Dose and duration dependent effect of aqueous extract of *Oreocnide integrifolia* (Gaud.) Miq. on systemic and pancreatic manifestations of diabetes: Studies on *in-vivo* streptozotocin model of rats.

Introduction: Diabetes mellitus is characterized by an initial loss of glucose homeostasis resulting from defects in insulin secretion and/or insulin action or leading to impaired metabolism of glucose and other energy- yielding metabolites (Scheen, 1997). It is also accompanied by hyperglycaemia, dyslipidaemia, hypertension, decreased fibrinolytic activity, increased platelet aggregation, and severe atherosclerosis, all of which are potential risk factors.

Though several drugs targeting carbohydrate hydrolyzing enzymes (pseudosaccharides), release of insulin from pancreatic β-cells (sulphonylurea), glucose utilization (biguanides), insulin sensitization and PPARγ agonists (glitazones) are in clinical practice, there is a growing market for anti-diabetic agents. Many of these oral antidiabetic agents have been reported to show serious adverse effects (Sy *et al.*, 2000). The multifactorial etiology and multiple pathogenic manifestations of diabetes demands multi-modal therapeutic approach and, future therapeutic strategies need to focus on the efficacy of combination drugs. Management of diabetes and its side effects is still a challenge for the pharmaceutical world. Traditional knowledge of medicinal plants has become a recognized tool in search for the search of new sources of drugs and neutraceuticals (Sharma and Majumdar, 2003). In fact, metformin, one of the most

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prescribed glucose-lowering medicines currently used, is derived from a chemical isolated from a plant (Witters, 2001). World health organization has in fact recommended the use of herbal medicine especially in developing countries (WHO, 2002). Because of their perceived effectiveness and minimal side effects in clinical experience and effectiveness, herbal drugs are prescribed widely even when their biologically active compounds are not known (Valiathian, 1998).

The local communities residing in the biodiversity-rich areas of the North Eastern Region of India have traditionally used and relied on herbs for treating various ailments (Kayang et al., 2005). In many cases, local knowledge of medicinal plants remains poorly documented in scientific literature. These plants have found a prime place in the indigenous system of medicine and are in focus for evaluation of their active ingredients.

Oreocnide integrifolia (Gaud.) Miq (family Urticaceae) are trees of 5-20 m height having reddish brown branchlets and simple, alternate, spiral and clustered leaves at twig end, found at wet evergreen forests at 300-1400 m height. They are mainly distributed in India, China, Bhutan, Indonesia, Laos, Myanmar, Sikkim and Thailand (Chen et al., 2003). The roots of *Oreocnide integrifolia* are mixed with ginger powder and applied for treatment of rashes by Khasi and Jayantia tribes of Meghalaya (Kharkongor and Joseph, 1981; Begum and Nath, 2000). In north eastern states of India, it is popularly known as ukhajing (manipuri), bonrhea (assamese), gingsining (garo) and dieng teingbah (khasi), and an infusion prepared from the leaves is used as a decoction to alleviate diabetic symptoms. Based on our literature survey till date, there are no scientific reports and hence the present study in this behest was undertaken to evaluate the hypoglycemic and hypolipidemic potential of *Oreocnide integrifolia* leaf extract on streptozotocin induced experimental diabetes, to validate the folklore usage.

Materials and Methods:

Plant material and extraction: Fresh green leaves were collected during the month of October from Imphal district (Manipur) and authenticated by botanist Dr. Hemchand Singh, D.M College of Science, Manipur University. A voucher specimen (#344) of the herbarium has been deposited at the same department for future reference. The leaves were collected during the month of Sep-Oct, washed thoroughly and shade dried at room temperature. The dried leaves were subjected to size reduction to a coarse powder by using dry grinder and passed through (# 400) sieve. Two hundred grams of powder was mixed with 1 litre of Milli Q water (Millipore, Billerica, MA) and boiled for 30 min and then left to cool down to room temperature. The decoction was filtered (Whatmann # 01) using a suction apparatus and the filtrate was lyophilized and stored in a freezer at $-20°C$. The extractive value of the aqueous extract in terms of yield was about 16.9 % (w/w).

Experimental animals: Female *Charles foster* rats (200–230 g) were housed in clean polypropylene cages under controlled room temperature $(21\pm2°C)$.They were fed with commercially available rat chow (M/s Pranav Agro Ltd., Baroda) and provided with water *ad libitum.* The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) India, and approved by the Animal Ethical Committee of the Department of Zoology, The M.S University of Baroda, Vadodara (Approval No. 827/ac/04/ CPCSEA).
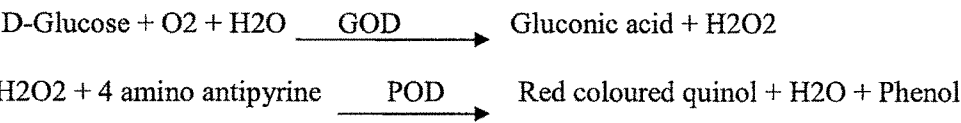
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Induction of Type I diabetes: Rats were rendered diabetic by single intraperitoneal administration of streptozotocin (60mg/kg) dissolved in 0.1 M Citrate buffer, pH 4.5. Seven days later, blood samples were collected and blood glucose levels determined to confirm induction of diabetes. Only those animals which showed fasting blood glucose levels higher than 250 mg/dl were used for the experiment.

Experimental procedure: Sixty-Six rats (30 normal and 36 diabetic rats) were divided into eleven groups of six animals each. Group I consisted of normoglycemic rats (NC) administered with vehicle alone while, Groups II, III, IV, and V (NOI100, NOI250, NOI500, NOI750) consisted of normoglycemic rats treated with 100, 250, 500 or 750mg/kg body weight of OI extract respectively. Group VI comprised of diabetic rats (DC) which received vehicle only while Groups VII, VIII, IX and X (DOI100, DOI250, DOI500 ,DOI750) consisted of diabetic rats treated with 100, 250, 500 or 750mg/kg body weight of OI extract respectively. Group XI (DMet) consisted of diabetic rats treated with metformin, an antidiabetic drug (50mg/kg). All animal received their respective drugs in 0.5% Sodium carboxymethylcellulose orally via gastric intubation for a period of 28 days.

Plasma Glucose (Trinder 1969):-

Glucose is an important source of energy. Glucose concentration fluctuates only in narrow range as insulin and its counter regulatory hormones in the body maintain glucose homeostasis.

Principle: This is an enzymatic method for estimation of serum glucose levels. The aldehyde group of glucose is oxidized by the enzyme glucose oxidase (GOD) in the presence of oxygen (air) to gluconic acid with the liberation of hydrogen peroxide (H_2O_2). Peroxidase splits H_2O_2 into H_2O and active oxygen, which reacts with phenol and a chromogen 4-amino antipyrene to form a pink coloured complex, which can be estimated colorimetrically at 505 nm.

$$\text{D-Glucose} + O_2 + H_2O \xrightarrow{\quad GOD \quad} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + 4 \text{ amino antipyrine} \xrightarrow{\quad POD \quad} \text{Red coloured quinol} + H_2O + \text{Phenol}$$

Reagents – commercially available kits contain following reagents:

Enzymes (glucose oxidase - peroxidase) with chromogen and phenol

Glucose standard (100mg/dl) (Range of standard –10 –50 µg)

Procedure

Reagents	Blank	Standard	Test
Enzyme reagent	1.0 ml	1.0 ml	1.0 ml
Glucose standard	-	0.01ml	-
Serum	-	-	0.01 ml

All the tubes were incubated for 15min at RT or for 10min at 37 C and then absorbance was read at 505 nm against blank. Calculation – Calculation was done according to the slope calculated from the standard graph. Units – mg/dl.

Fasting Insulin:-

Serum fasting insulin was quantitatively estimated by enzyme immunoassay method by using Mercodia rat insulin ELISA kit, Mercodia B, Uppsala, Sweden.

Principle:

Mercodia Rat Insulin ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample react with Peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to microtitration well. A simple washing step removes unbound enzyme labeled antibody. The bound conjugate is detected by reaction with 3, 3', 5, 5'-tetramethylbenzidine. The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically.

Reagents:

- **Coated plate:** It is made up of 96 wells.

- **Calibrators:** Kit contains 5 vials of rat insulin of different concentration which are used to prepare calibration curve.

- **Enzyme conjugate 11X:** It is Peroxidase conjugated mouse monoclonal anti-insulin.

- **Enzyme conjugate buffer**

- **Wash buffer 21X**

- **Substrate TMB**

- **Stop solution:** It is made up of 0.5M H_2SO_4.

Preparation of enzyme conjugate solution

Enzyme conjugate solution was prepared by mixing 600µl of enzyme conjugate 11X solution with 6ml of enzyme conjugate buffer. The solution is stable at 2-8°c for two months.

Preparation of wash buffer

Wash buffer was prepared by mixing 20ml of wash buffer 21X with 400ml of redistilled water. The solution is stable at 2-8°c for 4 weeks.

Procedure:

Each determination for calibrators and unknown samples was performed in duplicate. All the reagents and samples were brought to room temperature before use.

Add to anti-insulin wells	Calibrators	Unknowns
Calibrators	25µl	------
Unknown serum sample	------	25µl
Enzyme conjugate solution	50µl	50µl

After mixing, following steps were performed.

- Samples were incubated on a shaker for 2 hours at room temperature.
- Reaction volume was aspirated from each well. 350µl of wash buffer was added to each well. The solution was aspirated completely. The process was repeated 5 times.
- After the final wash, the plate was inverted and tapped firmly against absorbent paper.
- Then, 200µl of substrate TMB was added to each well. The reaction mixture was incubated for 15 minutes. 50µl of stop solution was added to each well. The plate was again placed on the shaker for 5 seconds to ensure mixing of substrate and stop solution.
- The absorbance was measured at 450nm in ELISA reader.
- The calibration curve was plotted as absorbance value against the known insulin concentration of calibrators.
- Concentration of the unknown samples was read from the calibration curve.

Haemoglobin and Glycosylated Haemoglobin (Parker *et al.*, 1981):-

Protein can universally bind non-enzymatically with glucose or other sugars present in the vicinity. The degree of glycation is directly proportional to the concentration of the sugar present in the surrounding medium. RBC has longer life span (120 days) as compared to other proteins like albumin (4 days). Therefore estimation of Glycosylated Haemoglobin (Gly Hb) gives an accurate reflection of mean plasma glucose concentration over this period.

Principle: This method is specific for ketoamine-linked hexoses which form furfural when heated under strong acidic condition. This furfural reacts with 2-thiobarbituric acid (TBA) and produces a bright yellow coloured compound, which can be estimated colorimetrically at 443 nm.

Reagents:

- 0.5 M Oxalic acid (Stable for 2 weeks at RT)

- 0.72g % Thiobarbituric Acid (TBA)(pH 6.0, stable for 1 weeks at RT)

- Saline (0.9g % NaCl)

- 40 % Trichloroacetic acid (TCA)

- Drabkin's reagent (pH 9.1) – 0.2g Potassium Ferricyanide [K3Fe(CN)6], 0.05g Potassium Cyanide (KCN), 0.14g Potassium dihydrogen phosphate (KH2PO4) in one litre of distilled water.

- Hb standard solution (60 mg/dl or 65 mg/dl) was procured from market.

- Fructose standard 0.2 µM (Range of the standard – 0.02 - 0.12 µM)

Procedure: Haemolysate preparation –RBC sediments were washed three times with 0.9% saline. Then packed cells were lysed by adding equal amount of distilled water and ¼ part CCl₄. Mixed well and centrifuged at 3000 rpm for 10 min. Haemoglobin (Hb) was estimated with Drabkin's reagent and adjusted to 10g Hb/dl.

Reagents	Control	Test
haemolysate	-	1ml
D/W	1ml	-
oxalic acid	0.5 ml	0.5ml
Keep in boiling waterbath for 1 hr		
chilled TCA	1ml	1ml
Centrifuge the tubes at 2000 rpm for 15min		
Supernatant	1ml	1ml
TBA	0.1ml	0.1ml
Incubated in water bath at 40°C for 30 min and read the absorbance at 443nm against the sample blank.		

Calculation – Calculation was done according to the slope calculated from the standard graph. Units – % of total Hb

Creatinine (Bonsnes and Taussky, 1945)

Creatinine is the end product of creatine metabolism. It is largely formed in the muscle by irreversible and non-enzymatic removal of water from creatine phosphate. It is a waste product and excreted out from the kidney. An increased serum creatinine level is virtually a diagnostic of renal disease.

Principle – Creatinine reacts with picric acid in alkaline medium to form a reddish yellow complex. Intensity of which is directly proportional to the concentration of creatinine in the specimen and can be measured at 540 nm.

Reagents –

- Saturated Picric acid
- NaOH (0.75M)
- Creatinine standard solution - Stock – 100mg/dl (Working – 10mg/dl, Standard range=10-50µg)

Sample - 1.5 ml picric acid was added to 0.5ml serum/plasma, and tubes were centrifuged at 2000 rpm for 10min. Supernatant was taken out for creatinine estimation.

Procedure –

Reagents	Control (ml)	Test (ml)
Supernatant	-	1.0
D/W	2.0	1.0
Picric acid	1.0	1.0
0.75M NaOH	1.0	1.0
Tubes kept for 20 min at RT and absorbance was recorded at 540nm		

Calculations – O.D. of test X concn of std / OD of std (Unit – mg/dl)

UREA KIT (DAM Method):

Principle: Urea in an acidic medium condenses with Diacetyl monoxime at 100°C to form a red coloured complex. Intensity of the colour formed is directly proportional to the amount of urea present in the sample.

Urea + Diacetyl monoxime → **Red Coloured Complex**
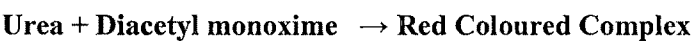
Contents

L1: Urea Reagent , L2: Acid Reagent , L3: DAM Reagent , S: Urea Standard

Addition sequence	Blank	Standard	Test
L1	1.0 ml	1.0 ml	1.0 ml
L2	1.0 ml	1.0 ml	1.0 ml
L3	1.0 ml	1.0 ml	1.0 ml
Distilled water	0.01 ml	---	---
S	---	0.01 ml	---
Sample	---	---	0.01 ml

Mix well and keep the test tubes in boiling water (100°C) for 10 minutes. Cool under running tap water and measure the absorbance of the Standard (Abs. S), and Test Sample (Abs. T) against blank.

CALCULATIONS

$$\text{Urea in mg/dl} = \frac{\text{Abs.T}}{\text{Abs.S}} \times 40$$

Assay of Total Cholesterol (TC):

Quantitative estimation of TC in serum was performed according to manufacturer's instructions provided in commercially available standard diagnostic kit (Span Diagnostics Pvt. Ltd., India) using Perkin Elmer Lambda UV-Vis Spectrophotometer.

Principle: Cholesterol esters are hydrolysed by Cholesterol Esterase (CE) to give free Cholesterol and Fatty acids. In subsequent reaction, Cholesterol Oxidase (CHOD) oxidizes the 3-OH group of free Cholesterol to liberate Cholest-4-en-3-one and Hydrogen Peroxide. In presence of Peroxidase (POD), Hydrogen Peroxide couples with 4 Aminoantipyrine (4-AAP) and Phenol to produce Red Quinoneimine dye. Absorbance of coloured dye is measured at 505 nm and is proportional to amount of Total Cholesterol concentration in the sample.
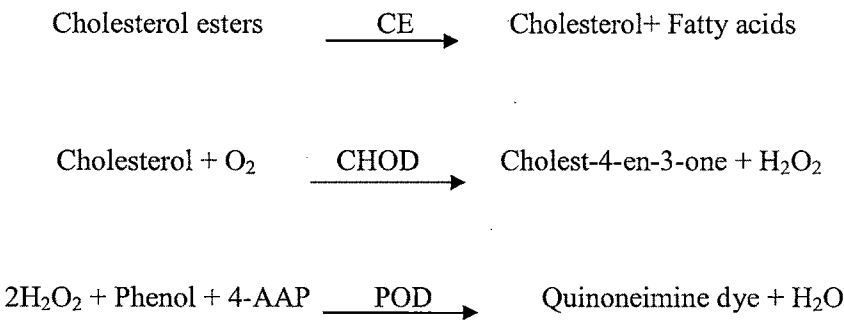
$$\text{Cholesterol esters} \xrightarrow{\quad CE \quad} \text{Cholesterol + Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\quad CHOD \quad} \text{Cholest-4-en-3-one} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + 4\text{-AAP} \xrightarrow{\quad POD \quad} \text{Quinoneimine dye} + H_2O$$

Procedure:

Pipette into 3 test tubes labeled Blank (B), Standard (S) and Test (T) as shown below:

	Blank	Standard	Test
Serum/plasma	--	--	10µL
Reagent 2	--	10µL	--
Regent 1	1000µL	1000µL	1000µL

Mix well. Incubate at 37° C for 10 minutes or at Room Temperature (15-30 ° C) for 30 minutes. Take absorbance of standard and test against blank at 505 nm and calculate results as per following formula.
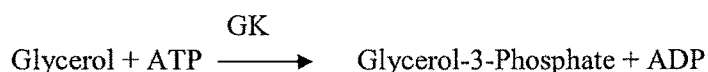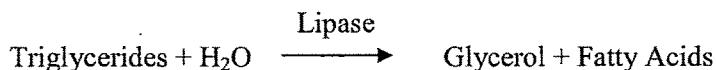
Calculation:

Cholesterol concentration (mg/dl) = Absorbance of Test / Absorbance of Standard × 200

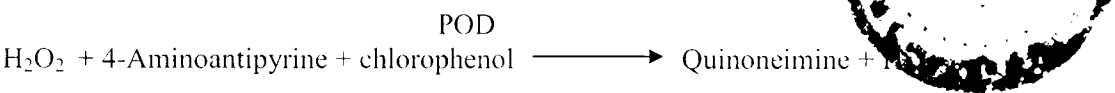## Assay of Triglycerides (TG):

Quantitative estimation of TG in serum was performed according to manufacturer's instructions provided in commercially available standard diagnostic kit (Reckon Diagnostics Pvt. Ltd., India) using Shimadzu 1800 UV-Vis Spectrophotometer.

Principle:

Triglycerides in the sample are hydrolyzed by microbial lipase to glycerol and free fatty acid (FFA). Glycerol is phosphorylated by adenosine 5-triphosphate (ATP) to glycerol 3-phosphate (G-3-P) in reaction catalyzed by glycerol kinase (GK). G-3-P is oxidized to dihydroxy acetone phosphate in a reaction catalyzed by the enzyme glycerol phosphate oxidase (GPO). In this reaction hydrogen peroxide (H_2O_2) is produced in equimolar concentration to the level of triglycerides present in the sample. H_2O_2 reacts with 4-aminoantipyrine and ADPS in the reaction catalyzed by peroxidases (POD). The result of this oxidative coupling is a quinoneimine purple colored dye. The absorbance of this dye in solution is proportional to the concentration of triglycerides in sample.
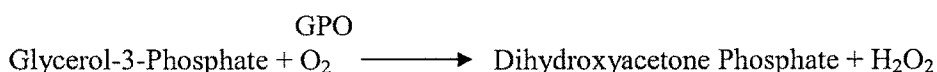
$$\text{Triglycerides} + H_2O \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{Fatty Acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-Phosphate} + \text{ADP}$$

$$\text{Glycerol-3-Phosphate} + O_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetone Phosphate} + H_2O_2$$

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$$POD$$
$$H_2O_2 + \text{4-Aminoantipyrine} + \text{chlorophenol} \longrightarrow \text{Quinoneimine} + $$

Procedure:

Pipette into 3 test tubes labeled Blank (B), Standard (S) and Test (T) as shown below:

	Blank	Standard	Test
Working Reagent (ml)	1.0	1.0	1.0
Standard (ml)	--	0.02	--
Sample (ml)	--	--	0.02

Mix & incubate at 37°C for 10 mins. & read absorbance of test & standard against reagent blank at 520 nm (500-550nm or GREEN filter).

Calculation:

Triglyceride (mg/dl) = Absorbance of Test/ Absorbance of Standard ×200

Assay of High Density Lipoprotein (HDL):

Quantitative estimation of HDL in serum was performed according to manufacturer's instructions provided in commercially available standard diagnostic kit (Span Diagnostics Pvt. Ltd.. India) using Perkin Elmer lamda UV-Vis Spectrophotometer.

Principle:

Low Density Lipoprotein (LDL) Cholesterol, Very Low Density Lipoproteins (VLDL) Cholesterol and Chylomicron fractions are precipitated by addition of Polyethylene Glycol 6000 (PEG). After centrifugation, the High Density Lipoprotein (HDL) Cholesterol fraction remains in the supernatant and is determined with CHOP-PAP method as described in Total Cholesterol estimation.

Procedure:

Precipitate other cholesterol using precipitating reagent.

Step: 1 HDL-Cholesterol separation

Pipette into test tubes	Test
Serum/plasma	200 µL
Reagent 3	200 µL

Mix well and keep at Room Temperature (15-30 ° C) for 10 minutes. Centrifuge for 15 minutes at 2000 rpm and separate clear supernatant. Use the supernatant for HDL-Cholesterol estimation.

Step: 2 HDL-Cholesterol estimation.

Pipette into test tubes	Blank	Standard	Test
Supernatant from step 1	--	--	100 µL
Reagent 4	--	100 µL	--
Reagent 1	1000 µL	1000 µL	1000 µL

Mix well. Incubate at 37°C for 10 minutes or at Room Temperature (15-30 °C) for

30 minutes. Take absorbance of standard and test against blank at 505 nm and calculate

results as per following formula.

Calculation:

HDL-Cholesterol concentration (mg/dl) = Abs of Test / Abs of Standard × 50

Assay of Low Density Lipoprotein (LDL):

Serum LDL cholesterol levels were calculated according to Friedewald's Formula:

$$LDL = TC - [HDL + (TG / 5)]$$

Assay of Very Low Density Lipoprotein (VLDL):

$$VLDL = TG/5$$

Estimation of Free Fatty acids (FFA):

FFA was estimated as per the method by Itaya and Ui 1965 modified by Hron and

Menahan 1981.

Reagents:

- Chloroform (200): Heptane (150): methanol (7) mixture (v/v)

- 2. Copper nitrate-triethanolamine reagent (Cu-TEA)

- 3. Diethyl dithiocarbamate solution (Colour reagent)

- 4. Activated silicic acid

- 5. 20mg palmitic acid was dissolved in 100ml of Chloroform:
  Heptane: methanol mixture-standard solution

Procedure:

About 0.5ml of LAE was added to 5.5ml of CHM (Chloroform heptane methanol) solvent. After adding 200mg of activated silicic acid the content were shaken well and centrifuged. Two ml of Cu-TEA (Copper triethanolamin) reagent was added and mixed well. The tubes were centrifuged to separate the two phases and 2ml of upper phase from each tube was transferred to another set of tubes. To all these tubes 1ml of 0.1% DDC (Diethyl dithio carbamate) colour reagent was added. The colour intensity was measured at 430nm using blue filter. It was expressed as mg/g wet tissue for tissue and mg/dl for serum.

Assay of Phospholipids (PL):

Phospholipid was estimated by the method of Bartlett (1959) by digestion with perchloric acid and the phosphorus liberated was estimated by the method of Fiske and Subbarow (1925).

Reagents:

- Perchloric acid

- Ammonium molybdate reagent (2.5% w/v): 2.5 gm of ammonium molybdate was dissolved in 100ml with 3M sulphuric acid.

- 1-Amino 2-naphthol 4-sulphonic acid (ANSA) reagent (0.25 %):

  (a) 0.25% w/v of ANSA reagent in 15% w/v of sodium metabisulphite and 20% w/v of sodium sulphite.

  (b) Accurately weighed 15 gms of sodium metabisulphite and 20 gms of solium sulphite were dissolved in 50 ml of distilled water separately. 250mg

of ANSA was dissolved in 50 ml of sodium metabisulphite and 50 ml of 20% w/v of sodium sulphite, mixed well and stored at room temperature.

- Standard Phosphorus: 35.1mg of potassium dihydrogen orthophosphate was dissolved in 100ml of distilled water. This contained 80μg of phosphorus/ml.

Procedure:

0.1 ml of sample (serum) was digested with 0.2 ml of perchloric acid over a sand bath. Digestion was continued until it was colourless. The liberated inorganic phosphorus of the digested sample was estimated.

4.3 ml of distilled water was added to the digested sample followed by 0.5 ml of ammonium molybdate. After 10 min, 0.2 ml of ANSA was added.

The tubes were shaken well and kept aside for 20 min and the blue color developed was read at 620 nm against water blank in a Shimadzu 1800 UV-Vis spectrophotometer. Standards and blanks were also treated similarly.

Calculations:

The total phospholipids were estimated by multiplying the value of phosphorus liberated with 25 and expressed as mg/g of wet tissue.

Determination of Inorganic Phosphorus (P_i):

Inorganic Phosphorus (P_i) was estimated by the method described by Fiske and Subbarow (1925).

Reagents:

- Ammonium molybdate reagent (2.5% w/v): 2.5 gm of ammonium molybdate was dissolved in 100 ml with 3 M sulphuric acid.

- 1-Amino 2-naphthol 4-sulphonic acid (ANSA) reagent (0.25 %): (a) 0.25% w/v of ANSA reagent in 15% w/v of sodium metabisulphite and 20% w/v of sodium sulphite. (b) Accurately weighed 15 gms of sodium metabisulphite and 20 gms of solium sulphite were dissolved in 50 ml of distilled water separately. 250mg of ANSA was dissolved in 50 ml of sodium metabisulphite and 50 ml of 20% w/v of sodium sulphite, mixed well and stored at room temperature.

- Standard Phosphorus: 35.1mg of potassium dihydrogen orthophosphate was dissolved in 100ml of distilled water. This contained 80μg of phosphorus/ml.

Procedure:

1ml of the supernatant was taken and the volume was made upto 5.0 ml with distilled water. To this, 1ml of 2.5% ammonium molybdate reagent and 0.5 ml of ANSA reagent were added. The color developed in 20 minutes was read using blank containing water instead of sample at 620 nm. A standard graph was prepared taking different concentrations of standard phosphorus (16-80μg). The values were expressed as μM of inorganic phosphorus liberated/mg protein/min.

Estimation of Glycogen (Seifter *et al.*, 1950):

Tissues were weighed to the accuracy of 0.1 mg on mono-pan balance and added to test tube containing 2 ml 30% KOH. The test tubes were kept in boiling water bath

until the tissue in the tube was completely digested. 2.0 ml of absolute alcohol was added to the tubes to precipitate glycogen. The contents were mixed thoroughly and kept in fridge for about 30 minutes to allow precipitous of glycogen to settle. The tubes were then centrifuged at 3000 rpm for 30 minutes and the supernatant was decanted. The process was repeated once more and finally the pellets were collected at the bottom of the tubes and were dissolved in known volume of distilled water. Different dilutions were made for the estimation of glycogen by the Anthrone method of Seifter et al. (1950). Anthrone reagent in concentrated H_2SO_4 hydrolysis glycogen into glucose which reduces the yellow colored anthrone to green color which is read at 620 nm on spectrophotometer.

Glycogen Phosphorylase (E.C. 2.4.1.1) (Cahill *et al.*, 1957).

Principle: Glycogen phosphorylase cleaves the phosphoric bond of α– 1,4 linkages between glucose molecules, to yield glucose -1-phosphate. The property of synthesizing glycogen from glucose-1-phosphate by liberating inorganic phosphorus is made use of in this procedure.

Procedure: In the sample tube were added 0.2 ml of sodium citrate buffer (0.1M pH5.9), 0.3 ml of potassium fluoride (0.154M), 0.05 ml of glucose-1-phosphate (0.2M) and homogenate (20 mg/ml). The incubation was carried out at 37°C for 30 minutes. The reaction was terminated by adding 1 ml of trichloroacetic acid (10%). In the control tubes all the contents were added along with trichloroacetic acid prior to incubation. The tubes were centrifuged at 3000 rpm for 30 minutes. The supernatant fluid was analyzed for phosphorus content according to the method of Fiske and Subbarow (1925) as

described below. To the supernatant fluid, 0.4 ml of sulphuric acid (10N) and 0.8 ml of ammonium molybdate (2.5%) were added and the tubes were allowed to stand for 10 minutes. After 10 minutes 0.4 ml of ANSA was added. After 10 minutes, the intensity of the blue color developed was read at 660 nm against the reagent blank. Appropriate standards were run along with each assay. The enzyme activity is expressed as μ moles of Pi released/ mg protein/ 15 minutes.

Glucose-6-Phosphatase (E.C. 3.1.3.9) (Harper 1963).

Principle: Glucose-6-phosphatase catalyses the reaction Glucose-6-phosphate + H_2O----------> glucose + phosphate. The rate of the reaction is measured by the increase of inorganic phosphate with time.

Procedure: In the sample tube were added homogenate (25 mg/ml in citrate buffer pH 6.5), and 0.1 ml of glucose-6-phosphate (0.08M). The incubation was carried out at 37°C for 15 minutes. The reaction was terminated by adding 2 ml of trichloroacetic acid. In the control tubes, all the reagents were added as above except for glucose-6-phosphate. The tubes were centrifuged at 3000 rpm for 30 minutes. The supernatant fluid was analyzed for phosphorus content according to the method of Fiske and Subbarow (1925) as described below. To the supernatant fluid, 5 ml of ammonium molybdate (2.5%) and 1 ml of ANSA were added. After 10 minutes, the intensity of the blue color developed was read at 660 nm against the reagent blank. Appropriate standards were run along with each assay. The enzyme activity is expressed as μ moles of Pi released/ mg protein/ 15 minutes.

Glycogen synthase: (E.C. 2.4.1.11). It was assayed by the method of Leloir and Goldenberg (1962).

Principle: In the presence of glycogen primer, glycogen synthase forms the glucose of uridine diphosphoglucose (UDPG) and C4 of the terminal glucose residue of glycogen liberating uridine diphosphate (UDP). The assay is based on the measurement of the amount of UDP formed from UDPG in the presence of glycogen and glucose-6-phosphate. The UDP estimation is carried out by using a preparation of pyruvate kinase (PK) which catalyses the transfer of phosphate from phosphoenolpyruvate (PEP) to UDP. The pyruvate is estimated colorimetrically.

Procedure: The following were added to the test and blank tubes.0.01ml of glycogen (40mg/ml), 0.01 ml of glycine buffer (0.75-pH 8.5) and glucose-6-phosphatase (0.05M) were added and mixed. 1ml of homogenate (10mg/ml) and 0.01ml of UDPG (25μm/ml) were then added to the test alone. The reaction was started by the addition of UDPG and incubated at 37°C for 10 min. The blank tube contained all components except UDPG. The tubes were kept in boiling water bath for 1 min. After incubation 0.01ml of UDPG was added in the blank tube and 0.025ml of PK (8IU) were added and the tubes were incubated at 37°C for 15 min. At the end of the incubation, the reaction was arrested by adding 0.15ml of dinitrophenyl hydrazine (0.1%). The contents of the tube were mixed and allowed to stand for 5 min at room temperature and 0.2ml of 10N sodium hydroxide was added for the maximum development of colour. 1.1ml of ethanol (95%) was then added and the tubes were centrifuged for 15 min at 3000 rpm. The optical density of the supernatant fluid was measured at 520 nm. Appropriate standards were run along with

each assay. Protein was estimated according to the method of Lowry et al. (1951). The enzyme activity was expressed as μ moles of UDP formed/mg protein/10min.

Estimation of Tissue Total Proteins (Lowry *et al.*, 1951) was used for the estimation of total protein.

Reagents:

- Sodium hydroxide (0.1M): 4 gms of sodium hydroxide was dissolved in 400 ml of distilled water and the final volume was made up to 1000 ml with distilled water.

- Lowry C reagent: (a) Copper sulphate in 1% sodium potassium tartarate (1% w/v). 0.5 gm of copper sulphate was dissolved in 1% sodium potassium tartarate (Prepared by dissolving 1gm of sodium potassium tartarate in 100 ml of distilled water). (b) Sodium carbonate in 0.1 M sodium hydroxide (2% w/v) 2gms of sodium carbonate was dissolved in 100 ml of 0.1 M sodium hydroxide. 2ml of solution (a) was mixed with 100 ml of solution (b) just before use.

- Standard Protein (Bovine serum albumin): 20 mg of bovine serum albumin was dissolved in 80 ml of distilled water and few drops of sodium hydroxide were added to aid complete dissolution of bovine serum albumin and to avoid frothing. Final volume was made up to 100 ml with distilled water and stored overnight in a refrigerator.

- Folin's phenol reagent: Folin's phenol reagent was diluted with distilled water in the ratio of 1:2. (i.e. 1ml of Folin's phenol reagent was mixed with 2ml of distilled water).

Procedure:

Diluted membrane fraction aliquots (0.1 ml) were taken in test tubes. To this, 0.8 ml of 0.1 M sodium hydroxide and 5 ml of Lowry C reagent was added and the solution was allowed to stand for 15 minutes. Then 0.5 ml of 1 N Folin's phenol reagent was added and the contents were mixed well on a vortex mixer. Colour developed was measured at 640 nm against reagent blank containing distilled water instead of sample. Different concentrations (40-200 µg) of standard protein (Bovine serum albumin) were taken and processed as above for standard graph. The values were expressed as mg of protein/ gm of wet tissue (mg/gm).

Hematoxylin and eosin staining:-

After the treatment period, the animals were sacrificed and the organs were excised, blotted free of blood and tissue fluids and preserved / fixed in 10 % formalin . Briefly, after 1-2 days the tissues were washed thoroughly in repeated changes of 70% alcohol and then dehydrated in ascending grades of alcohol (70-100%). After dehydration, the tissues were cleaned in xylene and embedded in paraffin wax. Sections of 5µ thickness were cut on a microtome and taken on glass slides coated with albumin. The sections were deparaffinized in xylene and downgraded through 100, 90, 70, 50, 30% alcohol and then finally in water. The haematoxylin-stained sections were stained with eosin for 2 minutes and were then quickly passed through ascending grades of alcohol, cleaned in xylene and mounted in DPX.

Gomori-Phloxine differential staining for pancreas:-

SOLUTIONS:

- **Bouins's Fluid**

- **0.3% Potassium Permanganate Solution**

- **5% Sodium Bisulfite Solution**

- **Chromium Hematoxylin Solution**

(To 100ml of chromium hematoxylin solution add 0.1g of potassium iodate. Boil until a deep blue. The mixture is ripe immediately and can be used as long as a film with a metallic luster forms on its surface in a Coplin jar.

- **1% Acid Alcohol**

- **0.5% Phloxine B Solution**

- **5% Phosphotungstic Acid**

STAINING PROCEDURE:

- Treat in Bouin's fluid for 12-24 hours.
- Rinse in running tap water for 15 minutes.
- Treat in potassium permanganate for 1 minute.
- Decolorize in sodium bisulfate.
- Rinse in running tap water for 10 minutes.
- Stain in chromium hematoxylin at 60°C for 1-2 hours.
- Rinse in acid alcohol for 1 minute.
- Rinse in running tap water for 5 minutes.
- Stain in phloxine B at 60°C for 2-3 hours.

- Rinse in distilled water.
- Treat in phosphotungstic acid for 1 minute.
- Rinse in running tap water for 5 minutes.
- Differentiate in 95% ethyl alcohol for approximately 1 minute.
- If section is too red, rinse in 80% alcohol for 15-20 seconds.

Blow dry. Mount.

Results: Differential pancreatic islet cell stain. Alpha cells -- pink; Beta cells -- blue; D cells from pink to red are indistinguishable from alpha cells.

Immunostaining and Confocal Microscopy:-

The pancreas were aseptically removed from the respective treatment groups and fixed in 4% fresh para-formaldehyde. The tissues were subsequently embedded in paraffin wax and sectioned at 5 mm thickness with a microtome (Leica, Wetzlar, Germany) and mounted on poly-L-lysine (Sigma) coated slides. Slides were de-paraffinized, downgraded in xylène and alcohol and blocked with 4% normal donkey serum and then incubated with antisera. Guinea pig anti-insulin antibody (Linco Research Inc, St. Charles, MO, USA), mouse antiglucagon (Sigma), were used at 1:100 dilutions. Alexa–Fluor 488 and Alexa–Fluor 546F (ab0)2 secondary antibodies (Molecular Probes, OR, USA) were used at 1:200 dilution. Hoechst 33342 was used to visualize nuclei. Primary antibodies were incubated overnight at 4°C, washed with calcium–magnesium-containing PBS 90 and then incubated with the secondary antibodies at 37°C for 1 h. Slides were washed extensively in PBS and mounted in Vectasheild (Vectorlabs).

Confocal images were captured using a Zeiss LSM 510 laser scanning microscope using a 63x1.3 oil objective with optical 95 slices ~0.8 mm. Magnification, laser and detector gains were set below saturation and were identical across samples.

Statistical analysis:-

Statistical evaluation of the data was done by one way ANOVA followed by Bonferroni's Multiple comparison test .The results are expressed as mean ± S.E.M using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego California USA.

Results:-

Plasma glucose (Table 1): - DC group recorded significantly elevated plasma blood glucose levels throughout the study period ($p < 0.001$). Administration of extract to diabetic rats showed significant reduction in blood glucose levels. Low doses of extract (DOT100 and DOI250 mg /kg) had no significant effect on plasma blood glucose throughout study period (Table 1). Higher doses of extract (DOI500 and DOI750 mg/kg) proved to be very potent as glycemic indices were significantly reduced especially between 7th and 28th days of study.

Plasma Hb, Urea, Creatinine, HbA1c and Insulin levels (Table 2; Fig. 1-6, 7-10):- Diabetic rats recorded significant decrement in haemoglobin content and increase in glycosylated haemoglobin (Fig. 8, 10) along with increase in Urea and Creatinine levels (Fig. 4, 6). Low doses of extract (DOI100 and DOI250 mg/kg of body weight) were not able to induce significant changes in the levels of haemoglobin and urea (Table 2) though; glycosylated haemoglobin recorded a significant decrement. Higher doses of the extract (DOI500 and DOI750 mg/kg) registered a significant increment in haemoglobin content with a concomitant decrement in glycosylated haemoglobin, Urea and Creatinine contents ($p < 0.05$). These results were comparable to metformin treated rats. OI extract administration did not have any significant effect on any of the parameter in NC rats (Fig. 3, 5, 7, 9). Grading of urine sugar level by Benedicts qualitative analysis revealed very high sugar content in DC rats, while it was in trace amounts in DOI750 rats. Insulin titre in DC animals was significantly lower (Fig.2), which was significantly improved by

treatment with 750 mg of extract. The DC Met animals showed slightly more elevated level compared to DOI750 but, still was lesser than in the control animals (Fig. 1).

Plasma Lipoproteins (Table 3; Fig. 11-16):- Diabetic animals recorded significant increase in plasma LDL-C (Fig. 12) and VLDL-C (Fig. 14), while HDL-C (Fig. 16) showed significant decrement. Low doses of extract (DOI100 and DOI250 mg/kg of body weight) did not have any significant effect in reversing the plasma lipoprotein levels, but higher doses of extract (DOI500 and DOI750 mg/kg of body weight) showed significant efficacy in ameliorating the altered plasma lipoprotein level (Table 3). These results were comparable with metformin treated diabetic rats.

Plasma lipid profile (Table 4; Fig. 17-24): - Diabetic animals recorded significant increase in plasma TC (Fig. 18) ($p<0.05$), TG (Fig. 20) ($p<0.05$), FFA (Fig. 21) ($p<0.05$) and PHL (Fig. 24) ($p<0.05$). Low doses of extract (DOI100 and DOI250 mg/kg of body weight) did not have any significant effect in reversing the plasma lipid profile, but higher doses of extract (DOI500 and DOI750 mg/kg of body weight) showed significant efficacy in ameliorating the altered lipid profile (Table 4). These results were comparable with metformin treated diabetic rats. OI extract did not have any significant effect on plasma lipid profile in control rats (Fig. 17, 19, 21, 23).

Liver and muscle glycogen and glucose metabolic enzymes (Fig. 25-31):

Liver glycogen (Fig. 26) contents decreased significantly ($p < 0.0001$) in the STZ treated group while OI extract treated groups recorded a significant increase ($p< 0.001$ &

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p<0.0001). Hepatic Glycogen phosphorylase (Fig. 25) and Glucose-6-phosphatase (Fig. 28) activity significantly increased ($p < 0.0001$, $p < 0.0001$) respectively in the STZ treated group. Rats treated with aqueous extract of OI showed significant decrease in enzymic activity compared to the diabetic group (p < 0.0001). Glycogen synthase (Fig. 27) activity in liver was decreased significantly ($p < 0.0001$) in diabetic group of animals and, animals treated with aqueous extract of OI showed significant increment ($p <$ 0.0001). Muscle glycogen (Fig. 30) content decreased significantly ($p < 0.0001$) in the diabetic group while a non-significant increase was seen in rats treated with low dose of OI extarct ($p > 0.05$). Diabetic animals supplemented with high doses of OI extract displayed a significant increment in glycogen content ($p < 0.001$). Glycogen phosphorylase (Fig. 29) activity in muscle increased significantly ($p < 0.0001$) in the STZ treated group. The decrease in the enzymic activity in both low and high OI dosed groups when compared with diabetic animals was highly significant ($p < 0.0001$). Muscle Glycogen synthase (Fig. 31) activity decreased significantly ($p < 0.0001$) in STZ group of animals while there was significant increase in DC rats treated with low doses of OI extract ($p < 0.01$).

Pancreas histology: Histoarchitecture of pancreas of diabetic animals showed marked islet disturbance, evident by wide intracellular spaces evident by hematoxylin and eosin (Fig. 32) and correlated with gomori phloxine staining (Fig. 33). Furthermore, diabetic animals displayed weak insulin immunopositivity (Fig. 34) depicting streptozotocin insult. OI extract treated animals (both doses) exhibited ameliorating effects, improved islet morphology and demonstrated increased expression of insulin positive beta cells.

Table: 1. Effect of OI extract on fasting blood glucose levels at 0, 7th, 14th, 21st and 28th days of treatment:

Groups	Treatments	Blood Glucose (mg/dl)				
		0th Day	7th Day	14th Day	21th Day	28th Day
I	NC	105.4±11.74	102.3±6.00	103.9±9.70	97.25±12.05	104.9±8.30
II	NC+ O.I 100	102.9±4.37	106.4±12.05	99.90±3.70	109.5±15.15	98.25±11.95
III	NC + O.I 250	110.6±9.34	107.5±11.15	104.1±3.80	102.7±6.40	110.3±5.35
IV	NC + O.I 500	105.7±9.30	106.6±15.95	106.7±6.25	103.0±6.05	110.6±5.35
V	NC + O.I 750	106.1±3.50	110.4±13.15	100.44±7.20	111.6±9.70	100.6±4.70
VI	DC	432.4±23.26	456.2±27.05	412.8±16.15	423.1±13.15	429.3±23.10
VII	DC + O.I 100	427.7±16.51	415.8±13.85	400.8±13.65	381.2±19.00	367.4±17.20
VIII	DC + O.I 250	433.3±12.99	412.6±19.35	394.6±16.40	366.5±20.70	351.3±16.10
IX	DC + O.I 500	441.6±24.27	337.7 ±15.80[a]	289.6±13.65[b]	232.6±12.35[c]	207.2±10.85[c]
X	DC + O.I 750	439.4±16.30	316.8±12.20[b]	277.3±17.25[c]	236.2±15.90[c]	186.6±17.35[c]
XI	DC+ Metformin	427.6±20.75	318.7±12.45[b]	241.4±19.90[c]	186.5±15.85[c]	147.6±14.70[c]

Groups II to V were compared with Group I
Groups VII to XI were compared with Group VI
a =p<0.05, b=p< 0.01and c= p<0.001

Table: 2. Changes in hemoglobin, insulin, urea and Creatinine contents in the blood of control and experimental rats treated with OI extract:

Groups	Treatments	Hb (gm/dl)	HbA1c (% of Hb)	Insulin (μIU/ml)	Urea (mg/dl)	Creatinine (mg/dl)
I	NC	13.20±0.20	6.40±0.20	25.61±0.21	25.60±3.20	24.50±2.30
II	NC+ O.I 100	13.50±0.20	6.40±0.10	24.57±0.18	24.80±2.10	26.25±1.05
III	NC + O.I250	12.90±0.30	6.60±0.10	24.81±0.61	25.60±1.40	24.30±2.10
IV	NC + O.I500	13.20±0.20	6.20±0.10	25.06±0.20	25.25±1.95	23.70±2.20
V	NC + O.I750	12.90±0.50	6.40±0.20	24.28±0.13	26.60±1.60	24.30±0.80
VI	DC	8.90±0.10	12.90±0.20	6.18±0.21	43.20±2.10	46.20±3.40
VII	DC + O.I100	8.90±0.40	10.35±0.25^c	8.08±0.24	40.90±2.20	42.10±0.70
VIII	DC + O.I250	9.70±0.20	9.85±0.15^c	11.51±0.19 ^{a}	33.70±3.10	32.46±2.10^a
IX	DC + O.I500	9.40±0.30	8.60±0.30^c	14.16±0.27 ^{b}	27.75±1.05^b	27.85±1.51^b
X	DC + O.I750	10.30±0.20	8.20±0.20^c	15.64±0.18 ^{c}	26.43±1.07^b	29.81±1.44^b
XI	DC+Metformin	12.40±0.20^c	8.70±0.20^c	18.64±0.30^c	26.12±1.11^b	34.34±1.01^a

Groups II to V were compared with Group I
Groups VII to XI were compared with Group VI
a =p<0.05, b=p< 0.01and c= p<0.001

Table: 3. Changes in serum lipoproteins in control and experimental animals treated with OI extract:

Groups	Treatments	Plasma Lipoprotein levels			Atherogenic index
		LDL-C (mg/dl)	VLDL-C (mg/dl)	HDL-C (mg/dl)	
I	NC	25.86±1.20	8.86±0.80	21.10±0.80	1.81
II	NC+ O.I 100	22.66±1.63	8.56±0.56	21.60±0.70	1.65
III	NC + O.I 250	31.24±1.59	9.64±0.58	20.10±1.20	2.07
IV	NC + O.I 500	26.99±1.96	8.40±0.74	21.95±0.35	1.85
V	NC + O.I 750	30.06±1.83	8.64±0.63	19.60±1.70	2.09
VI	DC	74.14±2.00	16.74±0.66	13.60±1.30	5.22
VII	DC + O.I 100	72.26±1.33	14.96±0.52	14.40±1.20	4.98
VIII	DC + O.I 250	64.99±2.01	13.44±0.42	15.25±1.05	4.38
IX	DC + O.I 500	45.52±1.45[c]	10.72±0.68[b]	20.40±1.10	2.66
X	DC + O.I 750	34.00±1.77[c]	8.70±0.31[c]	21.70±1.50[a]	2.17
XI	DC+Metformin	34.24±1.98[c]	8.54±0.48[c]	21.60±0.70[a]	2.19

Groups II to V were compared with Group I
Groups VII to XI were compared with Group VI
a =p<0.05, b=p< 0.01and c= p<0.001

Table: 4. Effect of OI extract on serum lipid profile in control and various experimental groups:

Groups	Treatments	TC (mg/dl)	TG (mg/dl)	FFA (mg/dl)	PHL (mg/dl)
I	NC	38.1±2.58	44.3±3.98	40.4±2.15	101.5±2.85
II	NC+O.I 100	35.7±2.45	42.8±2.82	40.1±1.14	103.4±1.21
III	NC + O.I 250	41.7±3.45	48.2±2.90	42.9±1.62	99.83±3.50
IV	NC + O.I 500	40.5±2.20	42.0±3.68	43.0±2.00	100.5±3.51
V	NC + O.I 750	41.0±1.33	43.2±3.13	41.2±1.47	102.3±3.01
VI	DC	71.0±2.95	83.7±3.31	76.6±2.35	134.6±3.38
VII	DC + O.I 100	71.7±1.30	74.8±2.58	72.2±1.84	128.0±2.66
VIII	DC + O.I 250	66.8±1.85	67.2±2.11	67.9±1.49	120.0±1.36
IX	DC + O.I 500	54.2±1.83[a]	53.6±3.38[a]	51.2±1.18[c]	107.8±2.49[b]
X	DC + O.I 750	47.0±1.31[b]	43.5±1.52[b]	43.5±1.46[c]	99.35±3.01[c]
XI	DC+Metformin	47.3±1.97[b]	42.7±2.41[b]	44.9±1.00[c]	94.83±3.49[c]

Groups II to V were compared with Group I
Groups VII to XI were compared with Group VI
a =p<0.05, b=p< 0.01and c= p<0.001

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Fig. 1. Effect of various doses of OI extract on Insulin titre in normal animals after 28 days of treatment
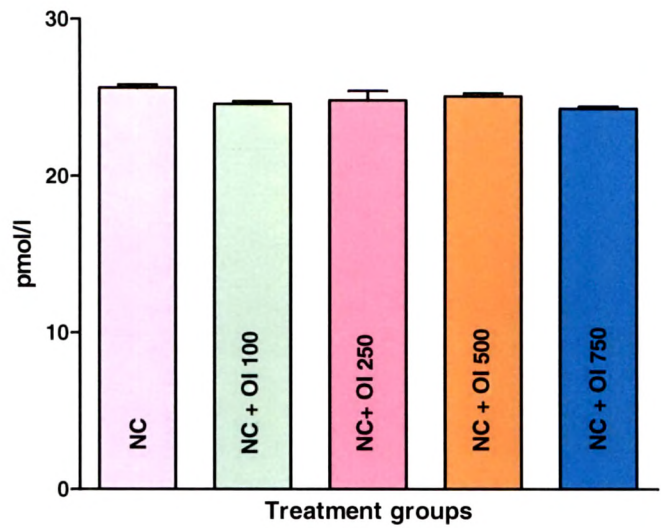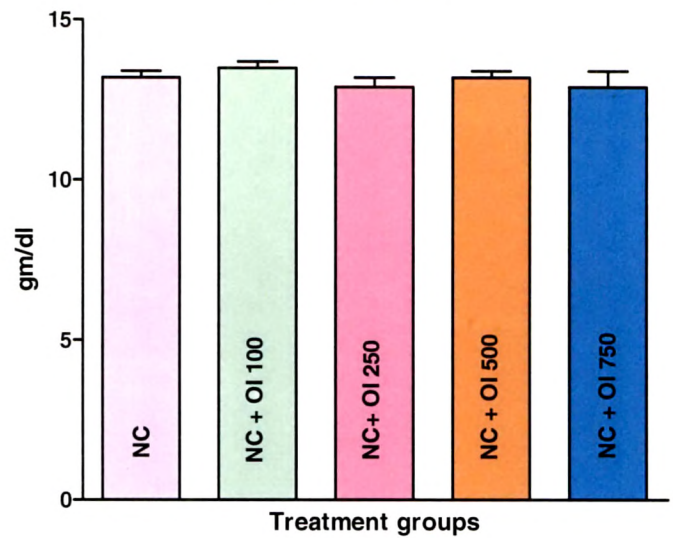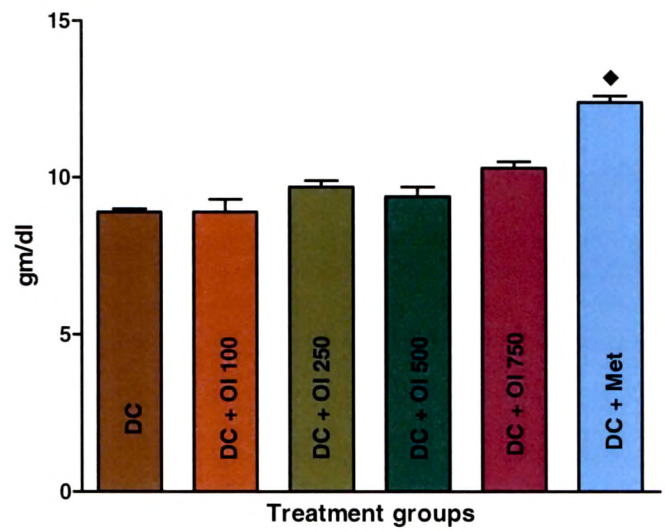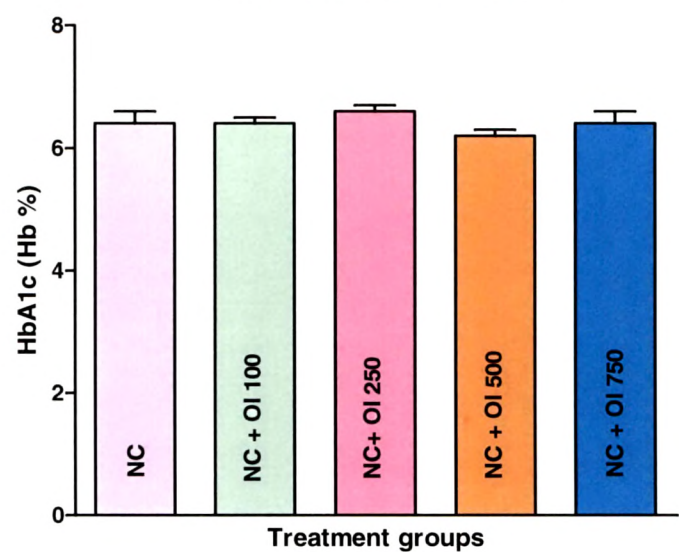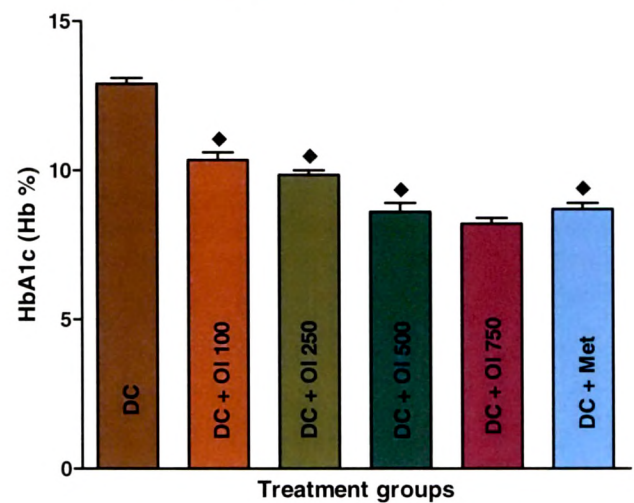

Fig. 2. Effect of various doses of OI extract on Insulin level in diabetic animals after 28 days of treatment

● = p < 0.05, ■ =p <0.01, ◆ =p < 0.001: Experimental groups compared to diabetic control

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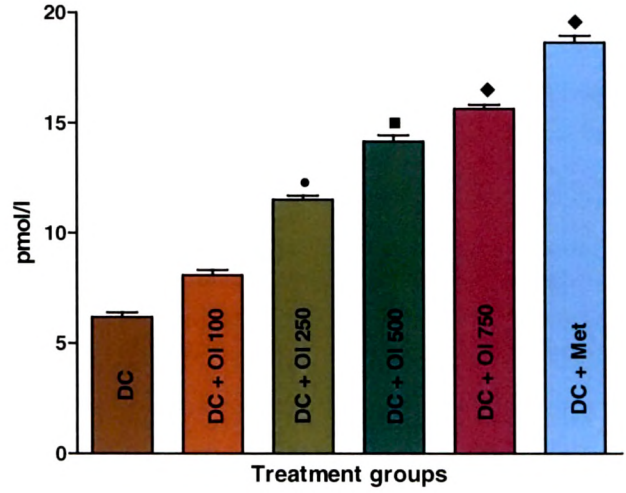
Fig. 3. Effect of various doses of OI extract on plasma urea level in normal animals after 28 days of treatment
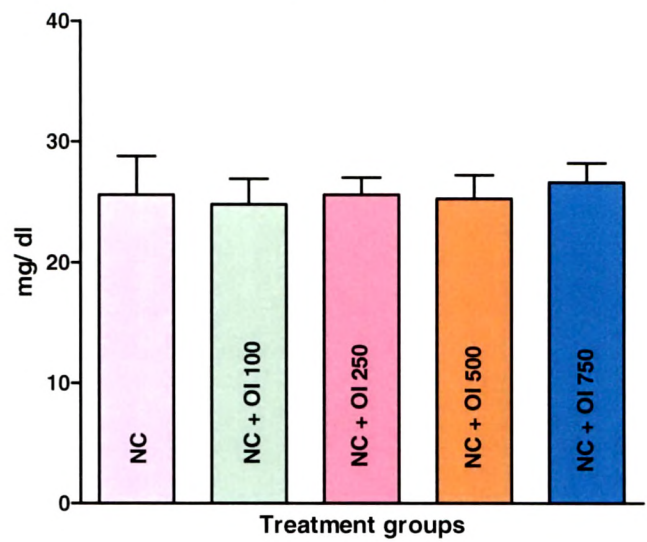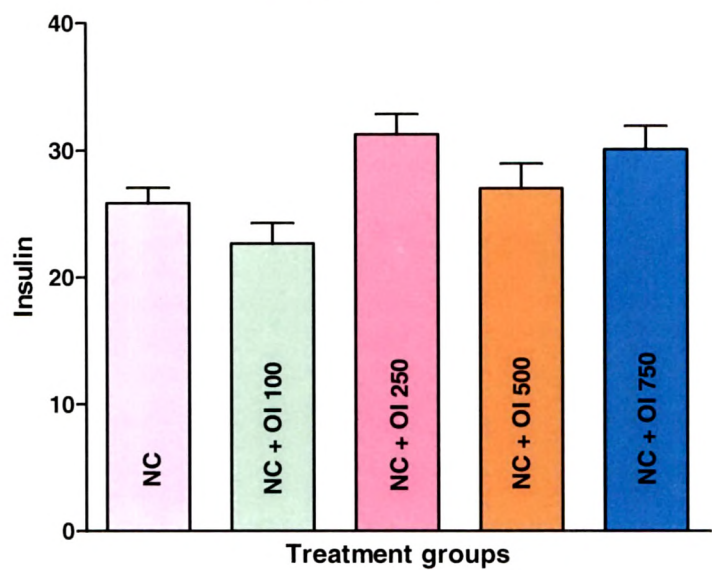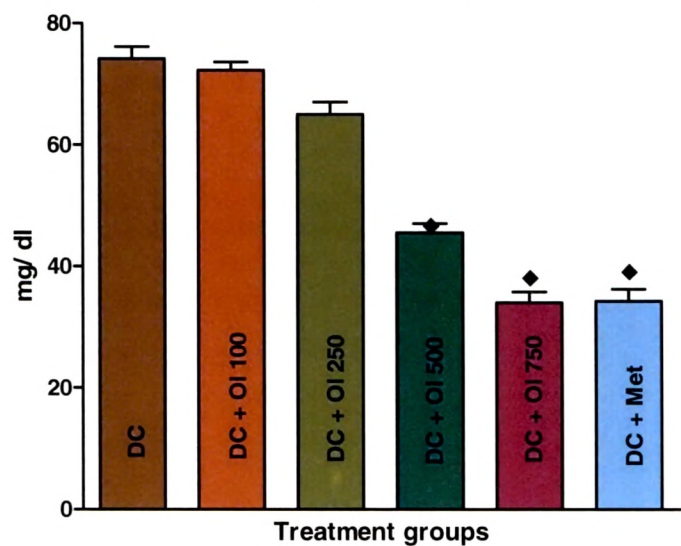

Fig. 4. Effect of various doses of OI extract on plasma urea level in diabetic animals after 28 days of treatment
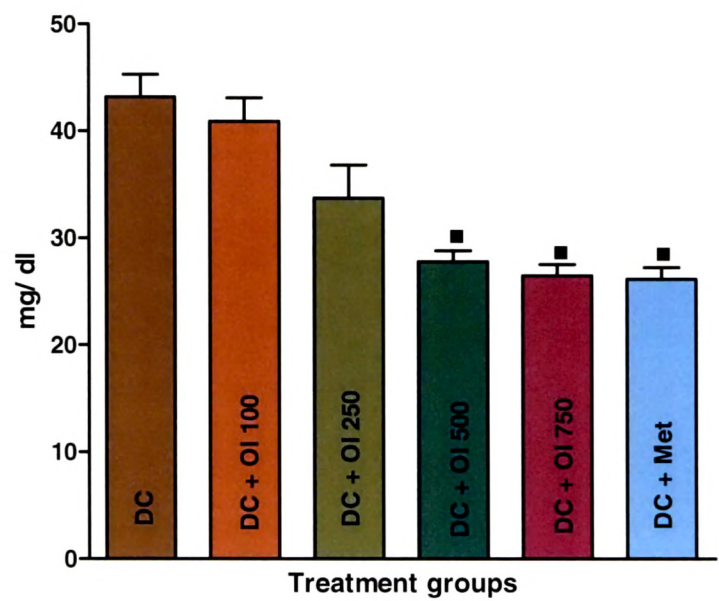
● = p < 0.05, ■=p <0.01, ◆=p < 0.001:Experimental groups compared to diabetic control

Fig. 5. Effect of various doses of OI extract on plasma creatinine level in normal animals after 28 days of treatment
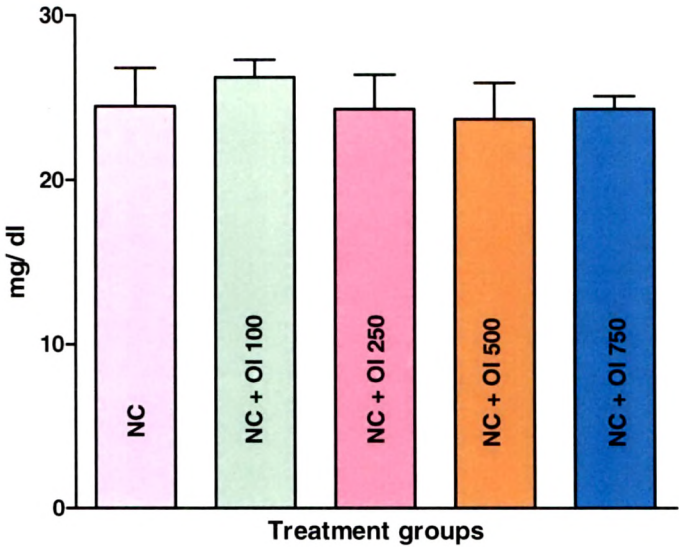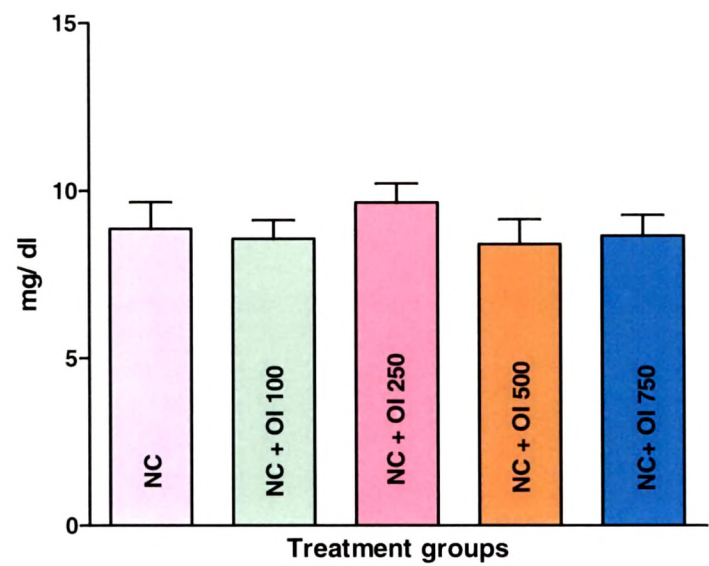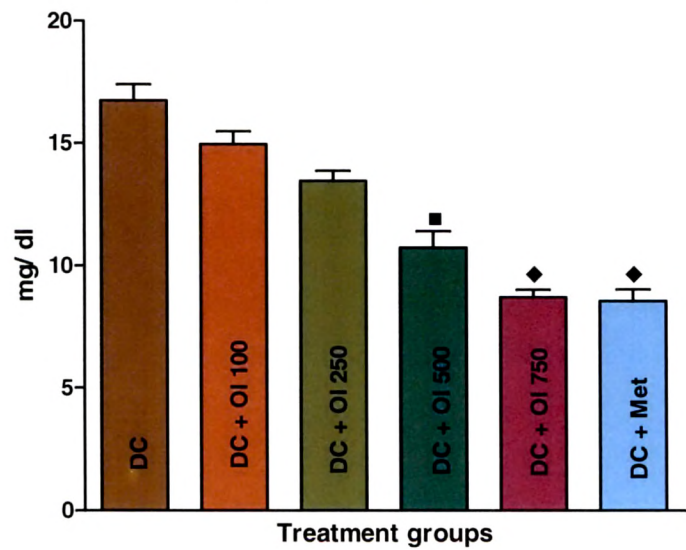

Fig. 6. Effect of various doses of OI extract on plasma creatinine level in diabetic animals after 28 days of treatment

● = p < 0.05, ■=p <0.01, ◆=p < 0.001:Experimental groups compared to diabetic control

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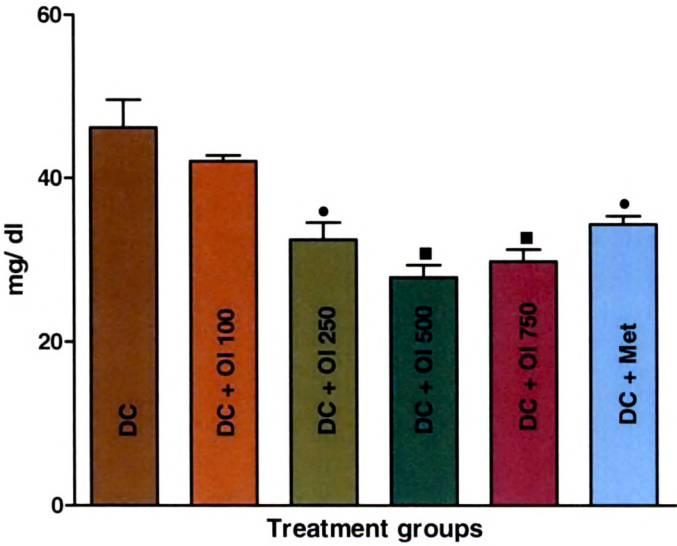
Fig. 7. Effect of various doses of Ol extract on hemoglobin content in normal animals after 28 days of treatment

Fig. 8. Effect of various doses of Ol extract on hemoglobin content in diabetic animals after 28 days of treatment

● = p < 0.05, ■=p <0.01, ◆=p < 0.001:Experimental groups compared to diabetic control

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Fig. 9. Effect of various doses of OI extract on glycosylated hemoglobin content in normal animals after 28 days of treatment

Fig. 10. Effect of various doses of OI extract on glycosylated hemoglobin content in diabetic animals after 28 days of treatment

● = $p < 0.05$, ■ = $p < 0.01$, ◆ = $p < 0.001$:Experimental groups compared to diabetic control

Fig. 11. Effect of various doses of OI extract on plasma LDL -C level in normal animals after 28 days of treatment
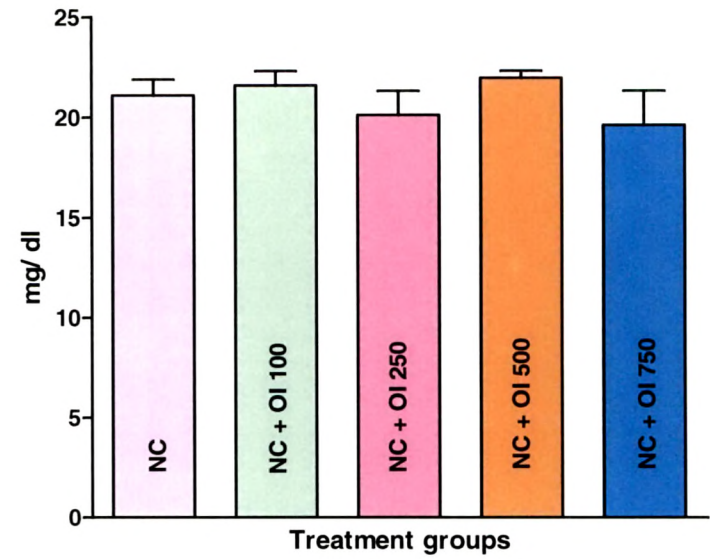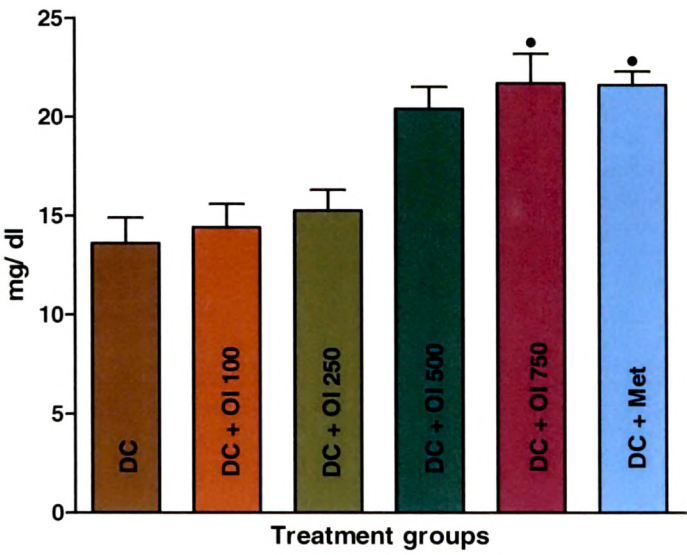

Fig. 12. Effect of various doses of OI extract on plasma LDL -C level in diabetic animals after 28 days of treatment

● = p < 0.05, ■=p <0.01, ◆=p < 0.001:Experimental groups compared to diabetic control

Fig. 13. Effect of various doses of OI extract on plasma VLDL -C level in normal animals after 28 days of treatment

Fig. 14. Effect of various doses of OI extract on plasma VLDL -C level in diabetic animals after 28 days of treatment

● = p < 0.05, ■=p <0.01, ◆=p < 0.001:Experimental groups compared to diabetic control

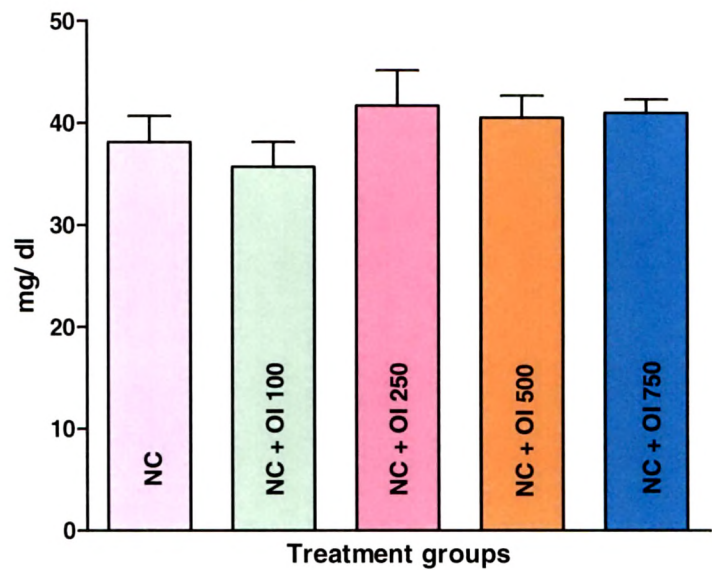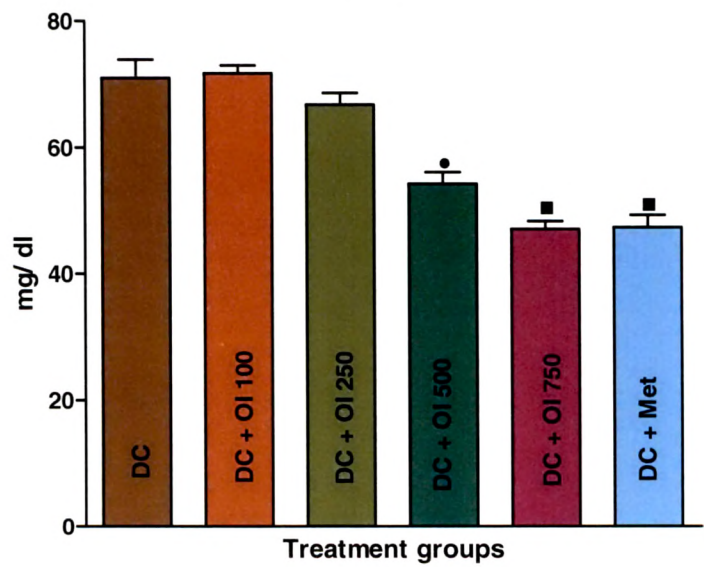Fig. 15. Effect of various doses of OI extract on plasma HDL -C level in normal animals after 28 days of treatment

Fig. 16. Effect of variuos dosses of OI extract on plasma HDL -C level in diabetic animals after 28 days of treatment

● = p < 0.05, ■=p <0.01, ◆=p < 0.001:Experimental groups compared to diabetic control

Fig. 17. Effect of various doses of OI extract on plasma total cholesterol level in normal animals after 28 days of treatment

Fig. 18. Effect of various doses of OI extract on plasma total cholesterol level in diabetic animals after 28 days of treatment

● = p < 0.05, ■=p <0.01, ◆=p < 0.001:Experimental groups compared to diabetic control

Fig. 19. Effect of various doses of OI extract on plasma triglyceride level in normal animals after 28 days of treatment
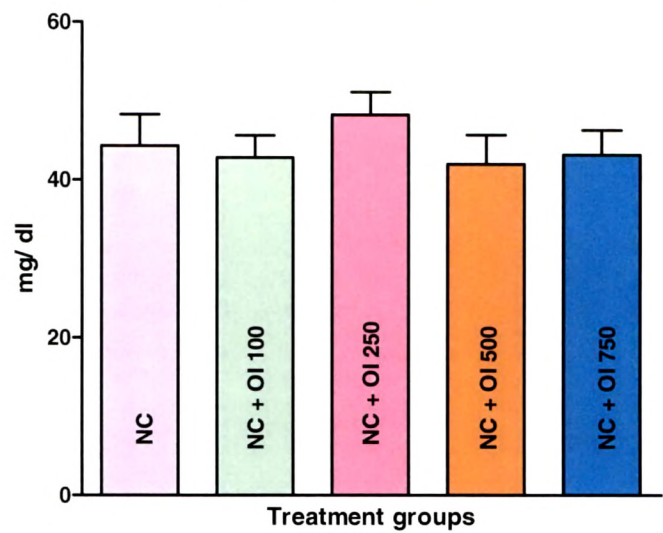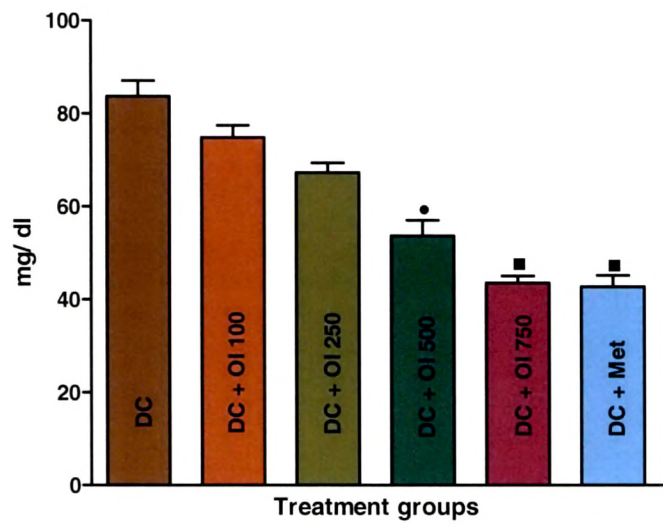

Fig. 20. Effect of various doses of OI extract on plasma triglyceride level in diabetic animals after 28 days of treatment

● = p < 0.05, ■=p <0.01, ◆=p < 0.001:Experimental groups compared to diabetic control

Fig. 21. Effect of various doses of OI extract on plasma free fatty acid level in normal animals after 28 days of treatment
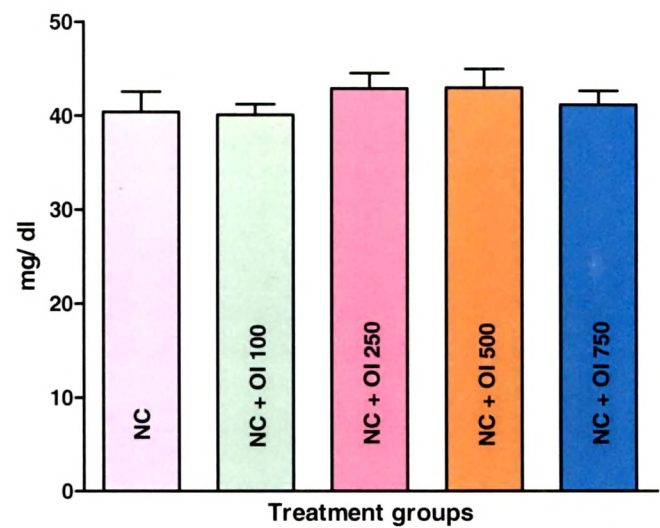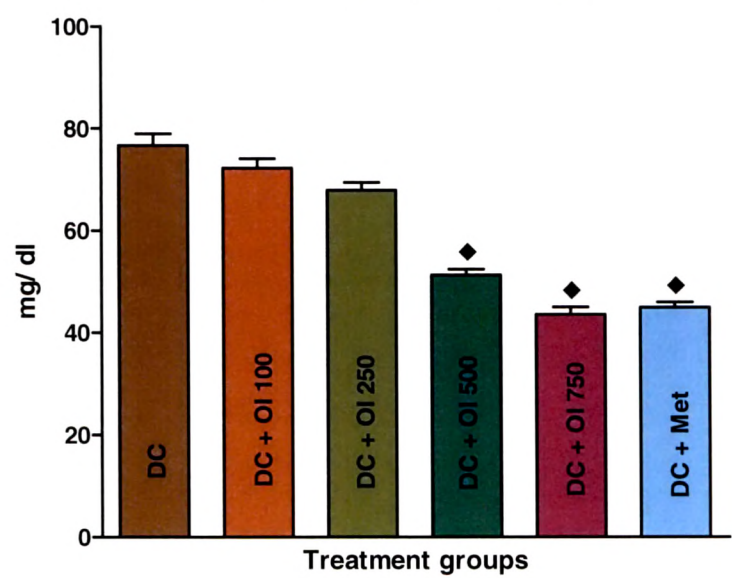

Fig. 22. Effect of various doses of OI extract on plasma free fatty acid level in diabetic animals after 28 days of treatment

● = p < 0.05, ■ =p <0.01, ◆ =p < 0.001:Experimental groups compared to diabetic control

Fig. 23. Effect of various doses of Ol extract on plasma phospholipid level in normal animals after 28 days of treatment
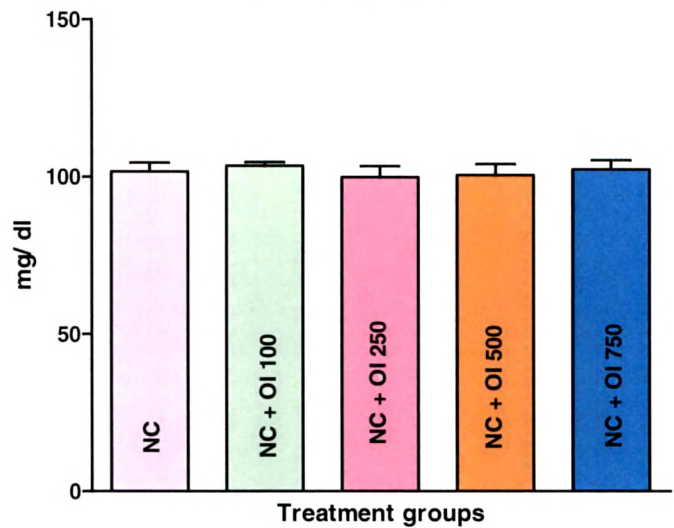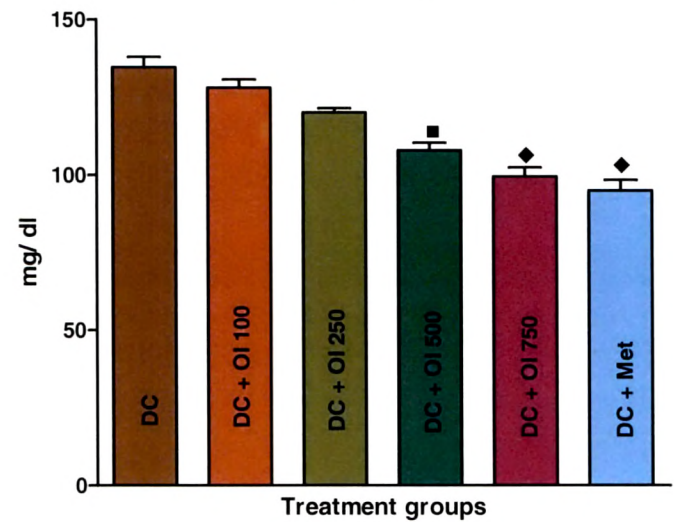

Fig. 24. Effect of various doses of Ol extract on plasma phospholipid level in diabetic animals after 28 days of treatment

● = $p < 0.05$, ■=$p < 0.01$, ◆=$p < 0.001$:Experimental groups compared to diabetic control

Fig. 25. Changes in hepatic Glycogen phosphorylase activity of control and experimental animals

● =p<0.01, ■=p<0.001, ◆=p<0.0001: Experimental groups compared to control group
α =p<0.01, β=p<0.001, δ=p<0.0001: Treated groups compared to diabetic group

Fig. 26. Changes in hepatic Glycogen content of control and experimental animals

● =p<0.01, ■=p<0.001, ◆=p<0.0001: Experimental groups compared to control group
α =p<0.01, β=p<0.001, δ=p<0.0001: Treated groups compared to diabetic group

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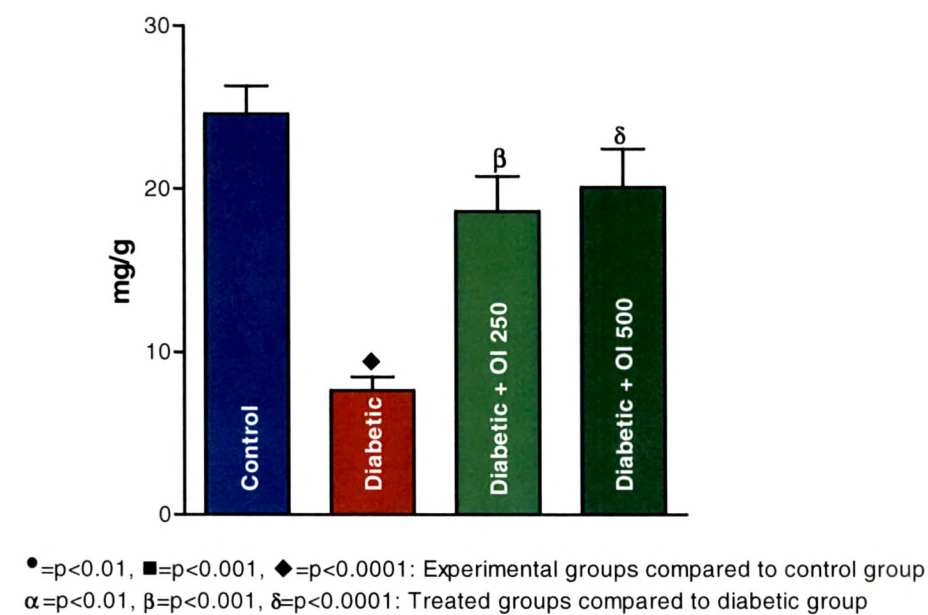
Fig. 27. Changes in hepatic Glycogen synthase activity of control and experimental animals

●=p<0.01, ■=p<0.001, ◆=p<0.0001: Experimental groups compared to control group
α=p<0.01, β=p<0.001, δ=p<0.0001: Treated groups compared to diabetic group
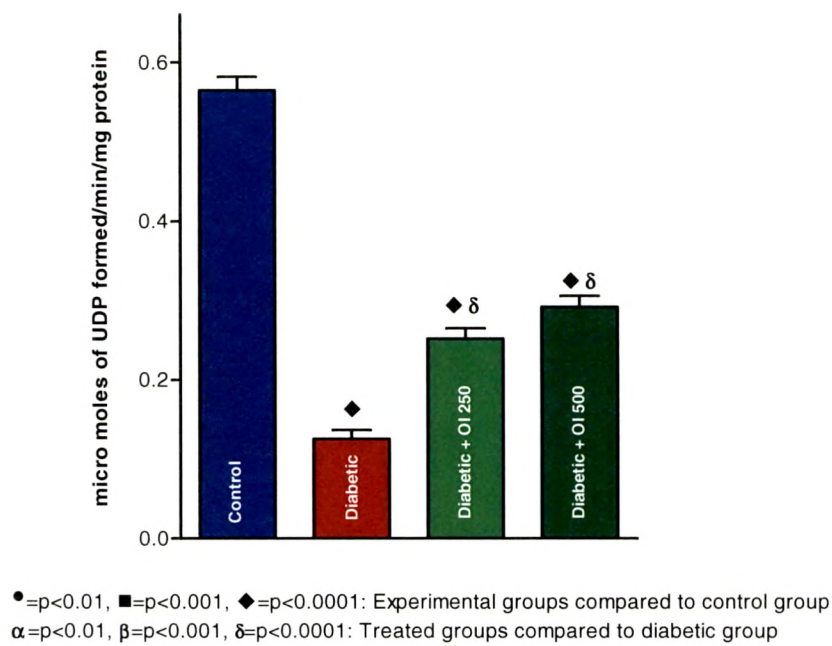
Fig. 28. Changes in hepatic Glucose-6- phosphatase activity of control and experimental animals

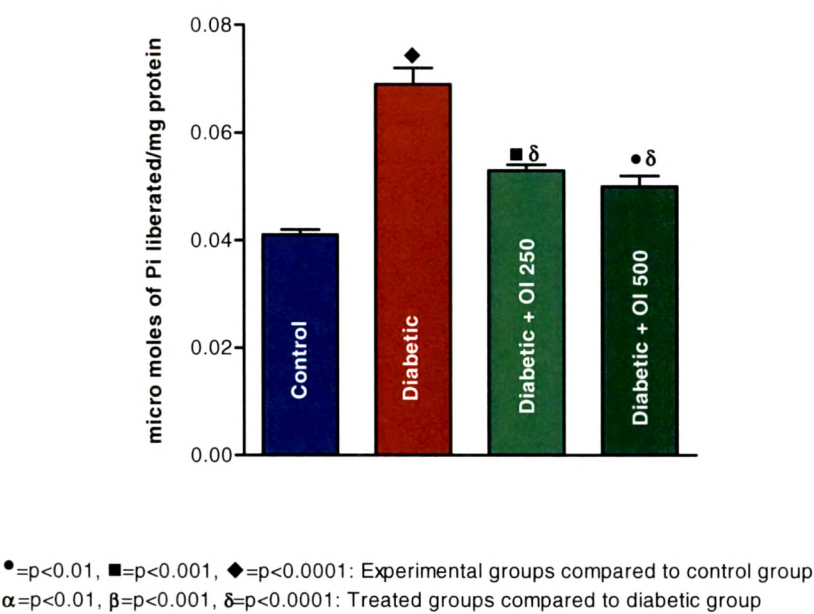●=p<0.01, ■=p<0.001, ◆=p<0.0001: Experimental groups compared to control group
α=p<0.01, β=p<0.001, δ=p<0.0001: Treated groups compared to diabetic group

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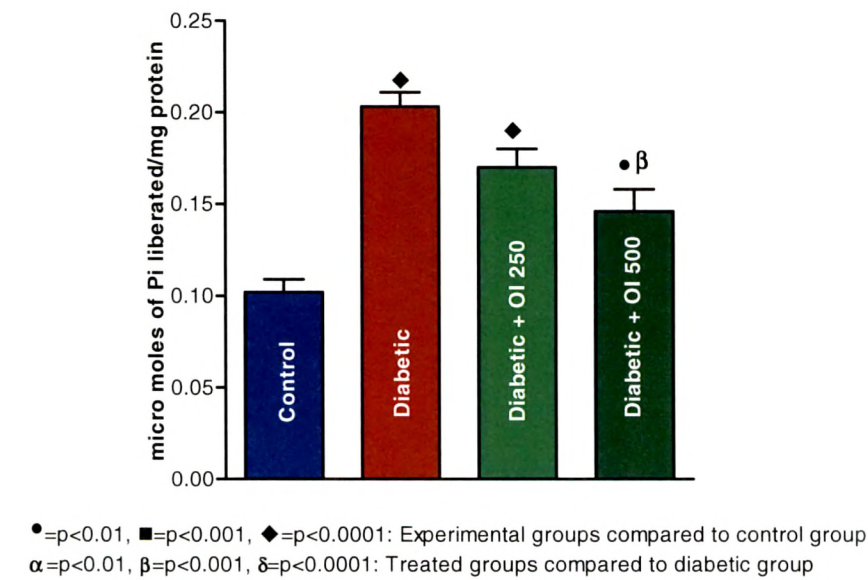
Fig. 29. Changes in muscle Glycogen phosphorylase activity of control and experimental animals

•=p<0.01, ■=p<0.001, ◆=p<0.0001: Experimental groups compared to control group
α=p<0.01, β=p<0.001, δ=p<0.0001: Treated groups compared to diabetic group
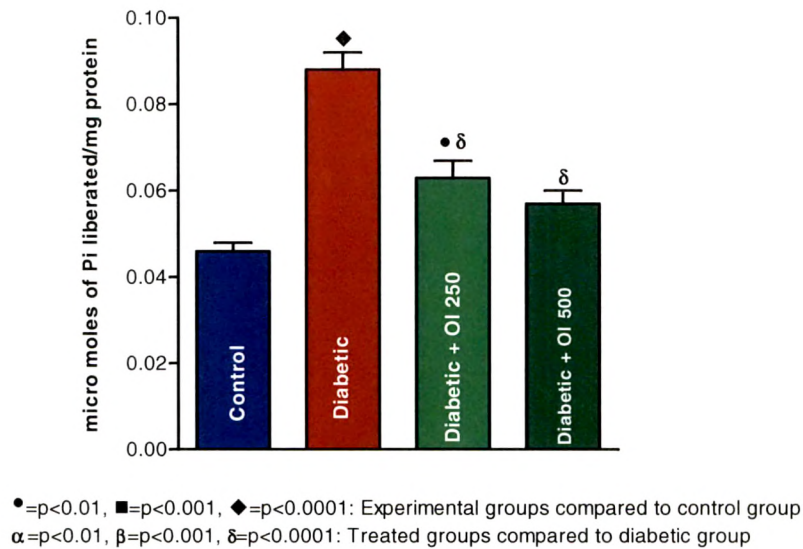
Fig. 30. Changes in muscle Glycogen content of control and experimental animals

•=p<0.01, ■=p<0.001, ◆=p<0.0001: Experimental groups compared to control group
α=p<0.01, β=p<0.001, δ=p<0.0001: Treated groups compared to diabetic group
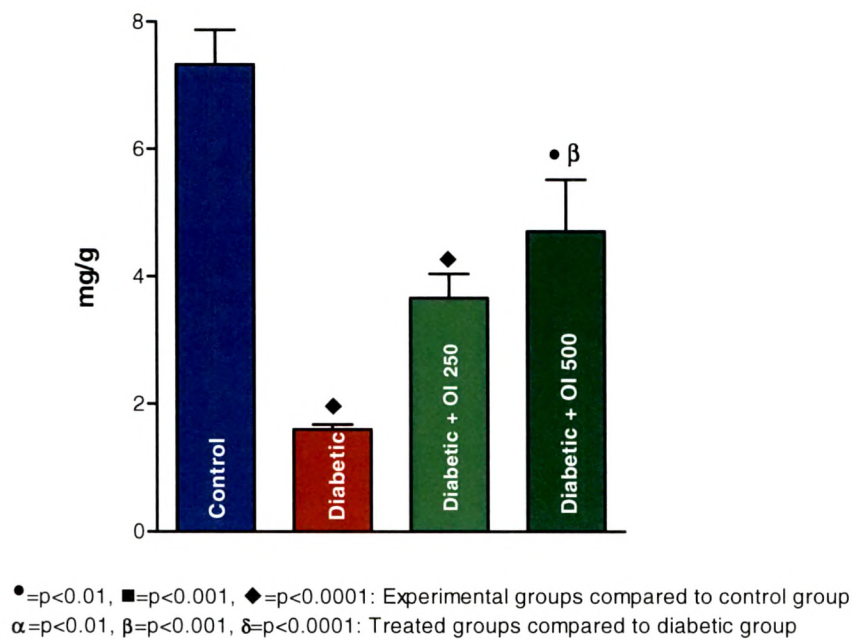
Fig. 31. Changes in muscle Glycogen synthase activity of control and experimental animals

●=p<0.01, ■=p<0.001, ◆=p<0.0001: Experimental groups compared to control group
α=p<0.01, β=p<0.001, δ=p<0.0001: Treated groups compared to diabetic group

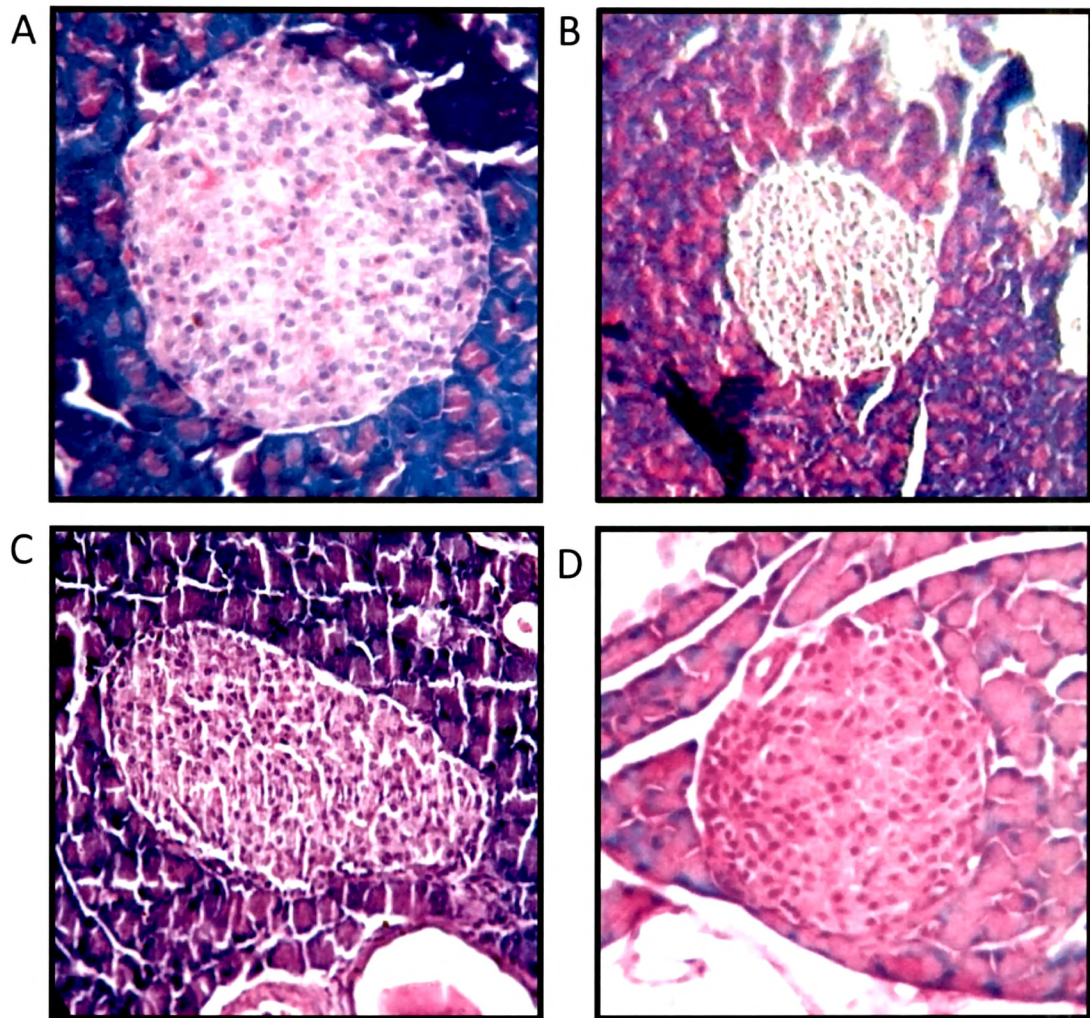Figure 32 : Images represent haemetoxylin and eosin stained sections of pancreas of A) Control animals showing intact islet architechture, B) Diabetic animals depicting islet cell destruction and wider intracellular spaces, C) Diabetic + OI 250 treated animals represent ameliorating effect of extract and D) Diabetic + OI 500 treated animals demonstrate well defined islet morphology . Magnification 200X

GOMORI PHLOXINE STAINING OF PANCREAS
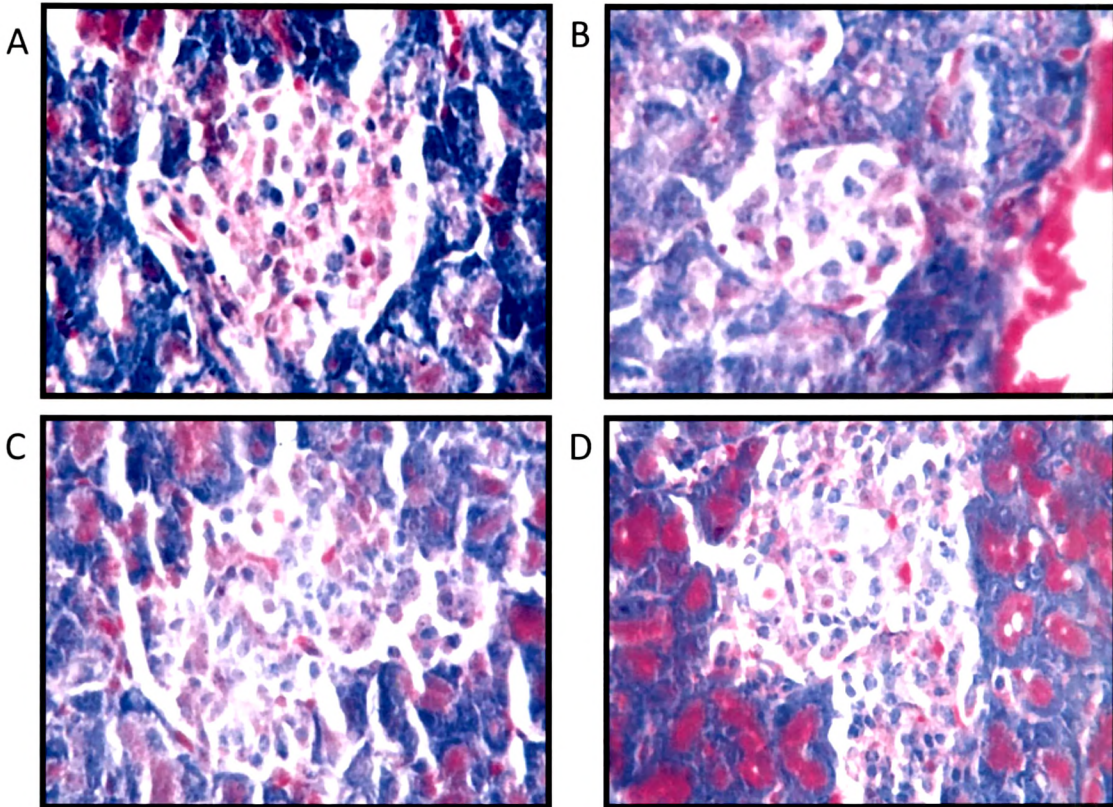
Figure 33 Images represent Gomori Phloxine stained sections of pancreas of A) Control animals showing blue stained nuclei representing beta cells and pink stained nuclei representing alpha cells and somatostatin cells. Magnification 200X, B) Diabetic animals showing reduced islet mass. Magnification 400X, C) Diabetic + OI 250 treated animals showing recovery against streptozotocin insult Magnification 200X and D) Diabetic + OI 500 treated animals demonstrate increased beta cell mass. Magnification 200X
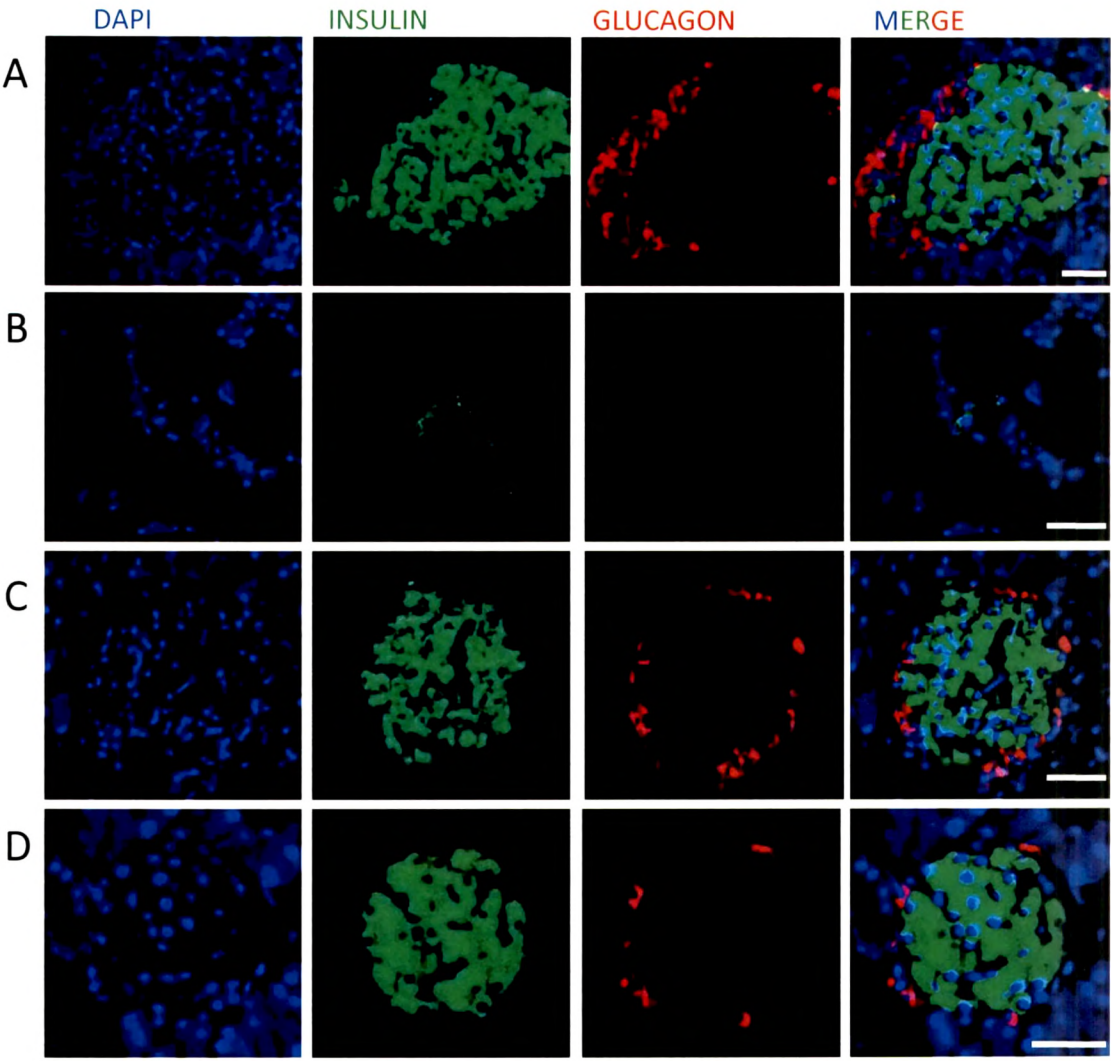
Figure 34 : Images represent immunostained section of pancreas of A) Control, B) Diabetic, C) Diabetic + OI 250 and D) Diabetic + OI 500. Guinea Pig anti insulin (green) and mouse glucagon (red) were used as primary antibodies while alexaflour 488 and alexaflour 546 as $F(ab')_2$ secondaries. Hoechst 33342 (Blue) was used to visualize nuclei. The slides were visualized by Laser Scanning Confocal Microscope (LSM 510 META, ZEISS, Germany) using Argon and Krypton Lasers. Optical slices were taken at ~ 0.8µm.Laser gains, pin hole setting and magnification were set identical across samples. Scale bar represents 100 µm.

Discussion:

Chronic hyperglycemia associated with diabetes is known to induce glycation of body proteins which can lead to secondary complications affecting eyes, kidneys, nerves and arteries (Sharma, 1993). Insulin-dependent diabetes mellitus or type 1 diabetes is an autoimmune disorder caused by destruction of insulin producing β-cells when auto aggressive T-lymphocytes infiltrate the islets and leads to hypoinsulinaemia and thus hyperglycemia

Streptozotocin (STZ) has been proposed to act as a diabetogenic agent due to its ability to destroy pancreatic beta cells by the generation of excessive free radicals (Szkudelski, 2001). Streptozotocin enters the pancreatic β-cell via the glucose transporter-GLUT2 and causes alkylation of DNA and impairment in glucose oxidation (Bolaffi et al., 1987) leading to decreased insulin biosynthesis and secretion (Nakatsuka et al., 1990).

Oreocnide integrifolia has been used in folklore medicine for the treatment of diabetes but no pharmacological studies have been carried out to validate the folklore claim. The findings of the present study indicate that an aqueous extract of *Oreocnide integrifolia* has significant hypoglycemic and hypolipidemic effect. The attributed anti-hyperglycemic effects of most of the plants is due to their ability to restore islet function by causing an increase in insulin output or by inhibiting the intestinal absorption of glucose or even by facilitation of metabolites in insulin dependent processes. Hence treatment with herbal drugs has an effect on protecting β-cells and smoothening out fluctuation in glucose levels (Jia et al., 2003; Elder, 2004). The hypoglycemic action of OI extract seen in the present study is still not clear but, the compound(s) present in the

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extract may potentiate glucose induced insulin release from pancreatic β-cells and decrease glucose levels. The observed increase in serum insulin level seen in extract treated rats clearly attest to increased hormonal output overcoming the suppressive effect of STZ. The histological and immunohistochemical revelations suggest a potent protective effect of OI extract on β-cells of STZ diabetic rats. The increased number of islet cells and even improved integrity of partially affected cells as seen in the routinely stained histological sections of pancreas as well as the increased insulin immunoreactivity in immunohistochemically stained sections are unambiguous documentary evidence of not only the protective effect of OI extract but, also its potential competence in promoting β-cell regeneration and/or proliferation. Many reports on other herbal preparations exhibited the insulinogenic and / or insulin secretomimetic potential as of OI extract observed herein (Agarwal *et al.*, 1996; Vats *et al.*, 2004; Dhanabal *et al.*, 2006; Grover *et al.*, 2006; Hannan *et al.*, 2006; Narendhirakannan *et al.*, 2006; Gayathri and Kannabiran, 2008). More robust appearance of islets with a greater mass of cells seen in the pancreas of OI extract treated diabetic rats seen herein, tends to provide evidence for the possible therapeutic potential of the extract to promote proliferation of surviving β-cells and/ or regeneration of islet β-cells. This inference finds validity not only from studies on in vitro evaluations (Chapter 5) and islet neogensis after pancreactomy (Chapter 4) but also from the reports of Chakravarthy *et al.*, (1980), Ahmed *et al.* (1991) and Homekam *et al.* (1997) of possible β-cell proliferation and /or regeneration by the use of *Pterocarpus marsupium* extract. Further, a recent study from our laboratory has shown recovery and robust appearance of islets in diabetic rats treated with a mixture of a polyherbal extract (Singh *et al.*, 2010). The OI extract induced increase in serum insulin

level is well reflected on the dose dependent decrease in diabetic hyperglycemia recorded herein. This could result in improved glucose tolerance and insulin action contributing to a reversal of diabetic glycemic dysregulation. The observed anti-hyperglycemic effect of OI extract may be taken to suggest a restoration of insulin mediated glucose disposal mechanisms disrupted by STZ induced diabetes. Pertinently, OI extract has been clearly shown to improve glucose tolerance and insulin response in diet induced Type II diabetic model (Chapter 3a).

The anti-hyperglycemic effect of OI extract can be related with decreased hepatic glucose output, enhanced glucose transport and utilization, uptake by peripheral tissues and increased glycogen synthesis as a package of glucose disposal mechanisms characteristic of non-diabetic state. The present study in fact reveals substantial recovery in hepatic and muscle glycogen stores on OI extract administration to diabetic rats. The recovery in tissue glycogen load is marked by decreased glycogenolysis as marked by the significant decrement in the phosphorylase activity and further, the recorded decrease in hepatic G-6-Pase activity attests to a moderated gluconeogenesis. Tissue glycogen load is essentially a reflection of insulin status as, this hormone promotes glycogenesis by activation of glycogen synthase and inhibition of glycogen phosphorylase. Decreased glycogen content noted in diabetic animals is relatable with the diminished activity of glycogen synthase concomitant to an increased activity of glycogen phosphorylase and these changes are the contributory factors towards diabetic hyperglycemia. OI extract has been found to be effective in reversing these diabetogenic changes in enzyme activity and restore hepatic and muscle glycogen contents. Glucose-6-phosphatase, a gluconeogenic enzyme, on increment in diabetic animals as seen herein and as noted by others

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(Horecker *et al.*, 1975; Minnassian and Mithieux, 1994; Raju *et al.*, 2001; Sharma *et al.*, 2008; Singh and Kakkar, 2009), contributes to decreased glycolytic flux. Insulin is a suppressor of gluconeogenic enzymes (Bacquer *et al.*, 1998) and in this context, the significant down regulation of glucose-6-phosphatase by OI extract in diabetic rats and the parallel increase in serum insulin level are self explanatory. It is quite likely that, increased protein catabolism would provide amino acids to hepatic gluneogenic pathway in diabetic animals (Rannels *et al.*, 1997) and, the OI extract must be exerting a sparing effect on proteolysis. Possibility of OI extract promoting insulin induced glucose uptake by muscles and adipose tissue by way of increased GLUT-4 expression and downstream signaling entities like IRS, PRPP kinase, PPAR gamma, PI 3 kinase etc can also be considered within the realm of possibilities as, insulin has been shown to upregulate their expressions (Okada *et al.*, 1994; Giavaldi *et al.*, 1995; Trakinidis *et al.*, 1995; Laville *et al.*, 1996; Desvergne and Wali, 1999), OI extract has also demonstrated such activation in Type II diabetic rat model (Chapter 3). Most of the plants with hypoglycemic properties have been found to contain metabolites such as glycosides, alkaloids, flavonoids etc (Loew and Kaszkin, 2002).

Glycosylated hemoglobin is known to increase in patients with diabetes mellitus (Koeing *et al.*, 1976) and, the increase has been found to be directly proportional to fasting blood glucose level (Jackson *et al.*, 1979). In diabetics, the excess of glucose present in blood reacts with hemoglobin to form glycosylated hemoglobin resulting in lowered total hemoglobin content (Sheela and Augusti, 1992). Higher doses of OI extract treated diabetic animals registered significant increase in HbA1c levels. Total hemoglobin decreased in the diabetic group, possibly due to the increased formation of

HbA1c whereas, the increase in hemoglobin levels in diabetic animals treated with OI extract may be due to decrease in blood glucose levels. An increase in glycosylated hemoglobin has been found to be directly proportional to fasting blood glucose levels (Llyod and Orchard, 1999). The significant reduction in glycosylated hemoglobin of extract-treated diabetic rats indicates its efficiency in glycemic control and the findings are consistent with the reported lowering of HbA1c levels in diabetic rats with many medicinal plants (Venkateshwaran and Pari, 2002).

The level of serum lipids is usually raised in diabetes, a change that serves as a prelude to coronary heart disease (Loci et al., 1994). Hyperlipidemia associated with diabetes mellitus results from accelerated de novo hepatic biosynthesis and release of VLDL-C without a corresponding increase in the rate of clearance from the blood by lipoprotein lipase, whose activity is dependent on high insulin: glucagon ratio (Harris and Crabb, 1982). OI extract treatment (500 mg/kg bw) lowered cholesterol, triglyceride and LDL-C levels, and increased HDL-C levels, which are known to play important role in cholesterol transport from peripheral tissues to the liver by a pathway termed 'reverse cholesterol transport'.

Insulin is a potent inhibitor of lipolysis and inhibits the activity of the hormone sensitive lipases in adipose tissue and suppresses the release of free fatty acids (Loci et al., 1994). STZ- induced diabetes shows increased plasma levels of cholesterol, triglyceride, free fatty acids and phospholipids, which are consistent with the findings of others (Murali et al., 2002), and are significantly lowered by treatment with OI extract. During diabetes, enhanced activity of hormone sensitive lipases results in increased release of free fatty acids into circulation by way of enhanced lipolysis (Agardh et al.,

1999). Higher free fatty acid concentration increases β-oxidation of fatty acids, generating more acetyl CoA and cholesterol moieties. On the other hand, glucagon, catecholamines and other hormones enhance lipolysis. The marked hyperlipemia that characterizes diabetic state may therefore be regarded as a consequence of the ininhibited actions of lipolytic hormones on the fat depots (Loci *et al.*, 1994). The underlying mechanism of lipid lowering role of OI extract may be by way of inhibited lipid absorption by saponins and phenolic substances present in the aqueous extract (Ansarullah *et al.*, 2010). Diabetes-induced hyperlipidemia is attributable to excess mobilization of fat from the adipose tissue due to the under utilization of glucose (KrishnaKumar *et al.*, 2000). The regression of diabetic state on OI extract administration increases the utilization of glucose, thereby depressing the mobilization of fat. Hypercholesterolemia could result in a relative molecular ordering of the residual phospholipids, resulting in a decrease in membrane fluidity. In normal condition, insulin increases the receptor-mediated removal of LDL-C and consequently, insulin deficiency during diabetes causes hypercholesterolemia. Hypercholesterolemia and hypertriglyceredemia have been reported to occur in diabetic rats (Bopanna *et al.*, 1997). Treatment with OI extract shows pronounced anti-hyperlipedimic effect in STZ diabetic rats. This could be due to an increase in HDL-C, indicating the participation of OI extract in hepatic mobilization of cholesterol from extra hepatic tissues and its catabolism. Further, reduction in cholesterol levels by treatment with OI extract is associated with a decrease in LDL fraction suggesting that, the cholesterol lowering property can result in rapid uptake of LDL-C through its hepatic receptors and its catabolism and elimination in the form of bile acids, as demonstrated by Khanna *et al.* (2002).

Postprandial elevation of triglyceride (TG)-rich lipoproteins (TRLs) is also a well-recognized feature of diabetic dyslipidemia and includes the accumulation of intestinally derived apolipoprotein B48 (apoB48)-containing lipoproteins (Lewis et al., 1991; Curtin et al., 1996; Mero et al., 1998). VLDL is normally converted to LDL via the action of post-heparin lipases. The result from the present study suggests that OI extract is able to restore at least partially, catabolism of β-lipoproteins, as also hypothesized by other workers with other plants (Campillo et al., 1994; Perez et al., 1999). The restoration of catabolic conversion of VLDL could be due to an increased stimulation of the lipolytic activity of plasma lipoprotein lipase.

The kidneys remove metabolic wastes such as urea, uric acid, creatinine and ions thereby contributing to maintenance of optimal chemical composition of body fluids. However, the concentrations of these metabolites increases in blood during renal diseases or renal damage associated with uncontrolled Diabetes mellitus (Almdal and Vilstrup, 1988). Increase in creatinine level is not only indicative of impairment of renal function (Hwang et al., 1997; Braulich et al., 1997) but also of the toxic effects of compounds on kidney in rats. In the present study, OI extract treated diabetic rats shows pronounced decrease in the plasma levels of creatinine and urea suggestive of the renoprotective effect of the plant extract. The renoprotective effect of OI extract is also very clearly indicated by the significantly lowered plasma levels of creatinine and urea present in higher amounts in DC rats. Apparently, OI extract has potent ability to afford protection against significant renal damage occurring under uncontrolled Diabetes mellitus (Almdal and Vilstrup, 1988; Hwang et al., 1997).

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Several phytomolecules including flavonoids, alkaloids, glycosides, saponins, glycolipids, dietary fibers, polysaccharides, peptidoglycans, carbohydrates, amino acids and others obtained from various plant sources have been projected as potent hypoglycemic agents (Mukherjee *et al.,* 2006) . Flavonoids are a heterogeneous group of ubiquitous plant polyphenols that exhibits a variety of pharmacological activities, including the anti-atherogenic effect, lipoprotein oxidation, blood platelet aggregation and vascular reactivity (Del Bas *et al.,* 2005; Peluso 2006). Triterpenoid and steroidal glycosides, referred to collectively as saponins, are bioactive compounds present naturally in many plants and known to possess potent hypoglycemic activity (Rao and Gurfinkel, 2000). Qualitative phytochemical analysis of *OI* extract from our lab revealed presence of flavanoids, phenolics, saponins, terpenoids, sugars and steroids. (Ansarullah *et al.,* 2010). Hence, the observed antidiabetogenic effects of OI extract principally at the level of carbohydrate metabolism as well as secondary on lipid metabolism could be accredited to the many active compounds present in the extract.

Conclusion: Overall, it can be concluded that, OI extract has potential anti-hyperglycemic and anti-hyperlipidemic effects along with competence to revert the metabolic derangements affecting carbohydrates and lipids and also bring about insulinogenic islet regeneration in STZ induced diabetic rats.