:	Normal Control + 1ml of Chitosan (300mg/kg BW in 1% glacial acetic acid)
Group III:	Diabetic control (1ml 1% glacial acetic acid)
Group IV:	Diabetic + 1ml of chitosan (300 mg/kg BW in 1% glacial acetic acid)
Group V:	Diabetic + 1 ml of glibenclamide (600µg/kg BW in 1% glacial acetic acid)

After 30 days, the animals were fasted for 12hour, anaesthetized between 8.00 am to 9.00 am using ketamine (24mg/kg b.w. intramuscular injection), and sacrificed by decapitation. Blood was collected in tubes with a mixture of potassium oxalate and sodium fluoride (1:3) for the estimation of plasma glucose and ethylene diamine tetra acetic (EDTA) for the estimation of various biochemical parameters. Pancreas were surgically removed, washed with cold physiological saline, cleared off adherent lipids and immediately transferred to ice-cold containers. Erythrocytes were also prepared for the estimation of biochemical preparations.

Biochemical estimations: Glucose was estimated by the method of Trinder using a reagent kit [16]. Hemoglobin (Hb) and glycosylated hemoglobin (HbA_{1c}) were estimated by the methods [17, 18] respectively. TBARS was estimated by the method [19]. The insulin in the rat plasma was measured by the method of Burgi [20]. Glucokinase, glucose-6-phosphatase, fructose-1, 6-bisphosphatase and glucose 6-phosphate de-hydrogenase (G6PD) were assayed by the methods of [21, 22, 23, 24] respectively. Glycogen content was determined as described by Morales *et al*., [25].

Histological Study: For histological study, three rats from each group were perfused with cold physiological saline, followed by formalin (10% formaldehyde). The pancreas were excised immediately and fixed in 10% formalin. Then dehydrated on treatment with a serious of different concentration of ethanol and embedded in paraffin wax. 3-5µm thick sections were cut using a microtome and stained with haematoxylin and eosin. The specimens were evaluated with light microscope. All histopathological changes were examined by pathologist.

Statistical analysis: Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's MULTIPLE Range Test (DMRT). Results were expressed as mean±S.D. from six rats in each group. *P*-values <0.05 were considered significant.

RESULTS AND DISCUSSION: Table 1 shows the dose dependent effect of chitosan (15 days) on blood glucose, TBARS and body weight in normal and STZ-diabetic rats. The blood glucose and TBARS level elevated and body weight decreased significantly in diabetic rats as compared to normal rats. Treatment with chitosan lowered plasma glucose, TBARS levels, and elevated body weight significantly as compared to STZ-diabetic rats. Since chitosan at a dose of 300mg/kg body weight decreased the plasma glucose towards normally and this dose was taken for further studies.

Table 2 shows the effect of 30 days oral administration of chitosan on body weight, blood glucose and water intake in normal and STZ-diabetic rats. The plasma glucose, water intake increased and body weight decreased significantly in diabetic rats as compared to normal rats. Treatment with chitosan at 300mg/kg b.w. reversed these parameters towards normalcy.

Table 3 shows the levels of plasma insulin, Hb and HbA1c. Plasma insulin and Hb decreased and HbA1c increased significantly in STZ-diabetic rats and these values reversed towards normalcy on treatment with chitosan and glibenclamide.

Table 4 shows the activities of carbohydrate metabolic enzymes and glycogen content in liver of normal and diabetic rats. Decreased activities of glucokinase and glucose 6-phosphate dehydrogenase and level of glycogen were observed in the liver of diabetic rats and

increased on treatment with chitosan and glibenclamide. **Table 5** shows the changes in the activities of gluconeogenic enzymes in the liver and kidney of normal and diabetic rats. Increased glucose 6-phosphatase and fructose 1, 6-bisphosphatase activities were observed in the liver and kidney of diabetic rats and these activities decrease on treatment with chitosan and glibenclamide.

TABLE 1: EFFECT OF CHITOSAN ON PLASMA GLUCOSE, TBARS AND BODY WEIGHT IN NORMAL AND STZ-DIABETIC RATS, AFTER 15 DAYS

S. No.	Name of the group	Plasma glucose (mg/dl)		TBARS (mmole/dl)		Body weight(g)	
		Day 0	Day 15	Day 0	Day 15	Day 0	Day 15
1.	Normal	66.53±2.13	70.30±5.08 [a]	0.175±0.01	0.169±0.01[a]	176.12±6.11	193.13±6.03[a]
2.	Normal +Chitosan	65.47±1.98	60.12±4.25 [a]	0.155±0.01	0.157±0.01[a]	180.91±5.95	190.11±6.16[a]
3.	Diabetic control	248.24±47	268.21±6.57 [b]	0.242±0.02	0.298±0.02[b]	182.20±6.54	171.48±7.03[b]
4.	Diabetic+ chitosan (100 mg/kg b.w.)	242.47±12.82	235.25±12.57 [c]	0.248±0.02	0.233±0.02[c]	187.66±3.78	179.28±7.15[a,b]
5.	Diabetic+ chitosan (200 mg/kg b.w.)	248.74±13.92	227.49±18.29 [c]	0.246±0.01	0.229±0.02[c]	184.39±6.09	181.38±5.16[a,b]
6.	Diabetic+ chitosan (300 mg/kg b.w.)	246.19±13.80	206.73±19.08 [d]	0.252±0.03	0.219±0.02[c,d]	183.57±6.98	187.16±5.20[a,b]
7.	Diabetic+ glibenclamide (600 µg/kg b.w.)	248.14±15.92	201.17±11.62 [d]	0.247±0.01	0.201±0.02[d]	184.22±6.71	191.16±8.61[a]

Values are mean ± SD for six rats in each group (n=6), Values not sharing a common superscript differ significantly at p<0.05 (DMRT)

TABLE 2: EFFECT OF CHITOSAN ON BODY WEIGHT, BLOOD GLUCOSE AND WATER INTAKE IN NORMAL AND STZ-DIABETIC RATS, AFTER 30 DAYS

S. No.	Name of the group	Plasma glucose (mg/dl)		Body weight(g)		Average water intake (ml/day)
		Day 0	Day 30	Day 0	Day 30	
1.	Normal	64.65±2.08	71.30±7.08[a]	178.33±5.16	199.89±8.20[a]	30.15±2.09[a]
2.	Normal +Chitosan (300 mg/kg b.w.)	63.02±4.98	70.42±7.25[a]	181.72±6.10	200.83±7.40[a]	29.11±1.74[a]
3.	Diabetic control	246.25±15.60	288.31±16.27[b]	183.59±5.56	156.48±3.37[b]	51.47±4.84[b]
4.	Diabetic+ chitosan (300 mg/kg b.w.)	248.29±17.80	143.33±12.08[c]	184.92±6.08	171.08±5.54[c]	40.67±2.62[c]
5.	Diabetic+ glibenclamide (600 µg/kg b.w.)	252.24±17.92	102.27±8.62[d]	186.37±7.14	191.84±7.57[a,c]	34.06±2.19[a]

Values are mean ± SD for six rats in each group (n=6), Values not sharing a common superscript differ significantly at p<0.05 (DMRT)

TABLE 3: EFFECT OF CHITOSAN ON PLASMA INSULIN, BLOOD HEMOGLOBIN, GLYCOSYLATED HEMOGLOBIN AND URINE SUGAR IN NORMAL AND STZ-DIABETIC RATS, AFTER 30 DAYS

S. No.	Name of the group	Plasma insulin (µU/ml)	Hemoglobin (g/100 ml)	Glycosylated Hemoglobin (mg/g of Hb)
1.	Normal	13.28±0.78[a]	12.76±0.67[a]	0.28±0.04[a]
2.	Normal +Chitosan (300 mg/kg b.w.)	13.62±0.83[a]	11.88±0.78[a]	0.21±0.02[b]
3.	Diabetic control	5.13±0.32[b]	6.75±0.43[b]	0.83±0.07[c]
4.	Diabetic+ chitosan (300 mg/kg b.w.)	9.12±0.91[c]	8.32±3.11[c]	0.36±0.05[d]
5.	Diabetic+ glibenclamide (600 µg/kg b.w.)	12.62±1.12[a]	12.41±0.75[a]	0.34±0.02[d]

Values are mean ± SD for six rats in each group (n=6), Values not sharing a common superscript differ significantly at p<0.05 (DMRT)

TABLE 4: EFFECT OF CHITOSAN ON THE ACTIVITY OF CARBOHYDRATE METABOLIC ENZYMES AND GLYCOGEN CONTENT IN THE LIVER OF NORMAL AND STZ-DIABETIC RATS, AFTER 30 DAYS

S. No.	Name of the group	Glucokinase (U*/h/mg/protein)	Glucose 6-phosphate dehydrogenease (U#/mg protein)	Glycogen (mg/100g tissue)
1.	Normal	0.246±0.011[a]	4.12±0.60[a]	47.03±3.65[a]
2.	Normal +Chitosan (300 mg/kg b.w.)	0.240±0.013[a]	4.43±0.40[a]	49.38±4.64[a]
3.	Diabetic control	0.101±0.015[b]	2.12±0.63[b]	17.43±4.85[b]
4.	Diabetic+ chitosan (300 mg/kg b.w.)	0.175±0.012[c]	2.68±0.42[c]	28.09±1.75[c]
5.	Diabetic+ glibenclamide (600 µg/kg b.w.)	0.158±0.016[c]	3.67±0.21[d]	42.34±4.65[d]

Values are mean ± SD for six rats in each group (n=6), Values not sharing a common superscript differ significantly at p<0.05 (DMRT), U* = µmoles of glucose phosphorylated

TABLE 5: EFFECT OF CHITOSAN ON THE ACTIVITY OF GLUCONEOGENIC ENZYMES IN THE LIVER AND KIDNEY OF NORMAL AND STZ-DIABETIC RATS, AFTER 30 DAYS

S. No.	Name of the group	Glucose 6-Phosphatase (Unit[@]/min/mg protein)		Fructose 1,6-bisphosphatase (Unit[$]/h/mg protein)	
		Liver	Kidney	Liver	Kidney
1.	Normal	0.127±0.012[a]	0.208±0.017[a]	0.341±0.012[a]	0.807±0.112[a]
2.	Normal +Chitosan (300 mg/kg b.w.)	0.114±0.016[b]	0.198±0.019[a]	0.366±0.046[a]	0.781±0.069[a]
3.	Diabetic control	0.348±0.014[c]	0.324±0.022[b]	0.614±0.025[b]	1.216±0.118[b]
4.	Diabetic+ chitosan (300 mg/kg b.w.)	0.199±0.017[d]	0.262±0.034[c]	0.484±0.027[c]	0.943±0.047[c]
5.	Diabetic+ glibenclamide (600 µg/kg b.w.)	0.140±0.015[a]	0.221±0.030[a]	0.331±0.036[a]	0.845±0.066[a]

Values are mean ± SD for six rats in each group (n=6), Values not sharing a common superscript differ significantly at $p<0.05$ (DMRT), $U^{@}$= µmoles of inorganic phosphorus liberated, $U^{\$}$ = µmoles of inorganic phosphorus liberated

In our study, histopathological examination of diabetic pancreas **(Figure 1: A-E)** showed growth of adipose tissue and shrinkage of islets in the pancreas. Administration of chitosan showed expansion in pancreatic islet cells and glibenclamide treatment showed normal islet cells supporting the biochemical analysis.

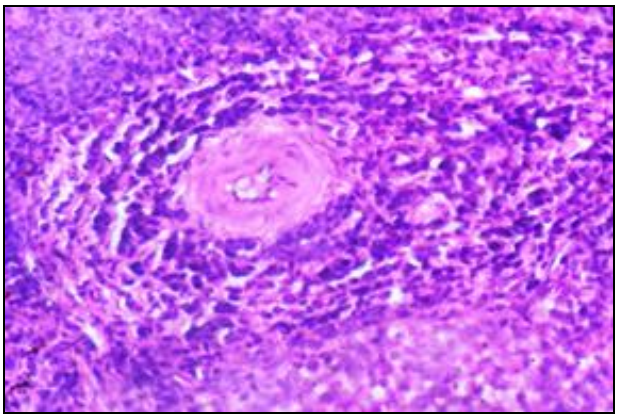

FIGURE 1(A): PANCREAS OF NORMAL RAT SHOWING ACINI AND ISLETS CELLS

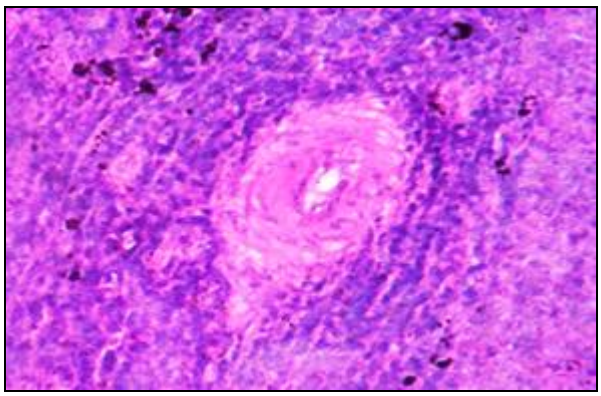

FIGURE 1(B): PANCREAS OF NORMAL RAT TREATED WITH CHITOSAN SHOWING NORMAL ISLETS CELLS AND ACINI

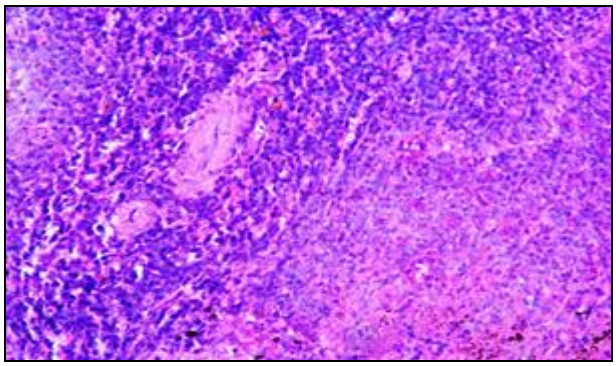

FIGURE 1(C): PANCREAS OF DIABETIC CONTROL RAT SHOWING GROWTH OF ADIPOSE TISSUE AND SHRINKAGE OF ISLETS

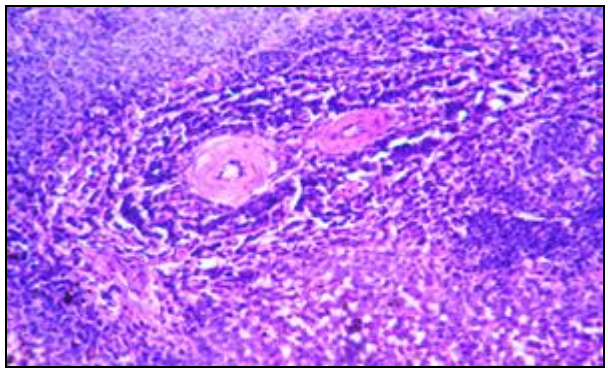

FIGURE 1(D): PANCREAS OF DIABETIC RAT TREATED WITH CHITOSAN SHOWING EXPANDED PANCREATIC ISLETS

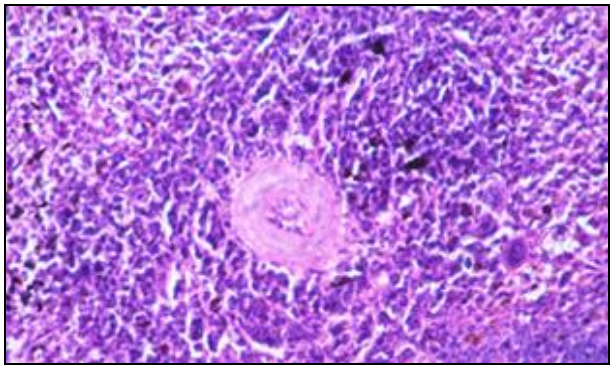

FIGURE 1(E): PANCREAS OF DIABETIC RAT TREATED WITH GLIBENCLAMIDE SHOWING NORMAL ISLETS WITH REDUCTION IN ADIPOSE TISSUE

FIGURE 1 (A-E): HISTOPATHOLOGICAL OBSERVATIONS IN THE PANCREATIC TISSUE OF CONTROL AND EXPERIMENTAL GROUPS OF RATS

Chitosan, the main component of crab and shrimp shells, is a polymer containing glucosamine units with high positive charge densities in acidic solutions. Although the exact mechanism of action is unknown, the positive charge of chitosan appears to interact with negative surfaces such as lipids. Its mechanism of absorption from the small intestine has not yet been well defined. It is thought that chitosan may be primarily absorbed after being transformed into oligosaccharides by chitosanase secreted from intestinal bacteria or by lysozyme in intestinal fluid. Consequently, chitosan exerts its antidiabetic action with the oligosaccharides, but not with the monosaccharide's [11].

It has been reported that administration of chitosan in patients with type 2 diabetes and hypercholesterolemia can effectively lower plasma TC and LDL-c concentrations without affecting plasma TG levels and glucose control [8]. However, Sandra *et al.* [26] studied in animal models where chitosan decreases TG concentrations [11, 12]. It is suggested that the blood glucose–lowering action of chitosan may be due in part to the TG and free fatty acid (FFA)–lowering action of this compound.

STZ-induced diabetes is characterized by severe weight loss [27] was observed in the present study. Chitosan and glibenclamide administration controlled the body weight loss in diabetic animals, though chitosan did not normalize the body weight completely. The decrease in body weight in diabetic rats might be the result of protein wasting due to unavailability of carbohydrate as an energy source [28]. The treated groups enhanced glucose metabolism and thus, improved the body weight in STZ-diabetic rats. The fundamental mechanism underlying hyperglycemia involves over production (excessive hepatic glycogenolysis and gluconeogenesis) and decreased utilization of glucose by the tissues.

Maintaining the blood glucose concentration within a narrow range is a critical physiological function which involves a dynamic equilibrium between endogenous glucose production and glucose utilization, requiring a series of cellular metabolic events, including a prominent role for hepatocytes [29].

Thus, significant antidiabetic effect of Chitosan may be due to is a natural, versatile and polymeric cationic character of the chitosan.

As a natural renewable resource, chitosan has both reactive amino and hydroxyl groups that can be used under mild reaction for biomedical application. Chitosan is an attractive agent for drug development given its function in the gastrointestinal tract and its intrinsic safety when taken orally [30]. The increase in insulin level could be due to increased pancreatic secretion from existing β-cells. Sandra *et al.* [26] reported Insulin sensitivity was increased significantly with the administration of chitosan and there were no adverse events with the interventions

The increased blood glucose stimulates nonenzymatic protein glycation namely, serum albumin, α-crystallin, collagen, low-density lipoprotein, hemoglobin etc., The first indication that a very simple chemical reaction between glucose and free amino groups on protein can lead to irreversible modification came with the characterization of glycated hemoglobin [31]. HbA_{1c} concentration is proportionately increased in diabetic patients with ambient hyperglycemic and reflects the extent as well as management of diabetic condition [32]. Several reports have been made on glycation-induced structural and functional modification of hemoglobin [33, 34]. Lower level of total hemoglobin observed in diabetic rats might be due to the increased formation of HbA_{1c} was found to increase in patients with diabetes mellitus to approximately 16% and the amount of increase was directly proportional to the fasting blood glucose level.

The liver is an important organ that plays a pivotal role in glycolysis and gluconeogenesis. A partial or total deficiency of insulin causes derangement in carbohydrate metabolism that decreases activity of several key enzymes including glucokinase, phosphofructokinsase and pyruvate kinase [35] is resulting in impaired peripheral glucose utilization and augmented hepatic glucose production. In our study, glucokinase activity was decreased in the liver of diabetic rats which may be due to a deficiency of insulin and treatment with chitosan and glibenclamide elevated the activity of glucokinase.

Chitosan administration increased insulin level which, in turn, activated glucokinase, thereby increasing the utilization of glucose leading to decreased blood sugar level.

Glucose 6-phosphatase and fructose 1, 6-bis phosphatase, are the regulatory enzymes in gluconeogenic pathway. Activities of these enzymes were increased significantly in diabetic rats [36] which might be due to the activation or increased synthesis of these enzymes contributing to the increased glucose production during diabetes. Treatment with chitosan and glibenclamide decreased the activity of gluconeogenic enzymes significantly, which might be due to decreased blood sugar level.

A decrease in the activity of glucose 6-phosphate dehydrogenase has been observed in diabetic rats [37]. Treatment with chitosan increased the activity of the enzyme, via increased secretion of insulin which increases the influxes of glucose into pentose monophosphate shunt in an attempt to reduce high blood glucose levels. This results in an increased production of the reducing agent, NADPH, which concomitant decrease in oxidative stress [38].

Glycogen is the primary intracellular storable form of glucose. Diabetes mellitus is known to impair the normal capacity of the liver to synthesize glycogen. The liver glycogen is markedly decreased in diabetic animals [39] which are in proportion to insulin deficiency [40].

Diabetic rats treated with chitosan brought back liver glycogen towards normalcy, which could be due to increased secretion of insulin. Thus, our results shows a sequential metabolic correlation between increased glycolysis and decreased glycogenesis stimulated by chitosan suggesting the possible biochemical mechanism via insulin secretion, though which glucose homeostasis is regulated.

CONCLUSION: In conclusion, chitosan possess a significant antihyperglycemic activity and can be used in the treatment of diabetes mellitus. Further studies are necessary to determine the mechanism of action of the chitosan.

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