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## FIRST REPORT ON EVALUATION OF ANTI-DIABETIC POTENTIAL OF CHITIN AND CHITIN-BASED DERIVATIVES AGAINST ALLOXAN INDUCED ALBINO RATS

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### Keywords:

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chito-oligosaccharides (COS).

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**ABSTRACT:** Crustacean's shells constitute the traditional and current commercial source of Chitin. These derivatives/molecules are potential resource as well as multiple functional substrates have generate attractive interest in various fields such as biomedical, pharmaceutical, food and environmental industries. In the present investigation, chitinous wastes were collected from the fresh water areas of Dehradun and Rishikesh of Uttarakhand State. The bacterium, *Bacillus* sp. isolated from soil produces chitinase enzyme responsible for degradation of chitin obtained from chitinous wastes. Further the chitinases enzyme was utilized to degrade the chitinous wastes into chito-oligosaccharides. Chitosan was prepared by deacetylation process and chito-oligosaccharides (COS) was prepared by enzymatic hydrolysis of chitosan. As per the solubility aspect of chitosan and chito-oligosaccharides, it was found that these are more soluble in comparison to chitin, the parent compound. The anti-diabetic potential of these chitin based derivatives was determined against alloxan-induced albino rats (diabetic model). In alloxan-induced diabetic rats, both the chitosan and chito-oligosaccharides (COS) both decremented blood sugar levels at the cessation of 1<sup>st</sup>, 2<sup>nd</sup> and 4<sup>th</sup> hours after dosing in acute studies. Simultaneously similar effect was observed during chronic studies, after 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> week during repeated dosing of the extracts in diabetic model at an interval of 7 days. It was thus observed that, Chito-oligosaccharides (COS) at dose of 50 mg/kg/day and chitosan at dose of 100 mg/kg/day showed significant hypoglycemic activity in comparison to standard anti-diabetic drug, glibenclamide (10 mg/kg).

**INTRODUCTION:** Chitin, a polysaccharide of animal origin, is obtained from waste material of seafood industries. It occurs in the skeletal material of crustaceans such as crabs, lobsters, shrimps, prawns and crayfish. Chitin is also the important component of exoskeleton of Arthropods Chitin is also forming the important composition of fungus. Chitosan is the deacetylated product formed by treatment of chitin with concentrated (50%) caustic alkali.

The regulatory and toxicological status of Chitosan has already been established. Chitin and Chitosan are described as a family of linear polysaccharides consisting of varying amounts of linked residues of  $\beta$  (1, 4) linked N-acetyl-2 amino-2-deoxy-D-glucose and 2-amino-2-deoxy-Dglucose residues. Chitin samples have a low amount of D units and hence the polymer is insoluble in acidic aqueous media.

Chitoooligosaccharides and their N-acetylated analogues are useful for applications in various fields because they have specific biological activities such as antimicrobial activity, antitumor activity, immuno-enhancing effects<sup>1</sup>. Some chito-oligosaccharides such as (GlcNAc) and (GlcNAc) have been reported to possess antitumor activity<sup>2</sup>.

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Chemical and biological methods for extraction of chitin and chitosan modification for application in the food industry were determined<sup>3</sup>. Macroporous artificial skin containing antibiotics was prepared by lyophilization of chitosan/PVA blendmer, which could protect the wound surfaces from bacterial invasions by suppressing bacterial proliferation effectively<sup>4</sup>. It was investigated that chitin and chitosan from crustacean waste can be used as food processing aid. It was reported that Chitosan is a potential biomaterial effective against typhoid<sup>5</sup>. The antibacterial<sup>6</sup>, antifungal<sup>7</sup> and plant growth regulation activities<sup>8</sup> were reported. Diabetes is a global prevalent endocrine disorder with an estimated worldwide prevalence of 246 million people in 2007 and forecasts to elevate to 300 million by 2025.

It is an endocrine predicated disorder of multiple etiologies characterized by hyperglycemia and hyperlipidemia. It is a kind of epidemic which is spreading at a faster rate day by day. The patients suffer from diabetes experience sundry complications, such as atherosclerosis, diabetic nephropathy and neuropathy. There are many oral hypoglycemic agents, such as sulfonylurea and biguanides, are available along with insulin for the treatment of diabetes, but these agents have paramount side effects. Today much research has been focused on medicinal plants and their molecules which can be utilized as anti-diabetic agent.

In view of the pharmacological and medical applications of chitin-based derivatives, the present study was thus focused on determination of anti-diabetic activity of chitosan and chito-oligosaccharides (COS) in alloxan-induced animal model. The objective was to establish chitin-based derivatives as natural and safe anti-diabetic agent.

## **MATERIALS AND METHODS:**

### **Chemicals:**

All the materials, reagents used for the study were procured from Ranchem and CDH. Specific chemicals and drugs viz. Alloxan/ALX (Sigma-Aldrich, USA); Glucose estimation kit (Span diagnostic Ltd., Surat, India); Glibenclamide tablets/GLB obtained from Aventis Pharma Ltd., Mumbai, India was utilized in this study.

### **Collection of chitinous wastes:**

The chitinous wastes of fresh water crustaceans were collected from the fresh water areas of Dehradun and Rishikesh (U.K), India and were washed properly in order to remove the sand debris present on the surfaces. The chitinous wastes were then after air dried and powdered material obtained was used as chitin.

### **Demineralization of chitinous wastes:**

The demineralization of chitinous wastes was performed<sup>9, 10</sup>. The chitinous wastes were treated with 1.75 N acetic acid at room temperature for about 12-15 hours. The ratio of waste to solvent were maintained (1:15 w/v). The demineralized material obtained were recovered by filtration and rinsing with de-ionized water and will be dried in forced hot air oven at 65<sup>0</sup>C.

### **Deproteinization and removal of lipids:**

The new and advanced methodology for deproteinization of proteins from demineralized chitinous wastes was designed by using deproteinization agents. This process can be performed either by using proteolytic enzymes such as proteinase-K dissolved in buffer containing 0.05 M Tris-base (pH, 6.5-9.1) in a ratio 1:20 (w/v) in flasks at various temperatures in incubator-shaker for about 72 h and adding mixture of solvents (phenol: chloroform, 1:1 ratio) again and again to the residue obtained and centrifuging the mixture until the residue gives no test for the presence of protein content. After repeating the procedure for 3-4 times, finally the residue was treated with 2N sodium hydroxide (1:25 w/v) at 70<sup>0</sup>C for 1 hour. The lipid content gets dissolved in phenol: chloroform mixture and was removed from the chitinous wastes. Greese spot test was performed in order to determine qualitatively the presence of lipid content if any present in the residual material<sup>11</sup>.

### **Preparation of colloidal chitin:**

The colloidal chitin was prepared by using 1g of fresh water crustacean's chitin in 1N HCl for 2 h at room temperature. The colloidal chitin prepared of each of the samples was washed several times with large volume of distilled water to adjust the pH to 7.0.

### **Isolation of microbe for screening of chitinases production:**

The soil was procured from Doiwala region of Dehradun (U.K), India by performing the serial dilution method and maintained on glycerol yeast medium plates at 37°C.

### **Chitinase Production:**

For the production of chitinase, microbial strain was grown in 100 ml of fresh medium (3% w/v chitin; 0.1% KH<sub>2</sub>PO<sub>4</sub>; 0.05% MgSO<sub>4</sub>.7H<sub>2</sub>O; 50mM Sodium Phosphate buffer, pH 6.0) in a 250 ml Erlenmeyer flask at 30°C. For reflecting the growth of the culture in this medium OD at 660 nm was taken using blank as medium in which no inoculum was added. The supernatant (enzyme) was collected from 3 day old cultures by centrifuging the mixture at 12,000 g for 20 minutes. The enzyme was concentrated by condensing the solution in order to reduce its volume<sup>12</sup>.

### **Preparation of Chito-oligosaccharides:**

For the preparation of chito-oligosaccharides, 1% colloidal chitin prepared from standard chitin, fresh water crab and fresh water prawn were dissolved in 0.05M Phosphate buffer (pH, 5.5). About 10 ml of enzyme (purified from Bacillus strain) was added in 15 ml of 1% of each of the colloidal chitin in a 100 ml flask; further flask was kept at 30°C for 3 h. The reaction was terminated by immersing the tubes in boiling water for 5 minutes. After performing centrifugation at 3000 rpm, the insoluble materials were collected and condensed to obtain the chito-oligosaccharides<sup>10</sup>. The chito-oligosaccharides extracts were dissolved in distilled water.

### **Preparation of Chitosan:**

The demineralized and deproteinized chitin material was subjected to concentrated sodium hydroxide at 40% w/v<sup>12</sup>. The deacetylated forms of chitosan obtained were solubilized in 2 M dilute acetic acid. Further the chitosan extracts were dissolved in distilled water.

### **Animals:**

Two to three month old either sex albino rats of body weight 180 - 210 g were obtained from Central animal house facility (CPCSEA Regd. no. 186/ CPCSEA, dated: 18 December, 2014)

acclimatized for seven days to faculty animal house, and maintained at standard conditions of temperature and relative humidity, with a 12-hour light dark cycle. Water and commercial rat feed *ad libitum* were provided. The current study was carried out with prior sanction from our Institutional Animal Ethical Committee and proposal no. 583.

### **Induction of diabetes to experimental animals:**

Alloxan was commonly utilized as an animal model of diabetes. The animals were fasted for 12h prior to the induction of diabetes with slight modification. Alloxan, freshly prepared in 0.5% Tween 80 was administered intraperitoneally at single dose of 140mg/kg body weight<sup>13</sup>. Development of diabetes was confirmed by measuring blood glucose concentration 5 days after the administration of ALX. Rats with blood glucose level of above 200 mg/dl were considered to be diabetic and used for the studies.

### **Experimental design:**

In the present experiment, a total of 24 rats (18 diabetic surviving rats; 6 normal rats) were used. The chitosan and chito-oligosaccharides extracts were diluted to prepare the specific doses. The rats were randomized into seven groups comprising of six animals in each group as given below.

**Group I:** Normal control (NC) rats received distilled water 10ml/kg body weight, p.o.

**Group II:** Diabetic rats received standard drug GLB (10 mg/kg p.o.), 5 days after ALX treatment.

**Group III:** Diabetic rats given Chitosan extracts prepared in distilled water (100 mg/kg p.o.), 5 days after ALX treatment.

**Group IV:** Diabetic rats given Chito-oligosaccharide, COS prepared in distilled water (50 mg/kg p.o.), 5 days after ALX treatment.

### **Biochemical assay:**

#### **Glucose levels:**

Blood samples were collected from retro-orbital plexus of each rat under mild anaesthesia at 0, 1, 2 and 4h (Acute study) as well as on 0<sup>th</sup>, 7<sup>th</sup>, 14<sup>th</sup> and 21<sup>st</sup> days after administration (Chronic study) of

chitosan and chito-oligosaccharides (COS) extracts. Blood glucose level was estimated by enzymatic glucose oxidase method. The reduction in blood glucose level was calculated with reverence to the initial level. The body weight of all animals was quantified on the 0, 7<sup>th</sup>, 14<sup>th</sup> and 21<sup>st</sup> days after 1h of treatment with the chitosan, chito-oligosaccharide extracts and GLB<sup>13</sup>.

**Glucose tolerance test**

Five days before the termination of the experiment, the oral glucose tolerance test (OGTT) was performed to evaluate the ability to respond appropriately to a glucose challenge. For this purpose, overnight fasted rats (control and treated rats) were feed glucose (2g/kg body weight) orally and blood was collected at 0, 30, 60 and 120 minutes interval from orbital sinus for glucose estimation using a glucometer.

**RESULTS AND DISCUSSION:**

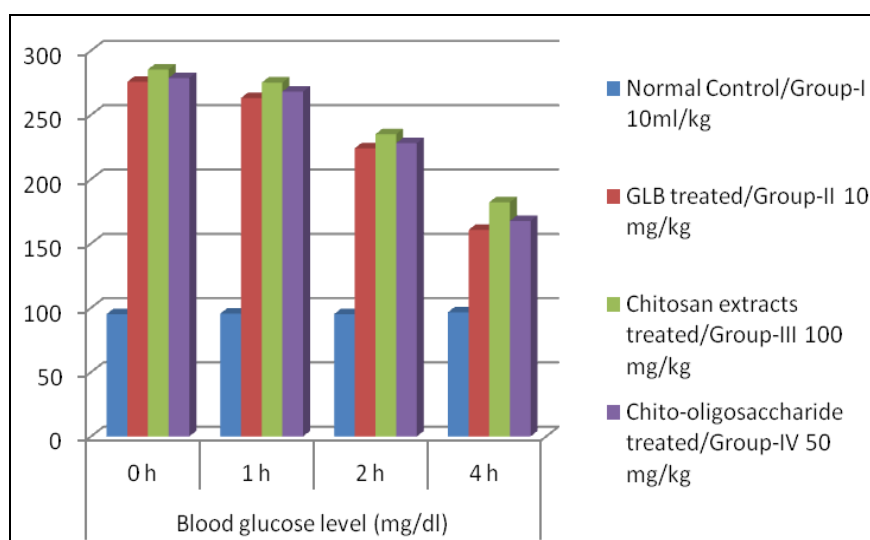
**Anti-diabetic activity in alloxan-induced diabetic rats (Acute study):**

It was found that, fasting blood glucose levels were within the range of 96–99 mg/dl in normal rats. Treatment with alloxan (140 mg/kg, i.p.) had increased the blood glucose level to a range of 250–295 mg/dl after 5 days. Single dose administration of chito-oligosaccharides (COS) (50mg/kg) and chitosan (100mg/kg) significantly at p<0.01 reduced the blood glucose level at the time intervals i.e. 2 and 4 hours after treatment, in alloxan-induced diabetic rats, while Glibenclamide/GLB (10mg/kg) significantly at p<0.05 reduces the blood glucose level at 1<sup>st</sup>, 2<sup>nd</sup> and 4<sup>th</sup> hour after single dose administration in alloxan-induced diabetic rats. Normal control showed no reduction in blood glucose level. The results are shown in **Table 1** and **Fig. 1**.

**TABLE 1: EFFECT OF CHITOSAN AND CHITO-OLIGOSACCHARIDE EXTRACTS IN ALLOXAN INDUCED ALBINO RATS**

| Treatments/Group                       | Dose      | Blood glucose level (mg/dl) |                 |                 |                 |
|--|-----------|-----------------------------|-----------------|-----------------|-----------------|
|  |           | 0 h                         | 1 h             | 2 h             | 4 h             |
| Normal Control/Group-I                 | 10ml/kg   | 95.12±0.45                  | 95.56±0.56      | 95.08±0.62      | 96.53±0.73      |
| GLB treated/Group-II                   | 10 mg/kg  | 275.67 ± 1.12*              | 263.16 ± 2.43** | 224.06 ± 1.20** | 160.53 ± 1.43** |
| Chitosan extracts treated/Group-III    | 100 mg/kg | 285.34 ± 1.12*              | 275.11 ± 2.12** | 235.01 ± 1.23** | 182.05 ± 0.86** |
| Chito-oligosaccharide treated/Group-IV | 50 mg/kg  | 278.67 ± 1.25*              | 268.12 ± 1.45** | 228.03 ± 0.88** | 167.52 ± 0.83** |

The data are expressed in mean ± S.E.M. n = 6 in each group. P values were analyzed using One way ANOVA [\*p<0.05- less significant; \*\*p<0.05- most significant



**FIG.1: EFFECT OF CHITOSAN AND CHITO-OLIGOSACCHARIDE EXTRACTS IN ALLOXAN INDUCED ALBINO RATS**

**Anti-diabetic activity in alloxan-induced diabetic rats (Chronic study):**

As per chronic study, it was observed that repeated dose administration of diabetic rats with GLB (10 mg/kg) and chitosan (100 mg/kg) and chito-

oligosaccharides (50 mg/kg) respectively in separate specific groups with an interval of 7 days had progressively reduced the blood glucose level in a dose dependent manner over a period of 3 weeks. The results were found to be most



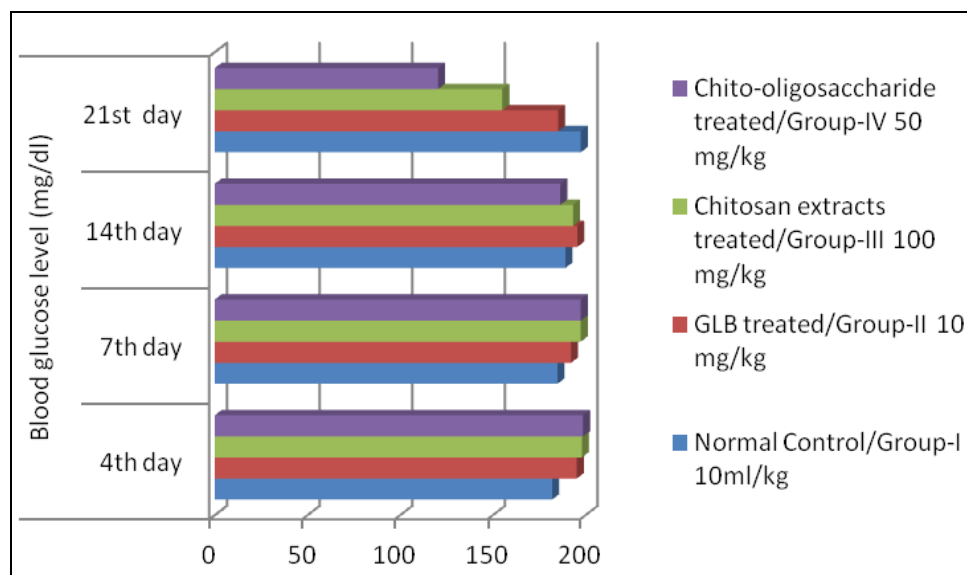
significant of chitosan and chito-oligosaccharides extracts at 4<sup>th</sup>, 7<sup>th</sup>, 14<sup>th</sup> and 21<sup>st</sup> days of treatment. It was observed that with the passage of time of dosing and performing repeated dosing of chitosan

and chito-oligosaccharides extract, the blood glucose level gets decreases in comparison to the positive control/GLB. The results are shown in **Table 2** and **Fig. 2**.

**TABLE 2: EFFECT OF CHITOSAN AND CHITO-OLIGOSACCHARIDE EXTRACTS IN ALLOXAN INDUCED ALBINO RATS**

| Treatments/Group                       | Dose      | Blood glucose level (mg/dl) |                     |                      |                      |
|--|-----------|-----------------------------|---------------------|----------------------|----------------------|
|  |           | 4 <sup>th</sup> day         | 7 <sup>th</sup> day | 14 <sup>th</sup> day | 21 <sup>st</sup> day |
| Normal Control/Group-I                 | 10ml/kg   | 182.12±0.45                 | 185±0.56            | 189.23±0.62          | 197.56±0.73          |
| GLB treated/Group-II                   | 10 mg/kg  | 195.45 ± 1.12*              | 192.34 ± 2.43**     | 195.67 ± 1.20**      | 185.34 ± 1.43**      |
| Chitosan extracts treated/Group-III    | 100 mg/kg | 198.16 ± 1.12*              | 197.56 ± 2.12**     | 193.34 ± 1.23**      | 155.23 ± 0.86**      |
| Chito-oligosaccharide treated/Group-IV | 50 mg/kg  | 198.82 ± 1.25*              | 197.56 ± 1.45**     | 186.53 ± 0.88**      | 120.52 ± 0.83**      |

The data are expressed in mean ± S.E.M. n = 6 in each group. P values were analyzed using One way ANOVA [\*p<0.05- less significant; \*\*p<0.05- most significant]



**FIG.2: EFFECT OF CHITOSAN AND CHITO-OLIGOSACCHARIDE EXTRACTS IN ALLOXAN INDUCED ALBINO RATS**

The study thus suggests that chitosan and chito-oligosaccharides at different doses are important anti-diabetic agents and showed no mortality in alloxan-induced albino rats. These molecules can be utilized to prepare anti-diabetic drugs as these are natural and safe. Further studies in this aspect can lead to determination of most optimum drug and duration required for earliest treatment of diabetes. This is the first kind of study performed to determine the anti-diabetic activity and standardizing dose of chitosan and chito-oligosaccharides (chitin-based derivatives) in treatment of diabetes. The results are satisfies the previous studies reported<sup>14-18</sup>.

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