CHAPTER 3

ANTIDIABETIC ACTIVITY OF γ-SITOSTEROL USING STREPTOZOTOCIN INDUCED DIABETIC RATS

3.1. Introduction

Diabetes mellitus is a clinical syndrome characterized by inappropriate hyperglycemia caused by a relative or absolute deficiency of insulin or by a resistance to the action of insulin at the cellular level (Akhilesh et al., 2011).

Diabetes has been a clinical model for general medicine. The primary defect in fuel metabolism results in widespread, multi-organ complications that ultimately encompass virtually every system of the body and every specialty of medicine. It has been said that to know diabetes is to know medicine and health care. Although from a clinical standpoint this may be true, our increasing knowledge of the pathophysiology of the syndrome, together with the mechanisms of long term complications, has placed diabetes research at the frontier of immunology and molecular biology (Debra, 1991).

Diabetes mellitus has been known since ages and the sweetness of diabetic urine has been mentioned in Ayurveda by Sushruta. Its pharmacotherapy however is over 80 years old. The word diabetes was coined by the Greek physician Aeretaeus in the first century A.D. In the 17th century, Willis observed that the urine of diabetics as wonderfully sweet as if imbued with honey or sugar. The presence of sugar in the urine of diabetics was demonstrated by Dobson in 1755 (Satoskar, 1999).

Diabetes mellitus is now recognized as a serious global health problem (King, 1993). Westernized cultures and populations experiencing rapid acculturation are showing a sharp rise in non-insulin-dependent diabetes mellitus (Bennett and Knowler, 1980; Zimmer, 1998). Various
epidemiological studies in India have shown that the prevalence and manifestations of diabetes are very high (Verma et al., 1986; Ramchandran et al., 1992; Ramaiya et al., 1990).

Traditional herbal drugs play an important part in the treatment of diabetes. If we were able to even identify some 5-6 herbal drugs that can reduce dose of insulin by increasing sensitivity, reducing insulin resistance, then we would have positively contributed in the treatment of diabetes (Akhilesh et al., 2011). Herbal medicines are often used as therapeutic remedies in combination with allopathic drugs (Ramesh, 2003).

Herbal medicines can be relevant today only if they are applied and tested within the framework of modern sciences and subjected to the rigorous criteria for quality, safety and efficacy. Only then, herbal products can be comparable with modern medicines and can bring necessary confidence in prescribing doctors (Akhilesh et al., 2011). The Pharmaceutical Research and Development Committee report of Ministry of Chemicals, Government of India also underscores the importance of traditional knowledge (Masheikar, 1999).

In this chapter we investigate the antihyperglycemic, antihyperlipidemic and antioxidant properties of \( \gamma \)-sitosterol isolated from \( L.\ nodiflora \) with special reference to various biochemical parameters such as plasma glucose, plasma insulin, blood parameter, carbohydrate metabolism, lipid metabolism, enzyme markers, glycoprotein levels and antioxidant status in normal and STZ-induced diabetic rats. In addition, histopathological study of pancreas, liver and kidney, immunohistochemical study of pancreas and insulin secretion experiments were carried out to assess the antidiabetic property of the \( \gamma \)-sitosterol from \( L.\ nodiflora \).
3.2. MATERIALS AND METHODS

3.2.1 Experimental animals

As given in 1.3.5.

3.2.2. Chemicals and biochemical measurements

Either molecular biology grade or extra pure analytical- reagent grade chemicals were used in the study. Streptozotocins, Bradford reagent, EDTA, Tween 80, DMSO (Dimethylsulfoxide) were obtained from Sigma- Aldrich, Bangalore. All other chemicals were of analytical grade. All spectrophotometric measurements were carried out using UV2010 Spectrophotometer (Hitachi, Germany).

3.2.3 Antibodies

Primary monoclonal antibodies (Mouse anti-EMA, anti-insulin) were purchased from ICN Biomedical, Costa Mesa, CA, USA.

3.2.4 Experimental and induction of diabetes:

As given in 1.3.6.

3.3. DOSE DETERMINATION

Totally 42 animals were utilized and the animals were randomly divided into 7 groups of six animals each as given below. Different doses (5, 10, 20 mg/kg /b.wt) of γ-sitosterol were suspended in vehicle solution 10% DMSO and administered orally using an intragastric tube; reference drug glibenclamide (600 μg/kg /b.wt) was suspended in 10% DMSO as vehicle solution and administered and blood glucose level was determined at 0, 60, 120 and 180 min.

Group 1 Normal rats + Vehicle alone (10% DMSO)
Group 2 Normal rats + 20 mg/kg /b.wt of γ- sitosterol
Group 3 Diabetic control + Vehicle alone (10% DMSO)
Group 4 Diabetic rats + 5 mg/kg /b.wt of γ -sitosterol
Group 5 Diabetic rats + 10 mg/kg /b.wt of γ- sitosterol
Group 6 Diabetic rats + 20 mg/kg /b.wt of γ-sitosterol

Group 7 Diabetic rats + Glibenclamide (600 µg/kg /b.wt)

All the doses were given 7 days after injection of STZ. No irritation or restlessness was observed after each drug or vehicle administration. No noticeable adverse effect (i.e., respiratory distress, abnormal locomotion and catalepsy) was observed in any animal after the drug administration. Fasting plasma glucose levels were estimated every week to ascertain the status of diabetes in different groups of rats. Oral administration of 20 mg/kg /b.wt of γ-sitosterol significantly reduced the plasma glucose level in STZ-induced diabetic rats when compared to other doses (5, 10 mg/kg /b.wt). This dose was fixed as effective dose and was selected for further biochemical study.

3.3.1. Experimental protocol for further biochemical study

The rats were divided into five groups with six rats in each group. 10% DMSO was used as vehicle. Single dose of γ-sitosterol (20 mg/kg /b.wt) was suspended in vehicle (10% DMSO) and was administered orally every day for 21 days.

Group 1 served as normal control (10% DMSO alone)

Group 2 Normal rats + 20 mg/kg /b.wt of γ-sitosterol in 10% DMSO

Group 3 Diabetic control (10% DMSO alone)

Group 4 Diabetic rats + 20 mg/kg /b.wt of γ-sitosterol in 10% DMSO

Group 5 Diabetic rats + glibenclamide 600µg/kg /b.wt in 10% DMSO

After 21 days of treatment, 12 h fasted animals were anaesthetized between 7 am to 8 am, using ketamine (24 mg/kg /b.wt, intramuscular injection) and sacrificed by decapitation. Blood was collected in two different tubes (i.e.), one with whole blood for serum separation and the
other with anticoagulant EDTA for plasma insulin assay. Tissues (Liver, kidney) were surgically removed, washed with cold physiological saline, cleared off the adherent lipids and immediately transferred to ice-cold containers and weighed for the estimation of various biochemical parameters.

3.4. PARAMETERS ANALYSED TO EVALUATE ANTIDIABETIC POTENTIAL

1. Body weight: Change in body weight assessed

2. Blood Parameters

   Whole blood
   i)  Hemoglobin (Hb).
   ii) Glycosylated haemoglobin (HbA$_{1c}$).

3. Plasma

   i)  Fasting Plasma Glucose.
   ii) Fasting Plasma Insulin.
   iii) Insulin secretion experiment.

4. Serum

   i.  Carbohydrate metabolizing enzymes: Glucokinase, Glucose 6-phosphatase, Glucose 6-phosphate dehydrogenase, Fructose 1, 6-bisphosphatase, Glycogen phosphorylase and Glycogen synthase.
   ii. Nonenzymic antioxidants: Ascorbic acid, α-tocopherol, Reduced glutathione (GSH).
   iii. Lipid Peroxidative markers: Thiobarbituric acid reactive substances (TBARS) and Lipid hydroperoxides (HP).
iv. Lipid Profile: Total Cholesterol (TC), Free Fatty Acids (FFA), Triglycerides (TG), Phospholipids (PL), High density lipoprotein (HDL), Low density lipoprotein (LDL), Very low density lipoprotein (VLDL).

v. Kidney function markers: Urea, uric acid and creatinine.

vi. Glycoprotein component analysis: Hexoses, Hexosamine, Sialic acid and Fucose.

vii. Protein profile: Total protein, Albumin, Globulin and A/G ratio

viii. Hepatic marker enzymes: Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Acid phosphatase (ACP), Alkaline phosphatase (ALP).

6. Tissue Parameters

   **Liver and Kidney**

   i) Carbohydrate metabolizing enzymes (As mentioned above)

   ii) Glycoprotein component analysis (As mentioned above)

   **Liver and Kidney**

   i) Lipid peroxidation markers (As mentioned above)

   ii) Enzymatic antioxidant (Superoxide (SOD), Catalase (CAT), Glutathione peroxidase (GPx) and Non-enzymatic antioxidant markers.

   **Liver and Kidney:**

   Lipid profiles (As mentioned above)

7. Histological and immunohistochemical study

   **Histology:** Pancreas, liver, kidney

   **Immunohistochemistry:** Pancreas

3.4.1. PROCESSING OF BLOOD AND TISSUE SAMPLES

3.4.1.1. Preparation of Serum and Plasma

   Fasting blood samples were collected from the various groups of experimental animals periodically and on the day of sacrifice in tubes with and without EDTA. Plasma and serum were
separated by centrifugation at 3000 rpm and were analyzed for various haematological and biochemical parameters.

3.4.1.2. Tissue homogenate preparation

Liver and kidney tissues (250 mg) were sliced into pieces and homogenized in appropriate buffer in cold condition (pH 7.0) to give 20 % homogenate (w/v). The homogenate was centrifuged at 1000 rpm for 10 min at 0°C in cold centrifuge. The supernatant was separated and used for various biochemical estimations.

3.4.1.3. Tissue sampling for Histopathological study

Three rats from each group were perfused with cold physiological saline, followed by formalin (10 % formaldehyde). The liver and pancreas were excised immediately and fixed with 10% formalin.

3.4.1.4. Immunohistochemical analysis

For immunohistochemical examination, pancreas was removed, washed in saline, kept in 10 % formalin and fixed in paraffin. This was washed in running water overnight and sectioned (5 μm in thickness). These were used for immunohistochemical analysis. The sections of control and treated rats were dewaxed, then incubated for 1hr at room temperature in 0.3% hydrogen peroxide in phosphate buffer solution (PBS), followed by 3 washings in PBS. Sections were then incubated for 16 hr, at 4 °C, in PBS containing 2% normal goat serum (NGS) and 0.5 % triton X-100 and washed in PBS at room temperature. Sections were incubated overnight at 4°C with the primary monoclonal antibody (Mouse anti-EMA, anti-insulin). Incubation was followed by 3 washes in PBS-2 % NGS. The primary antibodies were bounded by a biotinylated anti-mouse secondary antibody in PBS for 1hr at room temperature. Sections were then incubated in avidin-biotin complex linked to peroxidase. The peroxidase was visualized with 0.03%
diaminobenzidine hydrochloride and 0.005 % hydrogen peroxide in 0.1 M tris buffer. Sections were counter stained with hematoxylin.

3.5. BIOCHEMICAL DETERMINATION

3.5.1. Estimation of blood glucose

As given in 1.4.3

3.5.2. Estimation of plasma insulin

As given in 1.4.4

3.5.3. Estimation of haemoglobin

Haemoglobin in the blood was estimated by the method of Drabkin and Austin (1932).

Reagents

1. Drabkin’s reagent: 200 mg of Potassium ferric cyanide, 50 mg of Potassium cyanide and 1.0 g Sodium bi carbonate (Na₂CO₃) were dissolved in water and made up to one liter. The reagent had a pale yellow colour of pH 9.6 and was stored in brown bottle.

2. Cyanomethaemoglobin standard solution: 16 g/dL.

Procedure

To 0.02 ml of blood, 5.0 ml of Drabkin’s reagent was added, mixed well and allowed to stand for 10 min. The solution was read at 540 nm together with the standard solution against a reagent blank. Values were expressed as mg/dl for blood.

3.5.4. Estimation of glycosylated haemoglobin (HbA₁c)

Glycosylated haemoglobin in the blood was estimated by the method of Sudhakar Nayak and Pattabiraman, (1981).

Reagents

i) 1 M potassium oxalate in 2 M Hydrochloric acid (Oxalate-hydrochloride acid).
ii) 80% Phenol

iii) Conc. Sulphuric Acid (H₂SO₄)

iv) 40% Tri-chloro Acetic acid (TCA)

v) Saline

vi) Stock Solution: Stock standard was prepared by dissolving 100 mg of fructose in 100 ml of water.

vii) Working standard: Stock standard was diluted to get a concentration of 100 µg/ml.

**Procedure**

5 ml of blood was collected with EDTA and plasma was separated. The contents were centrifuged and the supernatant was discarded; the 0.5 ml of saline was added, mixed and processed for estimation. To 2 ml of aliquot, 4 ml of Oxalate hydrochloride solution was added and mixed. The contents were heated at 100°C for 4 h, cooled and precipitated with 2 ml of 40% TCA. The mixture was centrifuged and to 0.5 ml of supernatant, 0.5 ml of 80% Phenol and 3.0 ml of conc. H₂SO₄ were added. Working standard in the range of 10-50 µg was taken and made up to 1 ml, 0.05 ml of 80 % phenol and 3 ml of conc.H₂SO₄ were added to this. The colour developed was read at 480 nm after 30 min. The concentration of glycosylated haemoglobin was expressed as mg/g of hemoglobin.

**3.5.5. Estimation of glycogen**

Glycogen was extracted and estimated by the method of Morales *et al.*, (1975).

**Reagents:**

i) 30% Potassium hydroxide

ii) Absolute alcohol

iii) Anthrone reagent: 0.2 % of Anthrone in concentrated Sulphuric acid was prepared just before use.

iv) 1 M Ammonium acetate
v) Stock standard: 1 mg/ml – 100 mg of anhydrous D-glucose was dissolved in 100 ml of distilled water containing 0.01 % Benzoic acid.

vi) Working standard: 100 μg/ml – 10 ml of stock standard was diluted to 100 ml with distilled water.

**Procedure**

The alkali extract of the tissue was prepared by digesting 50 mg of fresh tissue with 3 ml of 30% Potassium hydroxide solution in boiling water bath for 15 min. The tubes were cooled and a drop of ammonium acetate was added to precipitate glycogen and left in a freezer overnight for complete precipitation. Glycogen was collected by centrifuging at 300 rpm for 20 min; the precipitate was dissolved with the help of heating and again the glycogen was precipitated with alcohol and 1 M ammonium acetate and centrifuged. The final precipitate was dissolved in saturated ammonium chloride solution by heating in a boiling water bath for 5 min. Aliquots of glycogen solution were taken up for suitable dilution and 4 ml of anthrone reagent was added by cooling the tubes in an ice bath. The tubes were shaken well, covered with marble caps and heated in a boiling water bath for 20 min. After cooling, the absorbance was read at 640 nm against water blank treated in a similar manner. Standard glucose solution was also treated similarly.

The glycogen content was calculated from the amount of glucose present in the sample, by multiplying with the factor of 0.91 and expressed as mg/100 mg of tissue.

**3.5.6. Estimation of tissue protein**

Protein in the tissues was determined after Trichloro acetic acid (TCA) precipitation by the method of Lowry et al., (1951).
Reagents

i. Alkaline copper reagent:

Reagent A: 2% Sodium carbonate in 0.1 N NaOH

Reagent B: 0.5 % Copper Sulphate in 1% Sodium potassium tartarate.

Reagent C: 50 ml of reagent A was mixed with 0.5 ml of reagent B just before use.

Folin’s Phenol reagent: Dilute 1:2 with distilled water.

Stock standard: 100 mg of Bovine Serum albumin/100 ml water.

Working Standard: 10 ml of the stock standard was diluted to 100 ml to get a working standard containing 0.1 mg/dl.

Procedure

0.5 ml of tissue homogenate was mixed with 0.5 ml of 10 % TCA and centrifuged for 10 min. The precipitate was dissolved in 1.0 ml of 0.1 N NaOH. From this, an aliquot was taken and 4.5 ml of alkaline copper reagent was added and allowed to stand at room temperature for 10 min. 0.5 ml of Folin’s phenol reagent was added and the blue color developed was read after 20 min at 640 nm. A standard curve was obtained with standard Bovine albumin and was used to assay the tissue protein level for enzyme activity. Values were expressed as mg/g of tissues.

3.5.7 CARBOHYDRATE METABOLIC ENZYMES

3.5.7.1. Glucokinase Assay (Hexokinase)

Hexokinase D was assayed by the method of Brandstrup et al., (1957).

Reagents

1. 0.05 M Glucose solution

2. 0.72 M ATP solution

3. 0.05 M Magnesium chloride (MgCl₂)
4. 0.0125 M Dipotassium hydrogen phosphate solution
5. 0.1 M Potassium chloride solution
6. 0.5 M Sodium fluoride solution
7. 0.01 M Tris-HCL buffer, pH 8.0

**Procedure**

The reaction mixture in a total volume of 5.0 ml contained the following: 1.0 ml of glucose solution, 0.5 ml of Magnesium chloride, 0.5 ml of Dipotassium hydrogen phosphate solution, 0.4 ml of Potassium chloride, 0.1 ml of Sodium fluoride solution and 2.5 ml of Tris-HCL buffer (pH 8.0). The mixture was preincubated at 37°C for 15 min. The reaction was initiated by the addition of 1.0 ml of tissue homogenate. 1.0 ml aliquot of the reaction mixture was taken immediately (Zero time) to tubes containing 1.0 ml of 10 % TCA. A second aliquot was removed after 30 min of incubation at 37°C and added to tubes containing 1.0 ml of 10 % TCA. The precipitated protein was removed by centrifugation and the residual glucose in the supernatant was estimated by the o-toluidine method of Sasaki and Matsui (1972) as described previously. A reagent blank was run with each test.

The difference between the two values gave the amount of glucose phosphorylated. The enzyme activity was expressed as µmol of inorganic phosphorous liberated/min/mg/protein.

**3.6.7.2. Glucose 6-Phosphatase Assay**

Glucose 6-phosphatase was assayed by the method of Koide and Oda, (1959).

**Reagents**

1. 0.1 M Maleic acid buffer, pH 6.5
2. 0.01 M Glucose 6-phosphate in distilled water
3. Ammonium molybdate solution; 2.5 g Ammonium molybdate dissolved in 100 ml of 3 N Sulphuric acid.

4. Aminonaphthol Sulphonic acid (ANSA) reagent: 500 g of ANSA was dissolved in 195 ml of 15 % Sodium bisulphite and 5 ml of 20 % Sodium sulphite was added to it. The solution was filtered and stored in a brown bottle.

5. 10% TCA

Procedure

The incubation mixture contained 0.3 ml buffer, 0.5 ml Glucose 6-phosphate and 0.2 ml tissue homogenate. This was incubated at 37°C for 1 h. 1 ml 10 % TCA was added to the tubes to terminate the enzyme activity, then centrifuged and the phosphate content of the supernatant was estimated by the method of Fiske and Subbarow (1925). To 1 ml of the aliquot of supernatant, 1 ml of ammonium molybdate and 0.4 ml ANSA were added.

The blue color developed was read after 20 min at 620 nm. A tube devoid of the enzyme served as control. A series of standards containing 8-40 µg of phosphorus was treated similarly along with a blank containing only the reagent. The enzyme activity was expressed as µmol of inorganic phosphorus liberated/min/mg of protein.

3.5.7.3. Fructose 1, 6-bisphosphatase

Fructose 1, 6-bisphosphatase was assayed by the method of Gancedo and Gancedo (1971).

Reagents

1. M Tris HCl buffer, pH 7.0
2. Substrate: 0.05 M Fructose 1,6-bisphosphatase
3. M Magnesium chloride (MgCl₂)
4. M Potassium chloride
5. M EDTA solution
6. 10% TCA
7. Molybdic acid: 2.5% Ammonium molybdate in 3 N sulphuric acid
8. ANSA reagent: As above
9. Phosphorus stock Standard: 35.1 mg of potassium dihydrogen phosphate was dissolved in 100 ml of distilled water (80 µg/ml).

**Procedure**

The assay medium in a final volume of 2.0 ml contained 1.0 ml buffer, 0.4 ml of substrate, 0.1 ml each of Magnesium chloride, 0.2 ml Potassium chloride, 0.1 ml of EDTA and 0.2 ml of enzyme source. The incubation was carried out at 37°C for 15 min. The reaction was terminated by the addition of 1.0 ml of 10% TCA. The suspension was centrifuged and the phosphorus content of the supernatant was estimated according to the method described by Fiske and Subbarow (1925). To 1 ml of an aliquot of the supernatant, 0.3 ml of distilled water and 0.5 ml of Ammonium molybdate were added. After 10 min, 0.2 ml of ANSA was added. The tubes were shaken well, kept aside for 20 min and the blue color developed was read at 620 nm. The values were expressed as µmol of inorganic phosphorous liberated/min/mg/protein.

**3.5.7.4. Hepatic Glycogen Synthase and Glycogen Phosphorylase Assay**

Hepatic Glycogen Synthase and Glycogen Phosphorylase were assayed by the method of Leloir and Goldermberg, (1962) and Cornblath *et al.*, (1963).

**Reagents:**

1. 0.05 M Tris HCl buffer, pH 7.5
2. 0.1M NADP⁺
3. 0.1 M Magnesium chloride (MgCl₂)
4. 0.1 M Potassium chloride
5. 0.001 M EDTA solution
6. 10 % TCA
7. Molybdic acid: 2.5 % Ammonium molybdate in 3 N sulphuric acid
8. Phosphorus stock Standard: 35.1 mg of potassium dihydrogen phosphate was dissolved in 100 ml of distilled water (80 µg/ml).

**Procedure**

The activity of glycogen synthase was estimated by coupling it with pyruvate kinase activity. It was measured by the amount of uridine diphosphate (UDP) formed from UDP-glucose in the presence of glycogen and glucose-6-phosphate. Pyruvate kinase catalyses the transfer of phosphate from phosphoenolpyruvate to UDP and the pyruvate liberated was estimated colorimetrically (Leloir and Goldemberg, 1962). The property of synthesizing glycogen from glucose-1-phosphate liberating inorganic phosphate is made use in the assay of glycogen phosphorylase activity (Cornblath *et al.*, 1963). The activity of glycogen synthase enzyme was expressed as µmoles of inorganic phosphate liberated/min/mg protein.

3.5.7.5. Glucose 6-phosphate Dehydrogenase Assay

Glucose 6-phosphate Dehydrogenase in the liver and kidney was assayed by the method of Ellis and Kirkman (1984).

**Reagents**

1. 0.05 M Tris HCl buffer, pH 7.5
2. 0.1 M Magnesium chloride (MgCl₂)
3. 0.1 M NADP⁺
4. 0.01% 2, 6-Dichlorophenol Indophenol (freshly prepared in Distilled Water)

5. 0.005% Phenacine Methosulphate (freshly prepared in Distilled Water)

6. 0.02 M Glucose 6-Phosphate solution (Substrate)

**Procedure**

The incubation in a total volume of 5.5 ml contained 1.0 ml of tris buffer, 0.1 ml of Magnesium chloride, 0.1 ml of NADP⁺, 0.5 ml of Phenazine methosulphae, 0.4 ml of the dye solution and the requisite amount of the enzyme extract. The mixture was allowed to stand at room temperature for 10 min to permit the oxidation of endogenous materials. The reaction was initiated by the addition of 0.5 ml of Glucose 6-phosphate. The absorbance was read at 640 nm against water blank at one minute intervals for 3.5 min in a UV spectrophotometer. The activity of the enzyme was calculated in units by multiplying the change in OD/Min by the factor 6/17.6, which is the molar extinction co-efficient of the reduced enzyme activity.

**3.5.8. LIPID PEROXIDATION PARAMETERS**

**3.5.8.1. Estimation of TBARS**

The concentration of TBARS in the plasma and tissues was estimated by the method of Nichans and Samuelson (1968). In this method, Malondialdehyde and other Thiobarbituric acid reactive substances (TBARS) react with Thiobarbituric acid in an acidic condition to generate a pink color chromophore which is read at 535 nm.

**Reagents:**

1. 15% TCA
2. 0.25N HCl
3. 0.375% TBA in hot distilled water
4. TBA-TCA-HCl reagent: Solution 1 and 3 were mixed in the ratio of 1:1:1 freshly prepared prior to use.

5. Stock standard: 4.8 molar solution of stock was prepared from 1, 1’3, 3’ Tetramethoxypropane purchased commercially.

6. Working standard: Stock solution was diluted to get a concentration of 48 nmol/mL.

Procedure

0.5 ml of plasma was diluted to 0.5 ml with double distilled water and mixed well, and then 2.0 ml of TBA-TCA-HCl reagent was added. The mixture was kept in a boiling water bath for 15 min; after cooling, the tubes were centrifuged at 1000 g for 10 min and the supernatant was estimated. A series of standard solution in the concentration of 2-10 nmol was treated in a similar manner. The absorbance of the chromophore was read at 535 nm against reagent blank. The values were expressed as mmol/ml of serum or nmol/100 g of tissues.

3.5.8.2. Estimation of lipid hydroperoxides

Lipid hydroperoxide in the plasma and tissues was estimated by the method of Jiang et al., (1992). Oxidation of ferrous ion (Fe2⁺) under acidic conditions in the presence of Xylenol orange leads to the formation of a chromophore with an absorbance maximum at 560 nm.

Reagents:

Fox reagent: 88 mg Butylated hydroxytoluene (BHT), 7.6 mg Xylenol orange and 9.8 mg Ammonium iron (II) sulphate were added to 90 ml methanol and 10 ml H₂SO₄ (250 mM) mixture.
Procedure

0.9 ml of Fox reagent was mixed with 0.1 ml of the sample, incubated for 30 min at room temperature and the absorbance read at 560 nm. Lipid hydroperoxides were expressed as nmol/ml of serum or nmol/100 g of tissues.

3.5.9. Non Enzymatic antioxidants

3.5.9.1. Estimation of reduced glutathione (GSH)

Reduced glutathione in the plasma and tissues was estimated by the method of Ellman (1959). This method was based on the development of yellow color when 5, 5’-dithio-bis (2-nitrobenzoic acid) (DTNB) was added to compounds containing sulphydryl groups.

Reagents

1. 0.1 M Phosphate buffer, pH 8.0
2. 5% TCA
3. Ellman’s reagent: 34 mg of DTNB in 10 ml of 0.1 % sodium citrate
4. 0.3 M Disodium hydrogen phosphate
5. Standard glutathione solution: 100 mg GSH in 100 ml water
6. Working standard: Stock was diluted to get a concentration of 100 µg/ml.

Procedure

250 mg of tissue was homogenized in phosphate buffer (0.1 M pH 7.0). 0.5 ml of homogenate or serum was pipetted out and precipitated with 2.0 ml of 5 % TCA. 2.0 ml of supernatant was taken after centrifugation and 1.0 ml of Ellman’s reagent and 4.0 ml of 0.3 M Disodium hydrogen phosphate were added. The yellow color developed was read at 412 nm. A series of standards (20-100 µg) was treated in a similar manner along with a blank containing 1.0
ml of buffer. The amount of glutathione was expressed as mg/dL of serum or nM of DTNB conjugated /mg protein.

3.5.9.2. Estimation of Ascorbic acid (Vitamin C)

Ascorbic acid in the plasma and tissues was estimated by the method of Roe and Kuether (1943). The ascorbic acid was converted to Dehydroascorbic acid by mixing with norit and then was coupled with 2, 4 Dinitrophenylhydrazine (DNPH) in the presence of thiorea, a mild reducing agent. The coupled Dinitrophenylhydrazine was converted into a red colored compound when treated with Sulphuric acid and read at 540 nm.

Reagents

1. TCA: 6 %
2. 2, 4 DNPH reagent: 2.0 g of DNPH was dissolved in 100 mL of 9 N Sulphuric acids. To this, 4.0 g of Thiourea was added and mixed.
3. Acid washed norit.
4. Sulphuric acid: 85 %
5. Stock ascorbic acid solution: 1 in 10 dilution of stock ascorbic acid solution with 4 % TCA to obtain a concentration of 0.1 mg/mL.
6. Working ascorbic acid solution: 1 in 10 dilution of stock ascorbic acid solution with 4 % TCA to obtain a concentration of 0.1 mg/mL.

Procedure

To 0.5 mL of sample, 1.5 mL of 6 % TCA was added and allowed to stand for 5 min and centrifuged. The supernatant was removed and 0.3 g of acid washed norit was added, shaken vigorously and filtered. This converted ascorbic acid to Dehydroascorbic acid. 2 mL of the
filtrate was taken and 0.5 mL of DNPH was added, stopped and placed in a water bath at 37 °C for exactly 3 h. It was removed, placed in ice-cold water and 2.5 mL of 85 % Sulphuric acid was added drop by drop. The contents of the tubes were mixed well and allowed to stand at room temperature for 30 min. A set of standards containing 20-100 μg of Ascorbic acid was taken and processed similarly along with a blank containing 2.0 mL of 4 % TCA. The color developed was read in a Spectronic 20 at 540 nm. The values were expressed as mg/dL of serum or mg/100 mg tissue.

3.5.9.3. Estimation of α -tocopherol (Vitamin E)

α- Tocopherol in the plasma and tissues was estimated by the method of Baker et al., (1980). The method involves the reduction of ferric ions to ferrous ions by α-tocopherol and the formation of a red colored complex with 2, 2’ Dipyridyl. Absorbance of the chromophore was measured at 520 nm.

Reagents

1. Petroleum ether : 60 - 80 °C
2. Double distilled ethanol.
3. 2, 2’ Dipyridyl solution: 0.2 % in double distilled ethanol.
4. Ferric chloride solution: 0.5 % in double distilled ethanol.
5. Stock standard: 10 mg of α-tocopherol is 100 mL of distilled ethanol.
6. Working standard: Stock solution was diluted with ethanol to a concentration of 10 μg/mL.

Procedure

To 0.5 mL of sample, 1.5 mL of ethanol was added, mixed and centrifuged. The supernatant was evaporated and to the precipitate, 3.0 mL of Petroleum ether, 0.2 mL of 2, 2’
Dipyridyl solution and 0.2 mL of Ferric chloride solution were added. This was mixed well and kept in dark for 5 min. An intense red color was developed. 4.0 mL of N-butanol was added to all the tubes and mixed well. Standard tocopherol in the range of 10-100 μg was taken and treated similarly along with a blank containing only the reagent. The color in the N-butanol layer was read in a Spectronic 20 at 520 nm. The values were expressed as mg/dL for serum or mg/100mg tissue.

3.5.10. Enzymatic antioxidants

3.5.10.1. Assay of superoxide dismutase

Superoxide dismutase in the tissues was assayed by the method of Kakkar et al., (1984). The assay is based on the inhibition of the formation of NADH Phenazine methosulphate, Nitroblue tetrazolium formazon. The reaction was initiated by the addition of NADH. After incubation for 90 sec, adding glacial acetic acid stopped the reaction. The color developed at the end of the reaction was extracted into n-butanol layer and measured in a Spectronic 20 at 520 nm.

Reagents

1. Sodium pyrophosphate buffer: 0.025 M, pH.8.3.
2. Absolute ethanol.
3. Chloroform.
4. N-butanol
5. Phenozone methosulphte (PMS): 186 μmol.
7. NADH: 780 μmol.
Procedure

Tissue was homogenized by using Sodium pyrophosphate buffer (0.025 M, pH 8.3). 0.5 mL of tissue homogenate or 0.5 mL of serum was diluted to 1.0 mL with water followed by addition of 2.5 mL of ethanol and 1.5 mL of chloroform (chilled reagents were added). This mixture was shaken for 90 min at 4 °C and then centrifuged.

The enzyme activity in the supernatant was determined as follows. The assay mixture contained 1.2 mL of Sodium pyrophosphate buffer, 0.1 mL of Phenozine methosulphate, and 0.3 mL of Nitroblue tetrazolium and appropriately diluted enzyme preparation in a total volume of 3 mL. The reaction was started by the addition of 0.2 mL NADH. After incubation of 30 °C for 90 sec, the reaction was stopped by the addition of 1 mL Glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 mL N-butanol. The mixture was allowed to stand for 10 min, then centrifuged and N-butanol layer was separated.

The color density of the chromogen in N-butanol was measured in a Spectronic 20 at 520 nm. The enzyme concentration required to inhibit the chromogen produced by 50% in one minute under standard conditions was taken as one unit. The specific activity of the enzyme was expressed as Unit/mg of protein for tissues.

3.5.10.2. Estimation of catalase

The activity of catalase in the tissues was determined by the method of Sinha (1972). Dichromate in acetic acid is converted to perchromic acid and then to chromic acetate, when heated in the presence of H₂O₂. The chromic acetate formed is measured at 620 nm. The catalase preparation was allowed to split, H₂O₂ for various periods of time. The reaction was stopped at different time intervals by the addition of dichromate - acetic acid mixture and the remaining H₂O₂ as chromic acetate was determined colorimetrically.
Reagents

1. Phosphate buffer: 0.01 M, pH 7.0
2. Hydrogen peroxide: 0.2 M
3. Potassium dichromate: 5 %
4. Dichromate-acetic acid reagent: 1:3 ratio of Potassium dichromate was mixed with glacial acetic acid. From this 1 mL was diluted again with mL of acetic acid.
5. Standard hydrogen peroxide: 0.2 mM

Procedure

Tissue homogenate was prepared by using phosphate buffer (0.01 M, pH 7.0). To 0.9 mL of phosphate buffer, 0.1 mL of tissue homogenate or 0.1 mL of serum and 0.4 mL of Hydrogen peroxide were added. The reaction was arrested after 30 sec interval by adding 2.0 mL of Dichromate-acetic acid mixture. The tubes were kept in a boiling water bath for 10 min, cooled and the color developed was read at 620 nm. Standards in the concentration range of 20-100 μmol were taken and preceded as for the test. The specific activity was expressed as mol of H₂O₂ consumed min⁻¹(mg protein)⁻¹.

3.5.10.3. Estimation of glutathione peroxidase

The activity of GPx in the tissues was measured by the method of Rotruck et al., (1973). A known amount of enzyme preparation was allowed to react with H₂O₂ in the presence of GSH for a specified time period. Then the remaining GSH content was measured.

Reagents

1. Tris buffer: 0.4 M, pH 7.0
2. Sodium azide solution: 10 mM
3. TCA: 10 %
4. EDTA: 0.4 mM
5. H₂O₂ solution: 0.2 mM
6. Glutathione solution: 2 mM

**Procedure**

The tissue was homogenized using tris buffer. To 0.2 mL of Tris buffer, 0.2 mL of EDTA, 0.1 mL of Sodium azide, 0.5 mL of tissue homogenate were added. To the mixture, 0.2 mL of GSH followed by 0.1 mL of H₂O₂ was added. The contents were mixed well and incubated at 37 °C for 10 min, along with a control containing all reagents except homogenate. After 10 min, the reaction was arrested by the addition of 0.5 mL of 10 % TCA. The tubes were centrifuged and the supernatant was assayed for GSH by the method of Ellman (1959). The activity was expressed as mol of CDNB-GSH conjugate formed min⁻¹(mg protein)⁻¹.

**3.5.11. ANALYSIS OF LIPID PROFILE**

**3.5.11.1. Extraction of lipids**

Serum and tissue lipids were extracted by the method of Folch *et al.*, (1957). The tissues were rinsed in cold physiological saline thoroughly and dried by pressing between the folds of filter paper. A known weight of tissues was homogenized with 2.5 mL of ethanol-either mixture (3:1 v/v) and contents were digested for about 2 h at 60-65 °C. The supernatant was collected; 3 mL of ethanol-ether mixture was added to the residue; it was digested further for a period of 2 h at 60-65 °C and then the supernatant was collected; then 1 mL of chloroform-methanol mixture (1:1 v/v) was added to the residue. It was again digested for 2 h at 60-65 °C and the supernatant was collected. The supernatant was pooled and made up to a specified volume. Aliquot of this
extract was then used for the estimation of cholesterol, free fatty acids, triacylglycerol and phospholipids. Serum was also processed similarly.

3.5.11.2. Estimation of total cholesterol

Total cholesterol in the plasma and tissues was estimated by the enzymic method described by Allain et al., (1974). Cholesterol esters were hydrolyzed by cholesterol esterase to free cholesterol and free fatty acids. The free cholesterol produced and pre-existing ones were oxidized by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. Hydrogen peroxide formed reacted with 4-aminoantipyrine and phenol in the presence of peroxidase to produce red colored quinoneimine dye. The intensity of color produced was proportional to the cholesterol concentration.

Reagents

1. Enzyme reagent: 4-aminoantipyrine, cholesterol esterase, phenol, cholesterol oxidase and horseradish peroxidase.

2. Cholesterol standard: 200 mg%

Procedure

To 10 µL of serum or 10 µL of lipid extract, 1.0 mL of enzyme reagent was added, mixed well and kept at 37 °C for min. 10 µL of cholesterol standard and distilled water (blank) were also processed similarly. The absorbance was measured at 510nm. Cholesterol concentration was expressed as mg/dL of serum or mg/100 g of tissue.
3.5.11.3. Estimation of HDL - cholesterol

HDL-cholesterol was estimated using the diagnostic kit based on the enzymic method described by Izzo et al., (1981). The VLDL and LDL fractions of plasma samples were precipitated using Phosphotungstic acid and then HDL in the supernatant was separated by centrifugation and measured for its cholesterol content.

**Reagents**

1. Precipitating reagent
2. Enzyme reagent
3. HDL-cholesterol standard: 50 mg %

**Procedure**

0.1 mL of plasma was mixed with 0.1 mL of precipitating reagent, allowed to stand at room temperature for 5 min and centrifuged at 2000-3000 rpm for 10 min in the clear supernatant; cholesterol was estimated as described earlier. The values were expressed as mg/dL of serum.

3.5.11.4. Estimation of VLDL- and LDL-cholesterol

These were calculated using the formula (Friedwald et al., 1972)

\[
\text{VLDL cholesterol} = \frac{\text{TG}}{5}
\]

\[
\text{LDL cholesterol} = \text{Total cholesterol} - (\text{HDL cholesterol} + \text{VLDL cholesterol})
\]

The values were expressed as mg/dL of serum.
3.6.11.5. Estimation of free fatty acids

Free fatty acids in the plasma and tissues were estimated by the method of Falholt et al., (1973).

Free fatty acids were extracted with chloroform-heptane-methanol mixture to eliminate interference from phospholipids and the extract was shaken with a high density copper reagent at pH 8.1. The copper soaps remained in the upper organic layer from which an aliquot was removed and copper content determined colorimetrically by treating with Diphenyl carbazide.

Reagents

1. Chloroform-heptane-methanol solvent (5:5:1)
2. Stock copper solution: 500 mM
3. Triethanolamine solution: 1 M
4. Sodium hydroxide solution:1M
5. Copper reagent (Cu-TEA solution): 10.0 mL of stock copper solution was mixed with 10.0 mL triethanolamine and 6.0 mL sodium hydroxide. To this 33 g of sodium chloride was added, made up to 100 mL and the pH was adjusted to 8.1.
6. Diphenylcarbazide solution: 0.03 M in ethanol
7. Standard palmitic acid: 2.0 mM in chloroform-heptane-methanol solvent (5:5:1).

Procedure

0.5 mL of lipid extract was evaporated to dryness and dissolved in 6.0 mL chloroform-heptane-methanol solvent and 2.5 mL of copper reagent were added. All the tubes were shaken vigorously for 90 sec and were kept aside for 15 min. The tubes were centrifuged and 3.0 mL of the copper layer was transferred to another tube containing 0.5 mL of Diphenyl carbazide and
mixed carefully. The color developed was read at 540 nm against a reagent blank containing 3.0 mL solvent and 0.5 mL Diphenyl carbazide. The free fatty acid content was expressed as mg/dL of serum or mg/100 g of tissues.

3.5.11.6. Estimation of triacylglycerol

Triacylglycerol in the plasma and tissues was estimated using the diagnostic kit based on the enzymic method described by McGowan et al., (1983).

Triacylglycerol in the sample was hydrolysed by microbial lipase to glycerol and free fatty acid. Glycerol was converted by glycerol kinase into glycerol 3-phosphate (G-3-p) which was oxidized by glycerol phosphate oxidase to Dihydroxyacetone phosphate and Hydrogen peroxide. In this reaction Hydrogen peroxide was produced in equimolar concentration to the level of triacylglycerol present in the sample. H_2O_2 reacts with 4-aminoantipyrine and 3, 5-dichloro-2-hydroxybenzene sulfonic acid in the presence of peroxidase to produce red quinoneimine colored dye. The intensity of this dye was proportional to the concentration of triacylglycerols in the sample.

**Reagents**

1. Triacylglycerol standard: 200 mg%

2. Enzyme reagent: Lipase, glycerol kinase, glycerol 3-phosphate oxidase, peroxidase, 4-aminoantipyrine, ATP, 3, 5-dichloro-2-hydroxybenzene sulfonate.

**Procedure**

To 10 μl of serum or 10 μL of lipid extract, 1.0 mL of enzyme reagent was added, mixed well and incubated at room temperature for 10 min. 10 μL of Triacylglycerol standard and
distilled water (blank) were also processed similarly. The absorbance was measured at 510 nm. The Triacylglycerol content was expressed as mg/dL of serum or mg/100 g of tissues.

3.5.12. ESTIMATION OF MARKER ENZYMES

Assay of aspartate aminotransferase

Serum aspirate aminotransferase was assayed by using the diagnostic kit based on the method of Reitman and Frankel (1957). AST catalyses the transfer of amino group from L-aspartate to α-ketoglutarate with the formation of Oxaloacetate and glutamate. Oxaloacetate was measured by converting it into pyruvate by treating with aniline citrate and then reacting the pyruvate with 2, 4 - Dinitrophenylhydrazine to form 2, 4-Dinitrophenyl hydrazone derivative which is brown colored in alkaline medium. The absorbance of this hydrazone derivative is correlated to AST activity.

Reagents

1. Buffered substrate: 2.66 g of DL-aspartate and 38 mg of α-ketoglutarate were dissolved in 20.5 mL of 1 N Sodium hydroxide, with gentle heating. This was made up to 100 mL with Phosphate buffer (0.01 M, pH 7.4).
2. Aniline-citrate reagent: 50 g of citric acid was dissolved in 50 mL of distilled water and mixed with equal volume of redistilled aniline.
3. Dinitrophenylhydrazine (DNPH) color reagent: 1.0 nM DNPH in 2.0 N Hydrochloric acids.
4. Sodium hydroxide: 0.4 N
5. Pyruvate standard: 2.0 nM
Procedure

0.5 mL of buffered substrate was added to 0.1 mL of serum and placed in a water bath at 37 °C. To the blank tubes, 0.1 mL distilled water was added instead of serum. Exactly an hour later, 2 drops of aniline citrate reagent and 0.5 mL of DNPH reagent were added and kept at room temperature for 20 min. Finally, 5.0 mL 0.4 N Sodium hydroxide was added. A set of standards also were treated in the same manner and read at 520 nm after 10 min. The results were expressed as IU/L of serum.

3.5.12.1. Assay of alanine aminotransferase

Serum alanine aminotransferase was assayed by using the diagnostic kit based on the method of Reitman and Frankel (1957).

ALT catalyses the transfer of amino group from L-alanine to α-ketoglutarate with the formation of pyruvate and glutamate. The pyruvate so formed, is allowed to react with 2, 4-Dinitrophenylhydrazine to produce 2, 4-Dinitrophenylhydrozone derivative which is brown colored in alkaline medium. The absorbance of this hydrozone derivative is correlated to ALT activity.

Reagents

1. Buffered substrate; 1.78 g of DL-alanine and 38 mg of α-ketoglutarate were dissolved in buffer. 0.5 mL of sodium hydroxide was added and the volume was made up to 100 mL with phosphate buffer (0.01 M, pH 7.4).

2. All other reagents were same as that used for the assay of aspartate transaminase.
**Procedure**

Procedure was the same as that used for the assay of aspartate transaminase except for the incubation time which was reduced to 30 min (60 min for AST). The results were expressed as IU/L of serum.

**3.5.12.2. Estimation of alkaline phosphatase**

Plasma alkaline phosphatase was estimated by using the diagnostic kit based on Kind & King’s method (1954). ALP catalyses disodium phenyl phosphate into phenol and disodium hydrogen phosphate at pH 10. Phenol so formed reacts with 4-aminoantipyrine in alkaline medium in the presence of oxidizing agent potassium ferricyanide to form a red colored complex whose absorbance is proportional to the enzyme activity.

**Reagents**

1. Buffered substrate: 0.01 M Disodium phenyl Phosphate dissolved in carbonate-bicarbonate buffer (0.1 M, pH 10).
2. Color reagent: 4-aminoantipyrine, sodium hydroxide and potassium ferricyanide.
3. Phenol standard: 10 mg%

**Procedure**

The incubation mixture, contained 1.0 mL of buffered substrate 3.1 mL of deionised water and 0.1 mL of serum, was incubated at 37°C. Exactly after 15 min, 2.0 mL of color reagent was added to all the tubes. The control tubes received the enzyme after the addition of color reagent. 0.1 mL of standard and 0.1 mL of distilled water (blank) were also treated simultaneously and the color developed was read at 510nm. The enzyme activity was expressed as IU/L of serum.
3.5.13. ESTIMATION OF NEPHRITIC MARKERS

3.5.13.1. Estimation of urea

Urea in the plasma was estimated by using the diagnostic kit based on the method of Fawcett and Scott (1960). Urea is hydrolyzed in the presence of water and urease to produce ammonia and carbon dioxide. Under alkaline conditions, the ammonia so formed reacts with hypochlorite and sodium salicylate in the presence of sodium nitroprusside to form a green colored chromophore. The intensity of the color produced is proportional to the concentration of urea in the sample.

Reagents

1. Buffered enzyme: Phosphate buffer, urease, sodium nitroprusside and ethylene diamine tetra acetic acid.
2. Color developing reagent: Buffer, sodium hypochlorite, sodium salicylate and sodium hydroxide.
3. Urea standard: 40 mg/dL

Procedure

To 1.0 mL of buffered enzyme, 10 μL of plasma added, mixed well and kept at 37°C for 5 min. 10 μL of standard and 10 μL of distilled water (blank) also processed simultaneously. To all the tubes, 1.0 mL of color developing reagent was added and mixed well. Exactly after 5 min of incubation at 37°C, 1.0 mL of distilled water was added and the color developed was read at 600 nm. The values were expressed as mg/dL of plasma and urine.
3.5.13.2. Estimation of Uric acid

Uric acid in the plasma was estimated by using the diagnostic kit based on the enzymic method described by Caraway (1955). Uric acid in the sample is oxidized by uricase to allantoin. In this reaction 1 mole of hydrogen peroxide is formed for every mole of uric acid oxidized. Hydrogen peroxide reacts with 3, 5-dichloro-2-hydroxybenzene sulfuric acid and 4-aminoantipyrine to give quinoneimine dye. Intensity of the color of this dye was proportional to the concentration of uric acid in the sample.

Reagents

1. Enzyme reagent: 4-Aminoantipyrine (4 mM), 3, 5-dichloro-2-hydroxybenzene sulfonate (2.0 mM), microbial uricase (150 U/L), horseradish peroxidase (10,000 U/L)
2. Standard uric acid: 5.0 mg/ 100 mL

Procedure

To 1 mL of the enzyme reagent, 25 μL of plasma was added and mixed by inversion. 25 μL of standard and 25 μL of distilled water (blank) also processed simultaneously. The tubes were incubated at 37°C for 5 min and the color developed was read at 510 nm. The values were expressed as mg/dL of plasma and urine.

3.5.13.3. Estimation of Creatinine

Creatinine in the plasma was estimated using the diagnostic kit based on the method of Tietz (1987) using Jaffe’s (1886) color reaction. The assay of creatinine has been based on the reaction of creatinine with alkaline picrate as described by Jaffe. Most of the contaminants
reacting with the Jaffe reagent produce a color at a lower rate than does creatinine. The initial rate of color formation is proportional to the concentration of creatinine in the sample.

**Reagents**

1. Saturated picric acid
2. Sodium hydroxide: 0.75 N
3. Creatinine standard: 2.0 mg/dL

**Procedure**

0.1 mL of plasma was added to a reagent mixture containing 0.5 mL picric acid solution and 0.5 mL of sodium hydroxide. The tubes were mixed well and incubated for 20 s. With the spectrophotometer adjusted to zero absorbance with distilled water. Reading was taken at 510 nm at 20 s ($A_1$) and exactly after 45 s ($A_2$). Change in absorbance ($A_2 - A_1$) was measured for test and standard which was used to determine the creatinine concentration in the test sample. The values were expressed as mg/dL of plasma.

**3.5.14. ESTIMATION OF PROTEIN PROFILE**

**3.5.14.1. Estimation of total protein and albumin**

Total protein and albumin in the serum were estimated by Biuret method (1953). Proteins from a purple colored complex with cupric ions in alkaline solution. The reaction takes its name from the simple compound biuret which reacts in the same way. The intensity of the purple colour is proportional to the amount of protein present in the sample.

**Reagents:**

1. Stock biuret reagent: 45 g of sodium potassium tartarate was dissolved in 400 mL of 0.2 N sodium hydroxide and 15 g of copper sulphate was added and stirred. 5.0 g
potassium iodide was then added, dissolved and make up to 1.0 L with 0.2 N sodium hydroxide.

2. Dilute biuret reagent: 200 mL of stock biuret reagent was diluted to 1 L with 0.2 N sodium hydroxide containing 5.0 g potassium iodide/L.

3. Standard egg albumin: 500 mg/100 mL distilled water (small quantity of alkali was added to dissolve albumin).

4. Sodium sulphite solution: 28%

**Procedure**

0.5 mL of serum was taken in a test tube and 9.5 mL of sodium sulphite solution was added and mixed. After mixing, 3.0 mL of the mixture was transferred into a tube for total protein estimation to which 5.0 mL of biuret reagent was added. To the rest of the mixture, 3.0 mL of ether was added, stoppered, shaken well for 20 s and then centrifuged for 5 min. 3.0 ml of the clear supernatant was taken for the estimation of albumin and treated with 5 mL of biuret reagent simultaneously, 2.0 mL of standard egg albumin were mixed with 1.0 of water and treated with 5.0 mL of biuret reagent. The purple color developed was read at 540 nm after 15 min using reagent blank. Values were expressed as g/dL of serum.

**3.5.14.2. Estimation of globulin**

Serum globulin concentration was calculated using the following formula after the estimation of total protein and albumin. Globulin = Total protein - albumin.

**3.5.15. ESTIMATION OF GLYCOPROTEIN COMPONENTS**

**3.5.15.1. Estimation of total hexoses**

Total hexoses in the serum and tissues were estimated by the method of Niebes (1972).
Reagents

1. Orcinol - sulphuric acid mixture: 1.6 g of orcinol was dissolved in 100mL of water.
   1.0 mL of this was mixed with 7.5 mL H$_2$SO$_2$: H$_2$O mixture (3:2 v/v). This was prepared fresh before use.

2. 5 mg of galactose and 5.0 mg of mannose were dissolved in 100 mL of water. This had a concentration of 100 μg/mL

Procedure

0.2 mL of the serum or homogenate was mixed with 8.5 mL of orcinol - H$_2$SO$_4$. The tubes were then heated at 80°C for 15 min, cooled and read at 540 nm after 20 min. standard and blank containing. 0.2 mL of 0.2 N H$_2$SO$_4$ were also processed similarly.

Total hexoses content was expressed as mg/dL of serum or mg/100 g of tissue.

3.5.15.2. Estimation of Hexoseamine

Hexosamine in the serum and tissues was determined by the method of Elson & Morgon (1933).

Reagents

1. Ethanol: 95 %

2. Hydrochloric acid: 3 N

3. Sodium hydroxide: 3 N

4. Acetyl acetone reagent: 1 mL of acetyl acetone in 50 mL of 0.5 N sodium carbonate, freshly prepared.

5. Ehrlich reagent: 0.8 g of p-dimethylaminobenzaldehyde (recrystallized as the hydrochloride) dissolved in 30 mL of methanol and 30 mL of conc HCl.
6. Glucosamine standard: 0.05 mg/mL of free glucosamine in water

**Procedure**

To 0.1 mL of serum or homogenate in a test graduated at 10 mL, 5 mL of 95 percent ethanol was added and mixed well, centrifuged for 15 min, decanted, and the precipitate was suspended in 3 mL of 95 % ethanol, centrifuged and decanted. To the precipitated protein 2 mL of 3 N HCl was added and hydrolysed in a boiling water bath with an air condenser for 4 h. The hydrolysate was neutralized with 3 N NaOH. 1 mL of the acetyl acetone was added to 1 mL of the aliquot, 1 mL of the water (blank) and 1 mL of standard. The tubes were capped with marbles to prevent evaporation and placed in a boiling water bath for 15 min. The tubes were cooled in a tap water. 5 mL of 95 % ethanol was added and mixed well. 1 mL of Ehrlich reagent was added mixed well and diluted to 10 mL with 95 % ethanol. Absorbance was measured at 530 nm after 30 min. Hexosamine content was expressed as mg/dL of serum or mg/ 100 g of tissue.

3.5.15.3. Estimation of Sialic acid

Sialic acid in the serum and tissues was estimated by the method of Welmer et al., (1952).

**Reagents**

1. TCA: 5%
2. Acid mixture: 90 mL of glacial acetic acid and 10 mL of concentrated sulphuric acid
3. Diphenylamine reagent: 1 g of diphenylamine recrystallized from ethanol was dissolved in 100 mL of mixture.
4. Sialic acid standard: 0.2 mg/mL
Procedure

4.8 mL of 5% TCA was added slowly to 0.2 mL of serum or homogenate, and 0.2 mL of orosomucoid standard in a separate tube. The test tube was placed in a boiling water bath for exactly 15 min with a glass marble to prevent evaporation; the tubes were cooled by immersion in water and filtered. 2 mL of clear filtrate was pipetted out in each tube; added 4 mL of DPA reagent into one of each pair of tubes and 4 mL of acid mixture without DPA was added into another tube. The reagent blank was prepared by adding 2 mL of 5% TCA and 4 mL of DPA reagent. The tubes were mixed well, capped with a glass marble and immersed in a boiling water bath for exactly 30 min. The tubes were cooled in water and the absorbance was determined at 530 nm with a reagent blank set at zero. Sialic acid content was expressed as mg/dL of serum or mg/100 g of tissue.

3.5.15.4. Estimation of Fucose:

Fucose in the serum and tissues was estimated by the method of Dische and Shettles (1948).

Reagents

1. Sulphuric acid reagent: conc H₂SO₄ and distilled H₂O were mixed in the ratio of 6:1
2. Cysteine hydrochloride reagent: 3% cysteine hydrochloride in water 0.1 N NaOH

Procedure

To 2.2 mL of serum or homogenate, 4.8 mL of sulphuric acid reagent was added and heated in a boiling water bath for 3 min. The sample was cooled and 0.1 mL of cysteine hydrochloride reagent was added; 0.5 mL of 0.1 N NaOH was also treated in the same way for
blank. After 25 min the optical density was measured at 393 and 430 nm. Fucose content was expressed as mg/dL of serum or mg/100 g of tissue.

3.5.16. Insulin secretion experiments

Non-fasting male Wistar rats (180–250 g) were euthanized by cervical dislocation and decapitation. Islets from rat pancreas were isolated by collagenase digestion method (Moskalewski, 1965). In all the in vitro experiments islets were pre-incubated with 0.5 ml of incubation buffer containing 11.1 mM glucose alone or in combination with various concentrations of γ-sitosterol (5, 10, 15 and 20 μg/kg /b.wt) at 37 °C for 30 min in a shaking water bath under 95% O₂ and 5% CO₂ atm. After the pre-incubation the buffer was removed, new additions were made in a similar fashion and incubated under similar conditions. Aliquots of 50 μl were removed from static incubation mixture at 0, 10 and 60 min and were frozen immediately until insulin assay was performed using ELISA method. For inhibition experiments, islets were pre-incubated with 0.25 mM diazoxide or 0.25 mM EGTA or 0.25 mM nimodipine along with and without 20 μg γ-sitosterol and 11.1 mM glucose for 30 min. Insulin released after 10 and 60 min was measured by ELISA method as indicated above.

3.5.17. HISTOLOGICAL EXAMINATION

3.6.17.1. Light microscopic studies-paraffin method

The microscopic study was done by the method of Humason, (1979)

Reagents:

1. Physiological saline (10%)
2. Bouin-Holland fixative
3. Ehrlich’s hematoxylin
4. Eosin
The tissues such as pancreas, liver and kidney from in treated and parallel experimental groups were blotted free of mucus, washed in physiological saline, cut into pieces of desired size and fixed in Bouin-Hollande fixative (10%) for 72 hrs. After fixation, the tissues were washed in 70% alcohol for two or three days to remove the excess picric acid and dehydrated in graded series of alcohol. The tissues were cleaned using xylene. The cleared tissues were infiltrated with molten paraffin at 58-60°C through three changes (20-30 min each) and finally embedded in paraffin. 3-5µm thick sections of all the tissues were obtained using the rotary microtome (Leica, Germany) and stained in Ehrlich’s hematoxylin with eosin as a counter stain. The slides were mounted using DPX mountant.

3.5.18.2. Immunohistochemical analysis of Pancreas:

Reagent:

1. Buffered formalin (10%)
2. Hydrogen peroxide (0.3%)
3. Hydrogen peroxide (0.005%)
4. 2% normal goat serum (NGS)
5. Triton X-100 (0.5%)
6. Primary antibody (primary antibody (Mouse anti-EMA, anti-insulin) Mouse anti-EMA, anti-insulin)
7. Secondary antibody (biotinylated anti-mouse antibodies)
8. Diaminobenzidine hydrochloride (0.03%)

For immunohistochemical examination, pancreas was removed, washed in saline, kept in 10% formalin and fixed in paraffin. The preparation was washed in running water overnight and then sectioned (5 µm in thickness). Sections were used for immunohistochemical analysis. The
sections of control and treated rats were dewaxed, then incubated for 1 hr at room temperature in 0.3 % hydrogen peroxide in PBS, followed by 3 washings in PBS. Sections were then incubated for 16 hr, at 4 °C, in PBS containing 2 % normal goat serum (NGS) and 0.5 % triton X-100 and washed in PBS. They were then incubated overnight at 4°C with the primary monoclonal antibody (Mouse anti-EMA, anti-insulin). Incubation was followed by 3 washes in PBS-2 % NGS. The primary antibodies were bounded by a biotinylated anti-mouse secondary antibody in PBS for 1 hr at room temperature. Sections were then incubated in avidin-biotin complex linked to peroxidase. The peroxidase was visualized with 0.03% diaminobenzidine hydrochloride and 0.005 % hydrogen peroxide in 0.1 M Tris -buffer. Sections were counterstained with hematoxylin (Hsu et al., 1981).

3.5.19. Statistical analysis

All the data were statistically evaluated using SPSS program; version 11.5 software package. The values were analyzed by one way analysis of varience (ANNOVA) followed by Student’s t-test. All the results were expressed in mean ± S.E.M for six rats in each group. \( p \leq 0.05 \) was considered significant.

3.6. RESULTS

The present study was undertaken to assess the possible antidiabetic effect of \( \gamma \)-sitosterol, the active principle isolated from \( L. \) nodiflora in normal and STZ-induced diabetic rats. The effective dose of \( \gamma \)-sitosterol was determined by checking its effect on fasting plasma glucose level, total haemoglobin, glycosylated haemoglobin, liver glycogen and the alteration in serum/plasma and tissue carbohydrate metabolizing enzymes, lipid peroxides, anti-oxidant profiles and lipid levels in STZ-induced diabetic rats. Immunohistochemical studies of the pancreatic samples were carried out under light microscope as well as transmission electron
microscope. Histopathological examination of liver and kidney samples were also carried out under light microscope.

3.6.1. Determination of effective dose of γ-sitosterol

Oral glucose tolerance test with different doses γ-sitosterol (Viz, 5, 10, 20 mg/kg /b.wt) was carried out. In STZ- treated diabetic rats, increased blood glucose level was observed after 60 minutes. Even after 120 minutes and at 180 min the blood glucose level in this group remained high. γ-sitosterol at 20 mg/kg /b.wt significantly decreased the plasma glucose levels (-45.07 %) at 180 min. At the same time γ-sitosterol at 5, 10 mg/kg /b.wt reduced the plasma glucose level by -13.75 %, -20.71 % respectively in treated groups (Table 1). Glibenclamide treated group also showed significant decrease (-40.72 %) in blood glucose levels. Oral administration of γ-sitosterol 20 mg/kg.bwt to normal rats did not show any significant effect.

3.6.3. Antihyperglycemic effect of γ-sitosterol

Table 2 shows the body weight and food intake of normal and STZ-induced diabetic rats. Body weight was decreased (-36.13 %) and food intake was significantly increased (54 %) in the diabetic control group when compared with normal group. Oral administration of γ-sitosterol at 20mg/kg /b.wt for 21 days significantly increased the body weight (7.6 %) and lowered the food intake (-106.50 %) in diabetic group. In the glibenclamide treated group also there was a significant increase (13.37 %) in body weight and a decrease in the food intake by -62.96 % when compared with zero day. Oral administration of γ-sitosterol at 20 mg/kg /b.wt to normal rats did not show any significant effect.

Table 3 shows the effect of oral administration of γ-sitosterol at 20 mg/kg /b.wt for 21 days on plasma glucose level in normal and STZ induced diabetic rats. In diabetic rats, plasma glucose level was increased significantly when compared with normal rats. Oral administration
of γ-sitosterol at 20 mg/kg /b.wt for 21 days treatment significantly decreased the plasma glucose levels (-44.31 %) compared with diabetic rats. Administration of glibenclamide also decreased the plasma glucose level significantly.

Table 4 shows the effect of oral administration of γ-sitosterol at 20 mg/ kg /b.wt on the levels of insulin, hemoglobin and glycosylated hemoglobin in normal and STZ- induced diabetic rats. Elevated level of glycosylated hemoglobin (108.85 %), decreased levels of plasma insulin (-90.32 %), decreased level of total hemoglobin (-58.30 %) were observed in diabetic rats when compared with normal rats. Oral administration of γ-sitosterol at 20 mg/kg /b.wt for 21 days prevented the elevation of glycosylated hemoglobin (-24.44 %) thereby increasing the level of plasma insulin (69.05 %) and total hemoglobin (66.96 %). Glibenclamide treated group showed decreased level of glycosylated hemoglobin (-11.76 %), increased levels of plasma insulin (70.86 %) and total hemoglobin (62.34 %) when compared with diabetic control. Oral administration of γ-sitosterol at 20 mg/kg /b.wt to normal rats did not show any significant effect.

Table 5 shows the liver and muscle glycogen content in normal and diabetic rats. There was a significant decrease in liver (-78.18 %) and muscle (-81.98 %) glycogen content in STZ-induced diabetic rats when compared to normal control rats. When γ-sitosterol (20 mg/kg b. wt) was administered orally to diabetic rats for 21 days it increased the muscle (76.86 %) and hepatic (77.15 %) glycogen content significantly when compared to diabetic control rats. Glibenclamide treated group showed significant increase in these parameters when compared with diabetic control rats.

Tables 6 and 7 show the effect of γ-sitosterol on the activities of hexokinase, glucose 6 phosphatase and Fructose 1,6 bisphosphatase in serum, liver and kidney in normal and diabetic rats. The activity of hexokinase was decreased while the activities of glucose 6 phosphatase and
Fructose 1, 6 bisphosphatase were increased in diabetic rats when compared to normal rats. Oral administration of \( \gamma \)-sitosterol and glibenclamide for 21 days showed more than one fold increase in the level of hexokinase in liver and kidney and in serum over 3 fold increase when compared with diabetic control.

Table 8 shows the activities of hepatic glycogen metabolizing enzyme levels and glucose -6- phosphate dehydrogenase levels in normal and experimental rats. The activities of glycogen synthase and glucose - 6- phosphate dehydrogenase were found to be decreased and the enzyme glycogen phosphorylase activity was found to be significantly increased in diabetic rats when compared to normal rats. Oral administration of \( \gamma \)-sitosterol at 20 mg/kg /b.wt for 21 days significantly increased the level of glycogen synthase (55.45 %), glucose -6- phosphatase (20.57 %) and significantly decreased the level of glycogen phosphorylase (- 22.10 %) when compared with diabetic control. Glibenclamide treated group significantly increased the glycogen synthase and glucose -6- phosphatase by 54.42 % and 17.91 % respectively and significantly decreased glycogen phosphorylase -24.99 % when compared with diabetic control.

Tables 9 and 11 show the effect of \( \gamma \)-sitosterol on total cholesterol (TC), Triglycerides (TG) and free fatty acid (FFA) in serum and tissues (liver and kidney) of normal and diabetic rats. The diabetic rats had elevated levels of TC, TG and FFA in the serum and tissues when compared with normal control. Treatment with \( \gamma \)-sitosterol decreased significantly TC (-41.28 %, -55.97 %, -54.61 %) level in serum, liver and kidney. The level of TG (-30.88 %, -64.63%, 54.61% and FFA (-53.14 %, -25.07 %, -45.96 %) decreased significantly in serum and tissues when compared with diabetic control. Glibenclamide treated group also showed significant decrease in TC, TG and FFA when compared with diabetic control.
Table 10 shows the levels of HDL, LDL and VLDL cholesterol in the serum of normal and diabetic rats. The diabetic rats had elevated levels of LDL and VLDL and decreased level of HDL. Oral administration of γ-sitosterol significantly increased HDL (68.89 %) level and significantly decreased LDL (-47.54 %) and VLDL (-32.34 %) levels compared to diabetic control. Glibenclamide treated group significantly increased HDL 68.98% and decreased LDL and VLDL (-47.52 %, -30.70 %) respectively when compared with diabetic control.

Table 12 represents the concentration of TBARS and hydroperoxides in serum, liver and kidney of normal and experimental rats. There was a significant elevation in tissue TBARS and hydroperoxides during diabetes, when compared to the corresponding normal control group. Administration of γ-sitosterol (20 mg/kg /b.wt) significantly decreased the lipid peroxidation in serum (-43.24 %), liver (-51.61%) and kidney (-68.52 %) of diabetic rats. Glibenclamide treated group also decreased the lipid peroxidation significantly in serum and tissues when compared with diabetic control. Oral administration of γ-sitosterol to normal rats did not show any significant effect.

Table 13 shows the serum non enzymatic antioxidant levels in normal and diabetic control rats. Decreased levels of Vitamin C and GSH and increased level of Vitamin E was observed in diabetic control when compared with normal control rats. Oral administration of γ-sitosterol at 20 mg/kg /b.wt for 21 days significantly increased the levels of Vitamin C (122.01%), and GSH (82.97 %) and decreased Vitamin E level by -45.29 % when compared with diabetic control. Glibenclamide treated group significantly increased Vitamin C (127.27 %), and GSH (84.87 %) and decreased Vitamin E level by -50.11% when compared to diabetic control.

Table 14 shows the levels of non-enzymatic antioxidants (Vitamin C, Vitamin E and GSH) in liver and kidney of normal and experimental rats. The Vitamin C and GSH levels were
significantly decreased where as the Vitamin E level in liver and kidney was increased in diabetic rats as compared to normal animals. Oral administration of $\gamma$-sitosterol for 21 days increased the levels of Vitamin C (138.88%, 123.52 %), and GSH (173.76 %, 129.33 %) and decreased level of Vitamin E (-39.25 %,-50.00 %) in liver and kidney respectively when compared to diabetic control. Glibenclamide treated group significantly showed the levels of Vitamin C, Vitamin E and GSH level in liver and kidney to be near normal when compared with diabetic control.

Tables 15 and 16 show the levels of antioxidant enzymes catalase (CAT), glutathione s-transferase (GST), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in liver and kidney in normal and experimental animals. CAT, GST, SOD and GPx activities were significantly decreased in STZ-induced diabetic rats when compared to normal rats. Treatment of STZ-induced diabetic rats with $\gamma$-sitosterol (20 mg/kg/b.wt) for 21 days resulted in a marked increase in CAT, GST, SOD and GPx activities in liver and kidney when compared to the activities of STZ-induced diabetic rats. Normal rats treated with $\gamma$-sitosterol (20 mg/kg/b.wt) showed no significant difference in the activities of enzymatic antioxidants.

Table 17 shows the levels of total proteins, globulins, albumin and albumin/globulin ratio in the plasma of normal and diabetic rats. The diabetic rats had decreased levels of plasma total proteins, globulins, albumin and albumin/globulin ratio when compared with normal rats. Oral administration of $\gamma$-sitosterol (20 mg/kg/b.wt) for 21days increased the level of total protein (53.37%), globulins (80.13%), albumin (150%) and albumin/globulin ratio (52.70 %); significant increase was also observed in glibenclamide treated group when compared with diabetic control group.
Table 18 shows plasma glycoprotein levels in normal and diabetic control groups. In diabetic control group increased levels of total hexose, hexose amine, fucose and decreased level of sialic acid was observed in diabetic control when compared with normal control. Oral administration of γ-sitosterol decreased the levels of total hexose (-148.92 %), hexoseamine (-45.16 %), and fucose (-24.46 %) and increased the sialic acid level by 149.18 % when compared with diabetic control. Glibenclamide treated group significantly decreased the level of total hexose (-83.70 %), hexose amine (-36.33 %), fucose (-18.11 %) and increased the level of sialic acid by 179.88 % when compared with diabetic control.

Table 19 shows the levels of Total hexose, Hexoseamine, Sialic acid, Fucose in liver and kidney of normal and diabetic rats. The diabetic rats showed increased levels of Total hexose, Hexoseamine and fucose and decreased level of sialic acid in liver and kidney when compared with normal control. Oral administration of γ-sitosterol for 21 days decreased the levels of total hexose (-47.18 %, -47.39 %) in liver and kidney, hexose amine (-47.28 %, -47.91 %) and fucose (-44.53 %, -50.02 %) and increased sialic acid level (88.37 %, 109.22 %) in liver and kidney respectively when compared with diabetic control. Glibenclamide treated groups improved positively the levels of these parameters significantly when compared with diabetic control.

Table 20 shows the activities of serum liver marker enzymes such as AST, ALT, ALP and ACP in normal control and diabetic control rats. The activities of AST, ALT, ALP and ACP increased significantly in diabetic rats. Oral administration of γ-sitosterol decreased the activities of AST (-37.65 %), ALT (-38.24 %), ALP (-23.40 %) and ACP (-30.39 %) when compared with diabetic control. Glibenclamide treatment for 21 days normalized the level of these parameters when compared with diabetic control.
Table 21 shows the levels of urea, uric acid, creatinine and albumin in the plasma of diabetic rats. In diabetic rats, we observed decreased levels of urea, uric acid and creatinine when compared with normal rats. Treatment with \(\gamma\)-sitosterol for 21 days decreased the levels of urea (-46.89 \%), uric acid (-36.51 \%) and creatinine (-38.26 \%) when compared with diabetic control. Glibenclamide treated group showed significantly decreased levels of urea (-43.70 \%), uric acid (-37.34 \%) and creatinine (-39.56 \%) when compared with diabetic control.

### 3.6.4. Insulin secretary assay:

**Effect of \(\gamma\)-sitosterol on glucose-induced insulin release:**

When pancreatic islets were incubated with 11.1 mM glucose in the presence of \(\gamma\)-sitosterol, a significant increase in insulin release was observed. There was a dose-dependent increase in insulin release when islets were incubated with various concentrations of \(\gamma\)-sitosterol for 10 and 60 min when compared with control group without the addition of \(\gamma\)-sitosterol (Fig. 3.1). Effects of diazoxide, EGTA and nimodipine on glucose and \(\gamma\)-sitosterol-induced insulin release were studied. Diazoxide, a potent K+-ATP channel opener, is a well-known inhibitor of glucose-induced insulin release. Incubation of pancreatic islets with 11.1 mM glucose and 20 \(\mu\)g \(\gamma\)-sitosterol in the presence of 0.25 mM diazoxide caused a 40\% inhibition in insulin release when compared with \(\gamma\)-sitosterol treated islets without diazoxide. When islets were incubated with 11.1 mM glucose, 20 \(\mu\)g \(\gamma\)-sitosterol and 0.25 mM EGTA or 0.25 mM nimodipine, no change was observed in the release of insulin as compared with \(\gamma\)-sitosterol treated islets without EGTA or nimodipine (Fig. 3.2). Decreased secretion of insulin was observed in the absence of \(\gamma\)-sitosterol.
3.6.5. HISTOLOGICAL STUDY:

Pancreas:

Histological examination of pancreas showed normal histology in normal rats and normal rats treated with γ-sitosterol. Diabetic pancreas showed shrinkage of islets and growth of adipose tissue. Diabetic pancreas treated with γ-sitosterol or glibenclamide reduced these changes. (Fig 3.3)

Liver:

Normal architecture of liver cells was observed in normal control and normal control treated with γ-sitosterol (20 mg/kg/b.wt). Histochemical changes in the liver of diabetic rats included hepatocytic nuclear condensation, portal triad with inflammation and sinusoidal dilation. Normal hepatocytes with mild inflammation were seen in liver treated with γ-sitosterol (20 mg/kg /b.wt) and glibenclamide (600 µg/kg /b.wt) (Fig. 3.4).

Kidney:

Histological examination of the sections of kidneys from normal control rat is shown in Figure 3.5. The normal kidney had a capsule at the left with underlying cortex which contained glomeruli and tubules. Numerous tubules (proximal and distal) were lying in the area adjacent to glomeruli. The kidney of diabetic control rats showed marked microscopic changes like multifocal clarifications and vacuolations compared to kidney of normal control rat. These changes were negligible in diabetic rats treated with γ-sitosterol (20 mg/kg/b.wt) and glibenclamide (600 µg/kg /b.wt).
3.6.6. Immunohistochemical study of pancreas

The pancreatic islets in the normal control showed numerous insulin-immunoreactive cells in both central and peripheral parts of the islets of Langerhans. The pancreatic islets administered with γ-sitosterol (20 mg/kg/b.wt) displayed similar characters. After the onset of diabetes only a few insulin-positive islets were observed. However administration of γ-sitosterol (20 mg/kg /b.wt) to diabetic rats enhanced the number of immunoreactive insulin-secreting cells in pancreatic islets (Fig. 3.6).

3.7. DISCUSSION:

The present study was undertaken to assess the possible antidiabetic effect of γ-sitosterol, the active principle isolated from L. nodiflora in normal and STZ-induced diabetic rats. Also the evaluations of chronic administration of effective dose of γ- sitosterol on fasting plasma glucose level, total haemoglobin, glycosylated haemoglobin, liver glycogen and the alteration in serum and tissue carbohydrate metabolizing enzymes, lipid peroxides, anti-oxidant profiles and lipid levels in STZ-induced diabetic rats were carried out. Light microscopic, transmission electron microscopic and immunohistochemistry studies of pancreas were carried out.

3.7.1. Determination of effective dose of γ- sitosterol

Based on our dose determination study, oral glucose tolerance test with 20 mg/kg/b.wt dose of γ- sitosterol significantly decreased the plasma glucose levels compared with 5 and 10 mg/kgbw. The plasma glucose lowering effect may be due to the insulin secretory potential of the active principle (γ- sitosterol). 20 mg/kg/b.wt dose of costunolide and eremanthin isolated from Costus speciosus had shown potential plasma glucose lowering effect (Eliza et al., 2010).
Adolfo et al., (2007) have reported that the water extracts of *Tournefortia hirsutissima* at dose of 20 mg/kg/b.wt significantly lowered the plasma glucose levels in diabetic rats.

### 3.7.1.1. Effect of γ-sitosterol on change of body weight and food levels.

STZ-induced diabetes is characterized by a severe loss in body weight (Al-Shamaorry et al., 1994) and increase in food intake (Szkudelski *et al.*, 2002). Body weight loss might be the result of protein wasting due to unavailability of carbohydrate as an energy source (Chen and Ianuzzo, 1982). Oral administration of γ-sitosterol improved body weight and decreased food intake in diabetic rats which might result from an improvement in glyceamic control. Similarly alcoholic extract of *Barleria prionitis* arrested the reduction of body weight in diabetic rats produced by alloxan (Reema dheer and Bhatnagar, 2010).

### 3.7.1.2. Antihyperglycemic effect of γ-sitosterol

Diabetes mellitus causes a disturbance in the uptake of glucose as well as glucose metabolism. The use of a lower dose of STZ (40 mg/kg/b.wt) produces an incomplete destruction of pancreatic β-cells even though the rats become permanently diabetic (Aybar *et al.*, 2001).

Streptozotocin (STZ)-induced hyperglycemia in animals is considered to be a good model for the preliminary screening of agents active against diabetes. In experiments with many animal species, streptozotocin produced permanent diabetes that impersonates the pathological status found in human diabetes (Saravanana and Ponmurugan, 2011). The mechanism by which STZ brings about a diabetic state includes selective destruction of pancreatic beta cells, leading to hypoinsulinemia which as a result decreases glucose uptake and produces hyperglycemia which are the characteristic features of diabetes mellitus (Marles and Farnsworth, 1995). After treatment with a low dose of STZ there should be many surviving β-cells, and regeneration is also possible (Gomes *et al.*, 2001). Hyperglycemia generates abnormally high levels of free
radicals by autooxidation of glucose and protein glycation; oxidative stress has been reported to be a causal factor of cardiovascular complications in STZ-induced diabetes (Okutan et al., 2005). The increased levels of plasma glucose in STZ induced diabetic rats was significantly lowered (-44.31 %) by γ-sitosterol administration for 21 days. The antihyperglycemic action of γ-sitosterol results from the potentiation of insulin release from existing β-cells of the islets of Langerhans.

The plasma glucose lowering activity was compared with glibenclamide, a standard hypoglycemic drug. Glibenclamide has been used for many years to treat diabetes, and to stimulate insulin secretion from pancreatic β-cells (Tian et al., 1998). The results of the present study show that glucose lowering effect of γ – sitosterol results from the plasma insulin release from pancreatic β-cells of the islets of Langerhans. It directly indicates that part of the antihyperglycemic activity of γ -sitosterol is through the release of insulin many folds, probably through β-cells stimulation resembling direct insulin secretagogue effect.

Insulin secretary effect of γ - sitosterol is directly related to the increased glucose level in STZ-diabetic rats. Li et al., (2004) have shown that sterols might stimulate the beta islets to secrete insulin and increase the sensitivity of insulin to uptake glucose. Similarly, Ng et al., (1986a) have reported that Charantin, a steroidal saponin, obtained from Momordica charantia, is known to have an insulin-like activity, and also stimulates the release of insulin and blocks the formation of glucose in the bloodstream, which may be helpful in the treatment of diabetes, particularly in non-insulin-dependent diabetes (Ng et al., 1986b). Michelet et al., (2011) reported that saponin and poly phenoic content of Zizyphus spina, improved glucose utilization in diabetic rats by increasing insulin secretion. Reema Dheer and Pradeep Bhatnagar (2010) also reported increase in serum insulin by leaf of Barleria prionitis.
3.7.1.3. Effect of γ- sitosterol on haemoglobin (Hb) and glycosylated haemoglobin (HbA1c) levels:

The typical characteristic of diabetes is the increase of serum glycated protein such as glycated haemoglobin (HbA1C), which is a parameter for glycemic control where glucose or other reducing sugars react with the amino residues of proteins to form Amadori products, for instance, glycated haemoglobin (Michel et al., 2011).

Hyperglycemia in diabetic patients is associated with alteration in carbohydrate, protein and lipid metabolism and modification in liver enzyme level. 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. Therefore estimation of glycosylated heamoglobin (HBA1C) gives an accurate reflection of mean plasma glucose concentration over this period and correlates best with the degree of glycemia (Danze et al., 1987). Oral administration of γ- sitosterol prevents a significant elevation in glycosylated haemoglobin thereby increasing the level of total haemoglobin in diabetic rats. The level of HbA1c is a reliable index of glycemic control in diabetes mellitus which reflects the average blood glucose concentration; decrease of glycosylated haemoglobin may be due to improved glycemic control (Balamurugan et al., 2011).

3.7.1.4. Effect of γ-sitosterol on key carbohydrate metabolic enzymes in serum and tissues (Liver and Kidney)

Insulin regulates blood glucose primarily by stimulating uptake of glucose into muscle and adipose tissue and by inhibiting hepatic glucose production. Insulin resistance plays an important role in the pathogenesis of type 2 diabetes.
One of the hallmarks of diabetes is the inability of insulin to inhibit hepatic glucose production (Hofmann et al., 1992). Glycolysis and gluconeogenesis are the two prime complementary events balancing the glucose load in our body. Hexokinase, glucose-6-phosphatase and fructose-1, 6-bisphosphatase are the key enzymes of glucose metabolism. They are markedly altered to produce hyperglycemia, which leads to pathogenesis of diabetic complications.

The gluconeogenic enzyme glucose-6-phosphatase is a crucial enzyme of glucose homeostasis because it catalyses the ultimate biochemical reaction of both glycogenolysis and gluconeogenesis (Mithievre et al., 1996). The increased activities of hepatic gluconeogenic enzymes, glucose-6-phosphatase and fructose-1, 6-bisphosphatase could be due to the activation or increased synthesis of the enzymes contributing to increased hepatic glucose production during diabetes (Kondeti et al., 2010). Increased hepatic glucose production in diabetes mellitus is associated with impaired suppression of the gluconeogenic enzyme fructose-1, 6-bisphosphatase. Activation of gluconeogenic enzymes is due to the state of insulin deficiency, because under normal conditions, insulin functions as a suppressor of gluconeogenic enzymes.

In the present study, we observed the increased levels of glucose-6-phosphatase and fructose-1, 6-bisphosphatase activity in diabetic rats compared to normal control. Oral administration of γ-sitosterol for 21 days to STZ-induced diabetic rats significantly reduced the activities of glucose-6-phosphatase and fructose-1, 6-bisphosphatase in serum and tissues (liver and kidney) and this may be due to a state of increased insulin concentration which reflects the insulin secretory effect of the active principle(s). The reduced activity of these enzymes can result in a reduction of blood glucose. The present findings indicate that γ-sitosterol has beneficial effects on glucose concentration as well as sequential metabolic correlation between
increased glycolysis and decreased gluconeogenesis stimulated by γ- sitosterol and suggests the possible biochemical mechanisms through which glucose homeostasis is maintained.

A decrease in the activity of glucose-6-phosphate dehydrogenase has been shown to slow down the pentose phosphate pathway in diabetic conditions (Abdel-Rahim et al., 1992). Oral administration of γ-sitosterol to diabetic animals significantly increased liver glucose 6-phosphate dehydrogenase activity via increased secretion of insulin. The increased activity of G-6-PDH indicates an improvement in glucose utilization by pentose phosphate pathway (Kondeti et al., 2010).

3.7.1.5. Effect of γ- sitosterol on glycogen, glycogen synthase and glycogen phosphorylase levels

Quantification of glycogen, the primary intracellular storage form of glucose in liver can be considered as an important indicator of diabetes mellitus. Glycogen level in various tissues especially in liver and skeletal muscle indicates direct reflection of insulin activity since it causes glycogen deposition by stimulating glycogen synthase and inhibiting glycogen phosphorylase (Kasetti et al., 2010). This results in an increased blood glucose level in diabetes mellitus. Since streptozotocin causes selective destruction of β-cells of islets of Langerhans, resulting in marked decrease in insulin levels, it could be predicted that glycogen levels in tissues (muscle and liver) decrease as the influx of glucose in the liver is inhibited in the absence of insulin (Golden et al., 1979). However, this alteration in muscle and hepatic glycogen content is normalized by insulin treatment (Vats et al., 2004). Oral administration of γ- sitosterol for 21 days to STZ-induced diabetic rats significantly increased hepatic glycogen levels in diabetic rats, possibly because of reactivation of the glycogen synthase system as a result of increased insulin secretion.
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3.7.1.6. Effect of γ- sitosterol on serum and tissue (Liver and Kidney) lipid profiles

Diabetes is associated with profound alterations in the plasma lipid and lipoprotein profile. In uncontrolled type 2 diabetes mellitus, there will be an increase in TC, LDL-C and VLDL-C and with decrease in HDL-C which contributes to the coronary artery disease (Zheng et al., 2011). Raised plasma FFA level plays a major role in the pathogenesis of insulin resistance and type 2 diabetes (Kovacs and Stumvoll, 2005). In addition, hypertriglyceridemia is also an important marker of insulin resistance (Schwartz, 2006). The abnormally high concentrations of serum lipids in the diabetic subjects are due mainly to the increase in the mobilization of free fatty acids from the peripheral fat depots, since insulin inhibits the hormone sensitive lipase. Insulin deficiency or insulin resistance may be responsible for dyslipidemia, because insulin has an inhibitory action on HMG-coA reductase, a key rate-limiting enzyme responsible for the metabolism of cholesterol rich LDL particles. Acute insulin deficiency initially causes an increase in free fatty acid mobilization from adipose tissue. This results in increased production of cholesterol rich LDL particle (Murali et al., 2002). Oral administration of γ- sitosterol normalized these effects, possibly by controlling the hydrolysis of certain lipoproteins and their selective uptake and metabolism by different tissues.

3.7.1.7. Effect of γ- sitosterol on plasma, tissue TBARS and Hydroperoxides levels

Hypoinsulinemia in diabetes increases the activity of the enzyme fatty acyl CoA oxidase, which initiates β-oxidation of fatty acids, resulting in lipid peroxidation (Horie et al., 1981). Circulating lipid peroxides were shown to be capable of initiating arteriosclerosis, which is a well known late feature of diabetes mellitus. Low levels of lipoxygenase peroxides stimulate the secretion of insulin, but when the concentration of endogenous peroxide increases, it may initiate uncontrolled lipid peroxidation leading to cellular infiltration and islet cell damage in diabetes
(Hessler et al., 1983). The cellular antioxidant status determines the susceptibility to oxidative damage and is usually altered in response to oxidative stress. Reduced antioxidant levels as a result of increased free radical production in experimental diabetes have been reported by many authors (Grankvist et al., 1981). We observed elevated levels of thiobarbituric acid reactive substances and hydroperoxides in plasma of STZ-induced diabetic rats.

A marked increase in the concentration of plasma TBARS indicates enhanced lipid peroxidation leading to tissue injury and failure of the antioxidant defense mechanism to prevent the formation of excess free radicals. Increased lipid peroxidation impairs membrane function by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptors. The products of lipid peroxidation are harmful to most cells in the body and are associated with a variety of diseases, such as atherosclerosis and brain damage (Acworth et al., 1997). In our study, significant increased levels of TBARS were observed in diabetic rats. Similarly Rajasekaran et al., (2005) have also reported increased lipid peroxide levels in diabetic rats.

Oral administration of γ- sitosterol and glibenclamide to diabetic rats for 21 days significantly decreased the plasma TBARS and hydroperoxides to near normal levels, which could be the result of improved antioxidant status.

3.7.1.8. Effect of γ- sitosterol on plasma and tissue non-enzymatic antioxidants levels

Vitamin C is a potent non-enzymatic antioxidant; it acts on oxygen free radicals and interacts with vitamin E (Garg and Bansal, 2000). In our study, vitamin E was increased in diabetic rats which could be due to increased membrane damage by ROS. Treatment with γ-sitosterol (20 mg/kg /b.wt) brought vitamin E to near normal levels which could be as a result of
decreased membrane damage as evidenced by decreased lipid peroxidation. Both Vitamin C and E are interrelated by recycling process (Packer et al., 1997). Recycling of tocopheroxyl radicals to tocopherol is achieved with vitamin C (Freisleben and Packer, 1993). In our study, vitamin C was decreased in diabetic rats. This might be due to increased utilization of vitamin C as an antioxidant defence against increased ROS or to a decrease in the GSH level, since GSH is required for the recycling of vitamin C (Wefers and Sies, 1988). Treatment with γ- sitosterol (20 mg/kg /b.wt) for 21 days brought back the level of vitamin C to near normal.

GSH is a major non-protein thiol in living organisms; this plays a central role in coordinating the body's antioxidant defense process. GSH is the first line of defense against prooxidant status (Ahmed et al., 2000). The elevated level of GSH protects cellular proteins against oxidation through glutathione redox cycle and also directly detoxifies ROS (Yu, 1994). STZ directly generates oxygen free radicals which induce lipid peroxidation (Spinias, 1999; Bassirat and Khalil, 2000). We observed a decrease in GSH in the liver and kidney of diabetic rats. Depletion of GSH levels enhances cellular damage caused by oxidative stress. Significant depletion of GSH in diabetic rats suggests its increased utilization against reactive oxygen species (Shanmugam et al., 2011). Oral administration of γ-sitosterol (20 mg/kg/b.wt) increased the level of GSH in the liver and kidney of diabetic rats.

3.7.1.9. Effect of γ- sitosterol on plasma and tissue enzymatic antioxidants levels

Enzymatic antioxidants such as SOD, CAT, GPx and GST play an important role in preventing cells from being exposed to oxidative damage (Sathishsekhar and Subramanian, 2005). In diabetes mellitus, high glucose can simply inactivate antioxidant enzymes SOD, CAT, GPx, etc. by glycating these proteins and hyperglycemia induces oxidative stress which in turn causes
lipid peroxidation (Yang et al., 2010). A decrease in the activity of these antioxidants can lead to an excess availability of superoxide anion \( \text{O}_2^- \) and hydrogen peroxide in biological systems, which in turn generate hydroxyl radicals, resulting in initiation and propagation of lipid peroxidation (Kumuhekar and Katyane, 1992). The increased activities of SOD and CAT in \( \gamma \)-sitosterol (20 mg/kgb.wt) treated diabetic rats suggested that this compound possessed free radical scavenging activity, which could exert a beneficial effect against pathological alterations caused by the presence of \( \text{O}_2^- \) and \( \text{OH}^- \). This confirmed that \( \gamma \)-sitosterol could help to scavenge \( \text{O}_2^- \), inhibit the generation of \( \text{OH}^- \), and reduce lipid peroxidation. This action could involve mechanisms related to antioxidant effect of \( \gamma \)-sitosterol on type 2 diabetes. The depletion of GSH, GPx and GST promotes generation of ROS and oxidative stress with cascade of effects thereby affecting functional as well as structural integrity of cell and organelle membranes (Raza et al., 2000). Oral administration of \( \gamma \)-sitosterol for 21 days increased the activities of GPx and GST in the liver and kidney of diabetic rats.

3.7.1.10. **Effect of \( \gamma \)-sitosterol and plasma and tissue glycoproteins levels.**

Glycoproteins are rich in extracellular matrix and they contribute a major source to the structure of the matrix (Wiese et al., 1997). These groups of macromolecules carry out numerous biological functions including lipid and hormone transport, hemoglobin binding, and blood coagulation. In diabetic state, abnormalities of glycoproteins metabolism is commonly observed. The increase in plasma glycoprotein components has been associated with the severity of diabetes. In this study, we have observed the increased levels of hexose, hexosamine, fucose and sialic acid in the plasma of streptozotocin induced diabetic rats. The secretion or shedding from cell membrane glycoconjugates into the circulation leads to the elevation of plasma glycoprotein components. STZ-induced diabetic rats exhibited a significant modification in the connective
tissue macromolecule. This is due to the depressed utilization of glucose by insulin-dependent pathways which leads to increase in the formation of hexose, hexosamine, sialic acid and fucose for the accumulation of glycoproteins (Saravanan et al., 2010). Impaired metabolism of glycoproteins plays a major role in the pathogenesis of diabetes mellitus (Begum et al., 1978). It has been reported that alterations occur in the concentrations of various membrane-bound carbohydrates (glycoproteins) in diabetes (Sharma et al., 1987). Raised levels of glycoprotein in diabetics may also be a predictor of angiopathic complications (Konukoglu et al., 1999). In recent times, many traditional medicinal plants have been tested for their efficacy against impaired glycoprotein levels in diabetes (Latha and Pari, 2004).

Sialic acid is a terminal component of the oligosaccharide side chains of glycoproteins and glycolipids, which are essential constituents of many hormones and enzymes present in serum and tissues. Serum sialic acid is almost completely bound to glycoproteins and lipids. The observation of serum sialic acid could be helpful since high concentration of serum sialic acid has been shown to be an important risk factor in the development of atherosclerosis and cardiovascular complications (Rahmana et al., 2009). Diabetic rats had increased level of sialic acid in the serum and liver tissues (Gavella et al., 2003) and reduced glomerular sialic acid in experimental rats (Cardenas et al., 1991) which resembles the present study. The elevated level of sialic acid in serum and liver in diabetic rats might be due to either enhanced sialic acid synthesis or sialidase activity. An increased activity of sialidase, an enzyme which catalyses the removal of sialic acid residues from sialoconjugates, might be responsible for the depletion of glomerular sialic acid (Barigos et al., 1986). Decrease in kidney sialic acid in diabetic rats may be due to its increased use for the synthesis of fibronectin. Fibronectins are large glycoproteins found in plasma, in extracellular matrix, and on cell surfaces. They promote cell-cell and cell-
matrix interactions and thus play a role in tissue construction and reconstruction (Hynes, 1986). The synthesis of fibronectin, which contains sialic acid residues in the core structure, was reported to increase significantly in various tissues of diabetic patients and animals (Schiller and Dorfman, 1957). Treatment with γ-sitosterol and glibenclamide had reversed the level of sialic acid towards normalcy in diabetic rats, which could be due to the regulation of sialidase activity by insulin through potentiation release from β-cells of the islets of Langerhan’s which might enhance glucose utilization (Saravanan et al., 2010) since insulin is more like mediator of sialic acid changes than any other alterations in plasma glucose levels (Pickup et al., 1995).

One pathway through which glucose is sensed sub acutely is hexosamine synthesis. It functions as physiologic glucose sensors that serve as an adaptive in diverting excess calories toward storage as fat (McClain, 1996). The amination of fructose 6-phosphate to glucosamine 6-phosphate is rate limiting and is catalysed by glutamine: fructose 6-phosphate aminotransferase (GFAT) (Marshall et al., 1991). Studies indicated that GFAT is a key regulatory enzyme in the hexosamine biosynthetic pathway, which plays an important role in regulation/development of diabetic vascular complications such as diabetic nephropathy (Nandini et al., 2003). Further, the hexosamine content of serum appears to be the most sensitive indication of the onset of complication in diabetes (Andreani and Gry, 1956).

In accordance with previous report (Ramesh et al., 2006), in our study, diabetic rats had elevated level of hexosamines which could be due to increased expression of GFAT and increased plasma glucose, and treatment with γ- sitosterol and glibenclamide had brought hexosamine towards normalcy which could be due to improved glycemic control, mediated via an increased insulin secretion.
Fucose (d-deoxy-L-galactose) is a characteristic constituent of many glycoproteins (Winzler, 1960) and is a mobile component of plasma glycoproteins of particular physiological and pathological significance (Dische, 1948). Alterations of enzymatic glycosylation processes, L-fucose metabolism and the fucosylation of acute phase serum proteins are detected in diabetes (McMillian, 1972). Further the level of fucose has been elevated in diabetic patients and animals (Radhakrishnamurthy et al., 1976; Ramesh et al., 2006).

In our study, diabetic rats had elevated level of fucose which could be due to elevated blood glucose level, which is in line with previous report (Sharma et al., 1987). Treatment with γ-sitosterol and glibenclamide had reversed fucose towards normalcy which could be due to improved glycemic control.

Protein bound hexoses in the cell membrane provide hydrophobic areas, whereas protein bound hexosamine provides cationic charges on the cell membrane surface and make the membrane more polar. The elevated level of hexoses in diabetic rats may be associated with disturbances with carbohydrate metabolism. Treatment with γ-sitosterol and glibenclamide had brought hexoses towards normalcy which could be due to improved glycemic control. Thus, our results have shown that treatment with γ-sitosterol reversed the levels of circulatory and tissue sialic acid, fucose, hexoses and hexosamine in STZ-diabetic rats which reflects the protective effect of γ-sitosterol from the risk of diabetic complications.

Oral administration with γ-sitosterol and glibenclamide to diabetic rats significantly reversed the changes of glycoproteins levels to near normal. The antihyperglycemic action of β-sitosterol, which medicated via an enhancement of insulin action, as evidenced by the increased
level of insulin in treated diabetic rats, may be responsible for the reversal of glycoprotein changes associated with diabetes.

3.7.1.11. Effect of γ-sitosterol on serum amino transferase levels

The increase in the activities of serum AST, ALT, ALP and ACP indicated that diabetes induced hepatic dysfunction (Zheng et al., 2011). An increase in these enzyme activities reflects active liver damage. Inflammatory hepatocellular disorders result in extremely elevated transaminase levels. The liver is necrotized in STZ-induced diabetic rats resulting in increased activities of serum enzymes including AST, ALT, ALP and ACP in plasma mainly due to the leakage of these enzymes from the liver cytosol into the blood stream (Balamurugan et al., 2011). On the other hand, treatment of the diabetic rats with γ-sitosterol caused reduction in the activity of these enzymes in plasma compared to the mean values of the diabetic group and this is in agreement with that of Eidia et al., (2006). The decreased hepatic marker level is an indication of the protective effect on liver (Kesari et al., 2007).

3.7.1.12. Effect of γ- sitosterol on nephritic markers (Urea, Uric acid, creatinine and albumin)

The diabetic hyperglycemia induces the elevation of plasma levels of urea, uric acid and creatinine, which are considered as the significant markers of renal dysfunction (Singh et al., 2012) which includes symptoms like polydipsia, polyuria and weight loss (ADA, 2005. Urea is the major nitrogen containing metabolic product of protein metabolism; uric acid is the major product of purine nucleotides, adenosine and guanosine; creatinine is endogenously produced and released into body fluids and its clearance measured as an indicator of glomerular filtration rate (Burtis and Ashwood, 1996). Increased levels of urea, uric acid and creatinine in diabetes mellitus causes renal damage due to abnormal glucose regulation, including elevated glucose and
glycosylated protein tissue levels, haemodynamic changes within the kidney tissue, and increased oxidative stress. The STZ-induced diabetic rats exhibited significantly higher urea, uric acid and creatinine levels (Kaleem et al., 2008).

Our data showed that uric acid levels were increased in diabetic rats. This may be due to metabolic disturbance in diabetes reflected in high activities of xanthine oxidase, lipid peroxidation and increased triglycerides and cholesterol (Madinov et al., 2000; Anwar and Meki, 2003). Moreover, protein glycation in diabetes may lead to muscle wasting and increased release of purine, the main source of uric acid as well as in activity of xanthine oxidase (Anwar and Meki, 2003). Elevation of the serum urea and creatinine, as significant markers, are related to renal dysfunction in diabetic hyperglycemia (Almadal and Vilstrup, 1988), and this is in agreement with the present result. Treatment with γ-sitosterol reversed these parameters to near normal level which could be due to decreased metabolic disturbances of other pathways such as protein and nucleic acid metabolisms as γ-sitosterol improved glycemic control.

3.7.1.13. Insulin secretion

To understand the mechanism of insulin release by γ-sitosterol a study was performed by incubating pancreatic islets with diazoxide, a potent K+ ATP channel opener and a known inhibitor of insulin release. γ-Sitosterol was able to partially reverse the inhibitory effect of diazoxide on glucose-induced insulin release at 10 min and 60 min. This suggested that γ-sitosterol afforded some protection to β-cells. When islets were incubated with EGTA (chelator of exogenous Ca2+) and nimodipine (inhibitor of Ca2+ influx) along with glucose there was no change in insulin release. This implies that γ-sitosterol does not require Ca2+ influx (Hisatomi et al., 1996; Komatsu et al., 1998). Since diazoxide is a potent K+ ATP channel opener, it stimulates the ROS production by opening of mitochondrial K+ ATP channel (Nagy et al.,
We think that \( \gamma \)-sitosterol works by the principle of closing the K+ ATP channel and releasing insulin from the islets of the pancreas. Hence \( \gamma \)-sitosterol may have antioxidant property. Moreover, Gupta et al. (2011) also reported the antioxidant property of \( \beta \)-sitosterol.

3.7.1.14. Effect of \( \gamma \)-sitosterol on histological parameters

3.7.1.15. Effect of \( \gamma \)-sitosterol on pancreatic islet of \( \beta \)-cells:

3.7.1.16. Light microscopic studies

In the type II diabetes mellitus, there is a gradual decrease in beta cell function and mass, induced by STZ. To prevent the loss of \( \beta \)-cell function and mass, \( \beta \)-cell must be stabilized/regenerated. This \( \beta \)-cell regeneration has been reported by several workers.

In STZ-diabetic rats, the islet is considerably reduced and shrunken and there is destruction of some \( \beta \)-cells with central hyalinization; a few cells showed pyknotic nuclei and the number of cells was decreased (Bora et al., 1985, 1989; Shanmugasundaram et al., 1981; Kavalali et al., 2003). Cardinal et al., (1998) reported that STZ affected cells displayed a variety of changes in their nucleus viz., nuclear membrane break down, cell membrane swelling and rupture, swollen mitochondria and endoplasmic reticulum (Aughsteen, 2000; Degrirmenci et al., 2005).

In the present study, the histology demonstrated that most of the islets were affected and showed observable changes in structures. The \( \beta \)-cells showed degranulation and swelling of the intracellular organelles. All these vital intracellular structures were affected inhibiting the synthesis and release of insulin. Oral administration of \( \gamma \)-sitosterol for 21 days effectively restored the pathological changes in STZ induced diabetic rat pancreatic tissues. In this context, treatment of STZ-induced diabetic animals with (−)-epicatechin and N-acetyl-l-cysteine (NAC)
well-known terpenoid, prevented hyperglycemia through reduced oxidative stress and restored β-cell function (Sheehan and Zemaitis, 1983). Shanmugasundaram et al., (1990) reported that sections of pancreatic islets of Gymnema sylvestre leaf extract-treated diabetic rats showed ability to regenerate the damaged endocrine tissue and increase β-cells numbers partially. Nagappa et al., (2003) showed the regeneration of beta cells in the pancreas of Terminalia catappa fruit extract-treated diabetic rats, due to β-carotene which is a constituent of Terminalia catappa fruit. Sharma et al., (2003) have reported that oral administration of Eugenia jambolana seed extract reversed the abnormalities in the islet of Langerhans of alloxan-induced diabetic rabbits.

3.7.1.17. Immunohistopathological study

Cytochemistry is the visualization of chemical compounds in tissues sections by staining. This predominant qualitative discipline has gradually developed into a quite sophisticated science employing a variety of micro-assays for the analysis of isolated tissue elements and tissue sections (Sternberger, 1986). Cytochemistry in this modern sense is indispensable in the exploration of complex tissues composed of a variety of cells (Gossner et al., 1963). The endocrine pancreas is such a tissue and it is the contribution of cytochemists that has enabled us to understand its specific function. Application of reliable and specific immunocytochemical techniques has allowed clear identification of the major islet cell type; insulin containing β-cells, glucagon containing β-cells and somatostain secreting β-cells (El-Nagger et al., 1993; Rebecca et al., 2004; Koyuturk et al., 2005).

Immunohistopathological staining of the islets of control rats revealed that the islets were well granulated, and the insulin positive β-cells from the majority of the islets cells were located
at the center (Rebecca et al., 2004). Islets from the diabetic animals showed lack of insulin response with degenerated β-cells (Sasaki et al., 1991; Higdon et al., 2001).

In the present investigation also, islets from untreated rats showed well granulated β-cells with majority of the β-cells being positive. Diabetic rats did not show any insulin-positive cells in the islets. Oral administration of γ-sitosterol to STZ induced diabetic rats showed several positive β-cells in the islets of Langerhans. This resulted in the preservation of β-cell mass and insulin secretory granules, which further potentiated the efficiency of γ-sitosterol. Thus apparently normal architecture of β-cells, as evident through electron microscopy, may be attributed to the residual β-cell mass restored by γ-sitosterol that may secrete insulin and alleviate mediated complications.

3.7.1.18. Possible mode of action of the γ-sitosterol isolated from L. nodiflora (whole plant)

γ-sitosterol appears to restore the damaged endocrine tissue and thereby stimulate the secretion of insulin in β-cells as revealed by insulin assay, and light microscopy. γ-sitosterol is a sterol; the hypoglycemic effect of sterol is similar to sulfonylurea. Charantin is a sterol which stimulates the release of insulin and blocks the formation of glucose in the bloodstream, which may be helpful in the treatment of diabetes, particularly in non-insulin dependent diabetes (Ng et al., 1986). The biochemical mechanism of action of the active principle seems to be through restoration of the damaged β-cells and thereby increasing the plasma insulin level.

The increased insulin in the plasma binds to a specific cell-surface receptor composed of two α-subunits and two β-subunits. Insulin binding to α-subunits activates the receptor tyrosine kinase leading to auto phosphorylation of tyrosine residues in several regions of the intracellular β-subunit. The phosphorylation of β-subunits is associated with increased tyrosine kinase activity
towards intracellular substrates, such as the insulin receptor substrate (IRS)-Proteins and PKc. These proteins act as ‘docking proteins’ and transmit the insulin signaling through protein-protein association and phosphorylation. Glucose transport into skeletal muscle is the first rate-limiting step for glucose utilization under physiological conditions.

The metabolic alterations found in the diabetic rats due to insulin deficiency were rectified. The increase in insulin secretion, decrease in plasma glucose and glycosylated hemoglobin, replenishment of glycogen in liver and increase in the activity of glycogen synthase and hexokinase, decrease in the activity of glucose-6-phosphatase, fructose 1, 6-bisphosphatase, decrease in total cholesterol and triglycerides, increase in HDL-cholesterol and decrease in urea and creatinine were observed. The oxidative stress was decreased due to the activation of antioxidant levels. The active principle γ- sitosterol did not cause any harmful effect on liver and kidney as evident from the biochemical studies. The acinar portion of the pancreas was also not affected as evident from the histological studies.

3.7.2. CONCLUSION

Our studies revealed that γ- sitosterol isolated from *L. nodiflora* can be considered as an important addition to the therapeutic armamentarium for the treatment of diabetes. The results of this study indicate that γ- sitosterol exhibited significant activity in decreasing the serum glucose level in STZ-induced diabetic rats. γ- sitosterol also showed improvement in lipid profile, plasma insulin, total haemoglobin, glycosylated haemoglobin, nephritic marker, liver glycogen, liver enzymes and carbohydrate metabolic enzyme in serum, liver and kidney in experimental diabetic rats. Histopathological and immunohistopathological studies revealed regeneration of β-cells of pancreas in γ- sitosterol treated diabetic rats. This confirmed the hypoglycemic potential of γ-sitosterol by stimulating the secretion of insulin in β-cells. The results were comparable with the
reference drug glibenclamide. From the above findings it is clear that \(\gamma\)-sitosterol possesses significant antioxidant, hepatoprotective, antilipidemic and glucose lowering effects.
REFERENCES


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Roe J M, Kuether C A. 1943. The determination of ascorbic acid in whole blood and urine through the 2, 4-dinitrophenylhydrazine derivate of dehydroascorbic acid. Journal of Biological Chemistry 147, 399-407.


induced by high fat diet and low dose STZ. Journal of Ethnopharmacology 137, 662–668.
Table 1: Effect of GTT of $\gamma$-sitosterol on blood glucose levels in diabetic rats using GTT.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Plasma glucose level (mg/dl)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (Min)</td>
<td>60 (Min)</td>
<td>120 (Min)</td>
<td>180 (Min)</td>
</tr>
<tr>
<td>Normal control</td>
<td>73.87 ± 2.25</td>
<td>79.96 ± 2.26</td>
<td>78.77 ± 2.07</td>
<td>77.84±1.97</td>
</tr>
<tr>
<td>Normal + $\gamma$-sitosterol 20 mg/kg/b.wt</td>
<td>77.08 ± 2.15$^b$</td>
<td>79.36 ± 1.86$^b$</td>
<td>76.90 ± 1.88$^b$</td>
<td>78.85±1.36$^b$</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>178.61 ± 2.79$^a$</td>
<td>361.79 ± 5.39$^a$</td>
<td>334.03 ± 2.86$^a$</td>
<td>307.53±5.04$^a$</td>
</tr>
<tr>
<td>Diabetic + $\gamma$-sitosterol 5 mg/kg/b.wt</td>
<td>181.63 ± 1.27$^a$</td>
<td>336.63 ± 2.07$^a$</td>
<td>288.10±1.28$^{ab}$</td>
<td>265.24 ±1.24$^b$</td>
</tr>
<tr>
<td>Diabetic + $\gamma$-sitosterol 10 mg/kg/b.wt</td>
<td>181.89 ± 1.27$^a$</td>
<td>309.60 ± 1.63$^{ab}$</td>
<td>269.38±1.26$^{ab}$</td>
<td>244.81±1.062$^{ab}$</td>
</tr>
<tr>
<td>Diabetic + $\gamma$-sitosterol 20 mg/kg/b.wt</td>
<td>181.00 ± 2.49$^a$</td>
<td>301.83 ± 3.11$^{ab}$</td>
<td>236.96±1.88$^{ab}$</td>
<td>168.90±1.17$^{ab}$</td>
</tr>
<tr>
<td>Diabetic + glibenclamide 600µg/kg/b.wt</td>
<td>178.32 ± 2.54$^a$</td>
<td>230.18 ± 1.12$^{ab}$</td>
<td>240.22±1.93$^{ab}$</td>
<td>182.29±1.36$^{ab}$</td>
</tr>
</tbody>
</table>

All values are (mg/dl) mean ± S.E.M for six animals; $^a$ values deviate significantly from diabetic control group by comparison with normal rats ($P \leq 0.05$); $^b$ values deviate very significantly from diabetic control groups.
Table 2: Effect of γ-sitosterol on body weight and food intake in STZ-induced diabetic rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight</th>
<th>Food intake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 day(g)</td>
<td>21st day(g)</td>
</tr>
<tr>
<td>Normal control</td>
<td>186.25 ± 8.98</td>
<td>203.75 ± 7.46</td>
</tr>
<tr>
<td>Normal + γ-sitosterol 20 mg/kg/b.wt</td>
<td>187.50 ± 7.77</td>
<td>203.75 ± 6.88 &lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>diabetic control</td>
<td>202.50 ± 12.99</td>
<td>148.75 ± 3.14 &lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + γ-sitosterol 20 mg/kg/b.wt</td>
<td>196.25 ± 4.26</td>
<td>210.00 ± 5.40 &lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide 600µ g/kg/b.wt</td>
<td>186.25 ± 3.721</td>
<td>215.00 ± 6.45 &lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M for six animals; <sup>a</sup>- values deviate significantly from diabetic control group (P≤0.05); <sup>b</sup>- values deviate very significantly from diabetic control.
Table 3: Effect of γ-sitosterol on Fasting plasma glucose level in STZ-induced diabetic rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Plasma glucose level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 day</td>
</tr>
<tr>
<td>Normal control</td>
<td>90.10 ± 3.80</td>
</tr>
<tr>
<td>Normal + γ-sitosterol 20 mg/kg/b.wt</td>
<td>92.15 ± 1.22b</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>370.67 ± 7.15a</td>
</tr>
<tr>
<td>Diabetic + γ-sitosterol 20 mg/kg/b.wt</td>
<td>343.28 ± 5.13a</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide 600µ g/kg/b.wt</td>
<td>356.86 ± 22.64a</td>
</tr>
</tbody>
</table>

All values are (mg/dl) mean ± S.E.M for six animals;

a values deviate significantly from diabetic control group by comparison with normal rats (P≤0.05); b values deviate very significantly from diabetic control groups.
Table 4: Effect of γ-sitosterol on plasma insulin and glycosylated hemoglobin in STZ-induced diabetic rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Plasma insulin (U/ml)</th>
<th>Haemoglobin (mg/dl)</th>
<th>Glycosylated haemoglobin (mg/g of Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>19.99 ± 1.27</td>
<td>12.85 ± 0.30</td>
<td>0.47 ± 0.006</td>
</tr>
<tr>
<td>Normal + γ-sitosterol 20 mg/kg/b.wt</td>
<td>19.95 ± 1.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.15 ± 0.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.45 ± 0.004&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>diabetic control</td>
<td>4.23 ± 0.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.90 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.94 ± 0.009&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + γ-sitosterol 20 mg/kg/b.wt</td>
<td>13.36 ± 1.08&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>14.26 ± 0.70&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.56 ± 0.011&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide 600µ g/kg/b.wt</td>
<td>14.52 ± 1.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.67 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.51 ± 0.006&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M for six animals;

<sup>a</sup> values deviate significantly from diabetic control group by comparison with normal rats (P≤0.05),
<sup>b</sup> values deviate very significantly from diabetic control groups.
Table 5: Effect of γ-sitosterol on liver and muscle glycogen content in STZ-induced diabetic rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Glycogen content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver glycogen (mg/100 mg wet weight)</td>
</tr>
<tr>
<td>Normal control</td>
<td>46.94 ± 0.25</td>
</tr>
<tr>
<td>Normal + γ-sitosterol 20 mg/kg/b.wt</td>
<td>45.98 ± 1.26&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>10.02 ± 0.55&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + γ-sitosterol 20 mg/kg/b.wt</td>
<td>43.86 ± 2.57&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide 600µ g/kg/b.wt</td>
<td>46.37 ± 1.25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M for six animals;

<sup>a</sup> values deviate significantly from diabetic control group by comparison with normal rats (P≤0.05);<sup>b</sup> values deviate very significantly from diabetic control groups.
<table>
<thead>
<tr>
<th>Group</th>
<th>Hexokinase (mg/dl)</th>
<th>Fructose 1,6-bisphosphatase (mg/dl)</th>
<th>Glucose 6 phosphatase (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>0.14±0.010</td>
<td>0.28±0.006</td>
<td>0.22±0.011</td>
</tr>
<tr>
<td>Normal + γ-sitosterol 20 mg/kg/b.wt</td>
<td>0.17±0.013</td>
<td>0.30±0.006</td>
<td>0.25±0.012</td>
</tr>
<tr>
<td>diabetic control</td>
<td>0.06±0.006</td>
<td>0.95±0.017</td>
<td>0.56±0.011</td>
</tr>
<tr>
<td>Diabetic + γ-sitosterol 20 mg/kg/b.wt</td>
<td>0.20±0.200</td>
<td>0.39±0.008</td>
<td>0.28±0.011</td>
</tr>
<tr>
<td>Diabetic +Glibenclamide 600µ g/kg/b.wt</td>
<td>0.16±0.010</td>
<td>0.46±0.009</td>
<td>0.25±0.008</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M for six animals;

a values deviate significantly from diabetic control group by comparison with normal rats (P≤0.05); b values deviate very significantly from diabetic control groups.
Table 7: Effect of γ-sitosterol on tissue carbohydrate metabolizing enzyme level in control and STZ-induced diabetic rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Tissue carbohydrate metabolizing enzyme level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hexokinase (m moles of Pi liberated/min/mg protein)</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>Normal control</td>
<td>126.15±0.62</td>
</tr>
<tr>
<td>Normal + γ-sitosterol 20 mg/kg/b.wt</td>
<td>123.81±1.17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>51.47±3.93&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + γ-sitosterol 20 mg/kg/b.wt</td>
<td>121.98±1.29&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide 600µg/kg/b.wt</td>
<td>112.10±1.47&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M for six animals;<sup>a</sup> values deviate significantly from diabetic control group by comparison with normal rats (P≤0.05);<sup>b</sup> values deviate very significantly from diabetic control groups.
Table 8: Effect of $\gamma$-sitosterol on liver glycogen metabolizing enzyme level in control and STZ-induced diabetic rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Glycogen synthase (µmoles of UDP formed/h/mg protein)</th>
<th>Glycogen phosphorylase (µmole of Pi liberated/h/mg protein)</th>
<th>Glucose 6 phosphate dehydrogenase (nmol NADP+ reduced/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>866.92±6.08</td>
<td>636.10±6.07</td>
<td>18.99±0.72</td>
</tr>
<tr>
<td>Normal + $\gamma$-sitosterol 20 mg/kg/b.wt</td>
<td>854.87±7.03$^b$</td>
<td>633.22±7.74$^b$</td>
<td>17.56$^b$±0.59</td>
</tr>
<tr>
<td>diabetic control</td>
<td>531.82±10.00$^a$</td>
<td>843.61±10.94$^a$</td>
<td>13.51$^a$±0.40</td>
</tr>
<tr>
<td>Diabetic + $\gamma$-sitosterol 20 mg/kg/b.wt</td>
<td>826.72±8.99$^{ab}$</td>
<td>657.91 ± 7.65$^b$</td>
<td>16.29$^b$±0.44</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide 600µg/kg/b.wt</td>
<td>821.24±13.20$^b$</td>
<td>632.74 ± 8.54$^b$</td>
<td>15.93$^b$±0.53</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M for six animals;

$^a$ values deviate significantly from diabetic control group by comparison with normal rats (P≤0.05); $^b$ values deviate very significantly from diabetic control groups.
Table 9: Effect of γ-sitosterol on lipid profile in STZ-induced diabetic rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum lipid profile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total cholesterol</td>
</tr>
<tr>
<td>Normal control</td>
<td>50.10 ± 2.00</td>
</tr>
<tr>
<td>Normal + γ-sitosterol 20 mg/kg/b.wt</td>
<td>52.45 ± 2.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>231.03 ± 5.17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + γ-sitosterol 20 mg/kg/b.wt</td>
<td>117.66 ± 9.16&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide 600µg/kg/b.wt</td>
<td>129.63 ± 3.94&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are mean (mg/dl) ± S.E.M for six animals;

<sup>a</sup> values deviate significantly from diabetic control group by comparison with normal rats (P≤0.05);
<sup>b</sup> values deviate very significantly from diabetic control groups
Table 10: Effect of γ-sitosterol on Serum lipid profile in STZ-induced diabetic rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum lipid profile</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LDL-Cholesterol</td>
<td>HDL-Cholesterol</td>
<td>VLDL-Cholesterol</td>
</tr>
<tr>
<td>Normal control</td>
<td>21.2550± .48371</td>
<td>51.38 ± 3.34</td>
<td>10.54 ± 0.34</td>
</tr>
<tr>
<td>Normal + γ-sitosterol 20 mg/kg/b wt</td>
<td>22.7200± .98044b</td>
<td>53.63 ± 1.25b</td>
<td>10.17 ± 0.23b</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>77.4725± 1.79533a</td>
<td>29.35 ± 2.14a</td>
<td>40.90 ± 0.34a</td>
</tr>
<tr>
<td>Diabetic + γ-sitosterol 20 mg/kg/b wt</td>
<td>41.4025± 1.22247ab</td>
<td>49.57 ± 2.67b</td>
<td>27.62 ± 0.18ab</td>
</tr>
<tr>
<td>Diabetic +Glibenclamide 600µ g/kg/b.wt</td>
<td>40.6500± 1.03977ab</td>
<td>49.57 ± 2.54b</td>
<td>28.34 ± 0.23ab</td>
</tr>
</tbody>
</table>

All values are mean (mg/dl) ± S.E.M for six animals;

^a values deviate significantly from diabetic control group by comparison with normal rats (P≤0.05); ^b values deviate very significantly from diabetic control groups
Table 11: Effect of γ-sitosterol on tissue lipid profile in normal and STZ-induced diabetic rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Tissue lipid profile</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TC</td>
<td>TG</td>
<td>Free fatty acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(mg/g wet tissue)</td>
<td>(mg/g wet tissue)</td>
<td>(mg/g wet tissue)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>Kidney</td>
<td>Liver</td>
<td>Kidney</td>
<td>Liver</td>
<td>Kidney</td>
</tr>
<tr>
<td>Normal control</td>
<td>7.19±0.13</td>
<td>6.34±0.21</td>
<td>5.83±0.21</td>
<td>4.81±0.42</td>
<td>8.48±0.15</td>
</tr>
<tr>
<td>Normal + γ-sitosterol 20 mg/kg/b.wt</td>
<td>7.57±0.21</td>
<td>6.47±0.45</td>
<td>5.66±0.20</td>
<td>4.75±0.27</td>
<td>8.12±0.11</td>
</tr>
<tr>
<td>diabetic control</td>
<td>15.72±0.83</td>
<td>13.33±0.63</td>
<td>15.61±0.91</td>
<td>8.11±0.15</td>
<td>26.36±2.09</td>
</tr>
<tr>
<td>Diabetic + γ-sitosterol 20 mg/kg/b.wt</td>
<td>6.92±0.32</td>
<td>6.05±0.11</td>
<td>5.52±0.16</td>
<td>4.05±0.20</td>
<td>8.97±0.18</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide 600µg/kg/b.wt</td>
<td>7.01±0.41</td>
<td>6.80±0.27</td>
<td>6.34±0.25</td>
<td>4.50±0.35</td>
<td>11.24±1.08</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M for six animals;

^a values deviate significantly from diabetic control group by comparison with normal rats (P≤0.05); ^b values deviate very significantly from diabetic control groups
Table 12: Effect of γ-sitosterol on tissue TBARS in normal and streptozotocin-induced diabetic male Wistar rats after 21 days.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum (nmol/mL)</th>
<th>Liver (nM/100 g tissue)</th>
<th>Kidney (nM/100 g tissue)</th>
<th>Serum (Values x105 Mm /DL)</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>0.20±0.007</td>
<td>0.64±0.02</td>
<td>1.43±0.01</td>
<td>8.4±0.21</td>
<td>66.60±1.37</td>
<td>54.50±1.11</td>
</tr>
<tr>
<td>Normal + γ-sitosterol 20 mg/kg/b.wt</td>
<td>0.19±0.005(^b)</td>
<td>0.62±0.02(^b)</td>
<td>1.37±0.01(^b)</td>
<td>8.9±0.29(^b)</td>
<td>66.66±1.69</td>
<td>55.61±1.65 (^b)</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>0.37±0.022(^a)</td>
<td>1.55±0.01(^a)</td>
<td>4.67±0.46(^a)</td>
<td>27.96±1.27(^a)</td>
<td>124.56±2.93</td>
<td>80.26±1.60 (^a)</td>
</tr>
<tr>
<td>Diabetic + γ-sitosterol 20 mg/kg/b.wt</td>
<td>0.21±0.005(^b)</td>
<td>0.75±0.01(^b)</td>
<td>1.41±0.007(^b)</td>
<td>12.16±0.16(^ab)</td>
<td>71.13±1.25</td>
<td>54.78±1.52 (^b)</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide 600 µg/kg/b.wt</td>
<td>0.20±0.004(^b)</td>
<td>0.70±0.01(^ab)</td>
<td>1.76±0.11(^ab)</td>
<td>10.27±0.21(^b)</td>
<td>72.30±1.35</td>
<td>56.06±1.90 (^b)</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M for six animals; \(^a\) values deviate significantly from diabetic control group by comparison with normal rats (P≤0.05); \(^b\) values deviate very significantly from diabetic control groups.
Table 13: Effect of γ-sitosterol on serum non-enzymatic antioxidants in normal and streptozotocin-induced diabetic male Wistar rat after 21 days

<table>
<thead>
<tr>
<th>Groups</th>
<th>serum non enzymatic antioxidant levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vitamin –C (mg/dL)</td>
</tr>
<tr>
<td>Normal control</td>
<td>1.87 ± 0.03</td>
</tr>
<tr>
<td>Normal + γ-sitosterol 20 mg/kg/b.wt</td>
<td>1.82 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>diabetic control</td>
<td>0.77 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + γ-sitosterol 20 mg/kg/b.wt</td>
<td>1.71 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide 600µ g/kg/b.wt</td>
<td>1.75 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M for six animals;
<sup>a</sup> values deviate significantly from diabetic control group by comparison with normal rats (P≤0.05);
<sup>b</sup> values deviate very significantly from diabetic control groups.
Table 14: Effect of γ-sitosterol on non-enzymatic antioxidants in normal and streptozotocin-induced diabetic male Wister rat tissues (Liver and kidney) after 21 days.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Normal control</th>
<th>Normal + γ-sitosterol 20 mg/kg/b.wt</th>
<th>Diabetic control</th>
<th>Diabetic + γ-sitosterol 20 mg/kg/b.wt</th>
<th>Diabetic + Glibenclamide 600 µg/kg/b.wt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vitamin - C (mg/100 mg tissue)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>1.92± 0.01</td>
<td>1.87± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.72 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.71± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.61± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.16± 0.01</td>
<td>1.72± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.51± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.14± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.11± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Vitamin- E (mg/100 mg tissue)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.63 ± 0.01</td>
<td>0.65 ± 0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.07± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.65 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.66± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.51 ± 0.04</td>
<td>0.47± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.8 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.4± 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.52± 0.00&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>GSH (nM of DTNB conjugated/mg protein)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>131.20 ±1.78</td>
<td>131.72 ±1.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>74.61 ± 1.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>129.10 ± 1.54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>130.94 ± 1.30&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kidney</td>
<td>124.36 ±1.53</td>
<td>118.97 ±1.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.21 ± 1.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>101.39 ± 3.13&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>104.55 ± 2.17&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M for six animals; 
<sup>a</sup> values deviate significantly from diabetic control group by comparison with normal rats (P≤0.05); <sup>b</sup> values deviate very significantly from diabetic control groups.
Table 15: Effect of γ-sitosterol on tissue Catalase and Glutathione s transferase in normal and streptozotocin-induced diabetic male Wistar rats after 21 days of treatment.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Catalase (mol of H2O2 consumed min(^{-1}) (mg protein)(^{-1}))</th>
<th>Glutathione s transferase (GST) (mol of CDNB–GSH conjugate formed min(^{-1}) (mg protein)(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Kidney</td>
</tr>
<tr>
<td>Normal control</td>
<td>95.16 ± 1.99</td>
<td>34.58 ± 1.91</td>
</tr>
<tr>
<td>Normal + γ-sitosterol 20 mg/kg/b.wt</td>
<td>95.97 ± 0.78(^{a})</td>
<td>35.28 ± 2.48(^{b})</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>65.76 ± 1.47(^{b})</td>
<td>16.85 ± 2.66(^{a})</td>
</tr>
<tr>
<td>Diabetic + γ-sitosterol 20 mg/kg/b.wt</td>
<td>91.52±0.97(^{b})</td>
<td>38.11 ± 2.41(^{b})</td>
</tr>
<tr>
<td>Diabetic +Glibenclamide 600µ g/kg/b.wt</td>
<td>93.95±1.32(^{b})</td>
<td>34.73 ± 1.94(^{b})</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M for six animals;

\(^{a}\) values deviate significantly from diabetic control group by comparison with normal rats (P≤0.05); \(^{b}\) values deviate very significantly from diabetic control groups.
Table 16: Effect of γ-sitosterol on tissue superoxide dismutase and Glutathione s transferase in normal and streptozotocin-induced diabetic male Wistar rats after 21 days of treatment.

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD (U/mg protein)</th>
<th>GPx (glutathione consumed min⁻¹ (mg protein)⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Kidney</td>
</tr>
<tr>
<td>Normal control</td>
<td>9.05 ± 0.17</td>
<td>15.30 ± 0.21</td>
</tr>
<tr>
<td>Normal + γ-sitosterol 20 mg/kg/b.wt</td>
<td>8.97 ± 0.12ᵇ</td>
<td>15.15 ± 0.40ᵇ</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>3.68 ± 0.25ᵃ</td>
<td>7.58 ± 0.19ᵃ</td>
</tr>
<tr>
<td>Diabetic + γ-sitosterol 20 mg/kg/b.wt</td>
<td>7.32 ± 0.23ᵇ</td>
<td>14.01 ± 0.31ᵇ</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide 600µ g/kg/b.wt</td>
<td>6.92 ± 0.20ᵇ</td>
<td>13.72 ± 0.34ᵇ</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M for six animals;

ᵃ values deviate significantly from diabetic control group by comparison with normal rats (P≤0.05);ᵇ values deviate very significantly from diabetic control groups.
Table 17: Effect of γ-sitosterol on serum protein profile in normal and STZ – induced diabetic rats after 21 days of treatment.

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum protein profile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total protein (g/dL)</td>
</tr>
<tr>
<td>Normal control</td>
<td>8.22±0.22</td>
</tr>
<tr>
<td>Normal + γ-sitosterol 20 mg/kg/b.wt</td>
<td>7.40±0.19&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>4.89±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + γ-sitosterol 20 mg/kg/b.wt</td>
<td>7.50±0.15&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide 600µ g/kg/b.wt</td>
<td>7.15±0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M for six animals;
<sup>a</sup> values deviate significantly from diabetic control group by comparison with normal rats (P≤0.05);<sup>b</sup> values deviate very significantly from diabetic control groups.
Table 18: Effect of γ-sitosterol on plasma glycoprotein in STZ-induced diabetic rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total Hexose (mg/dL)</th>
<th>Hexoseamine (mg/dL)</th>
<th>Sialic acid (mg/dL)</th>
<th>Fucose (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>94.98 ± 1.80</td>
<td>74.44 ± 1.38</td>
<td>7.00 ± 0.10</td>
<td>15.73 ± 0.14</td>
</tr>
<tr>
<td>Normal + γ-sitosterol 20 mg/kg/b.wt</td>
<td>93.63 ± 1.62(^b)</td>
<td>73.17 ± 2.11(^b)</td>
<td>8.47 ± 0.13(^b)</td>
<td>15.65 ± 0.10(^b)</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>148.92 ± 0.06(^a)</td>
<td>113.36 ± 1.49(^a)</td>
<td>4.30 ± 0.09(^a)</td>
<td>32.33 ± 0.35(^a)</td>
</tr>
<tr>
<td>Diabetic + γ-sitosterol 20 mg/kg/b.wt</td>
<td>94.14 ± 2.51(^b)</td>
<td>77.31 ± 1.18(^b)</td>
<td>6.60 ± 0.12(^b)</td>
<td>17.07 ± 0.15(^b)</td>
</tr>
<tr>
<td>Diabetic +Glibenclamide 600 µg/kg/b.wt</td>
<td>97.21 ± 2.24(^b)</td>
<td>87.31 ± 2.93(^b)</td>
<td>7.92 ± 0.35(^b)</td>
<td>16.31 ± 0.35(^b)</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M for six animals;
\(^a\) values deviate significantly from diabetic control group by comparison with normal rats (P≤0.05);\(^b\) values deviate very significantly from diabetic control groups.
Table 19: Effect of γ-sitosterol on liver glycoprotein in normal and STZ-induced diabetic rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total hexose (mg/100g)</th>
<th>Hexoseamine (mg/100g)</th>
<th>Fucose (mg/100g)</th>
<th>Sialic acid (mg/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Kidney</td>
<td>Liver</td>
<td>Kidney</td>
</tr>
<tr>
<td>Normal control</td>
<td>30.41±1.22</td>
<td>26.73±0.92</td>
<td>7.80±0.14</td>
<td>15.45±0.55</td>
</tr>
<tr>
<td>Normal + γ-sitosterol 20 mg/kg/b.wt</td>
<td>27.50±1.52&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.04±0.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.75±0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.08±0.65&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>53.75±1.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52.39±0.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.02±0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.51±0.90&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + γ-sitosterol 20 mg/kg/b.wt</td>
<td>28.39±0.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.57±0.86&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.50±0.18&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>15.89±0.40&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide 600µg/kg/b.wt</td>
<td>29.69±0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.29±0.66&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.65±0.18&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>15.82±0.69&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M for six animals;
<sup>a</sup> values deviate significantly from diabetic control group by comparison with normal rats (P≤0.05);<sup>b</sup> values deviate very significantly from diabetic control groups.
Table 20: Effect of γ sitosterol on plasma liver marker enzymes in normal and STZ-induced diabetic rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum liver markers enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AST (U/dl)</td>
</tr>
<tr>
<td>Normal control</td>
<td>31.71±1.73</td>
</tr>
<tr>
<td>Normal + γ-sitosterol 20 mg/kg/b.wt</td>
<td>31.56±1.82</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>67.06±0.91</td>
</tr>
<tr>
<td>Diabetic + γ-sitosterol 20 mg/kg/b.wt</td>
<td>41.81±1.97</td>
</tr>
<tr>
<td>Diabetic + Glibenclamide 600μg/kg/b.wt</td>
<td>42.82±2.24</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M for six animals;

a values deviate significantly from diabetic control group by comparison with normal rats (P≤0.05), b values deviate very significantly from diabetic control groups.
Table 21: Effect of γ-sitosterol on plasma kidney marker in normal and STZ-induced diabetic rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma kidney marker</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urea</td>
</tr>
<tr>
<td></td>
<td>(mg/dl)</td>
</tr>
<tr>
<td>Normal control</td>
<td>22.18±0.88</td>
</tr>
<tr>
<td>Normal + γ-sitosterol 20 mg/kg/b.wt</td>
<td>21.09±0.88$^b$</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>45.74±2.09$^a$</td>
</tr>
<tr>
<td>Diabetic + γ-sitosterol 20 mg/kg/b.wt</td>
<td>24.29±1.61$^b$</td>
</tr>
<tr>
<td>Diabetic +Glibenclamide 600µ g/kg/b.wt</td>
<td>25.75±1.13$^b$</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M for six animals;
$^a$ values deviate significantly from diabetic control group by comparison with normal rats (P≤0.05); $^b$ values deviate very significantly from diabetic control groups.
Concentration of ϒ-sitosterol (µg)

Fig 3.1 Concentration dependent effect of ϒ-sitosterol on glucose induced insulin release from rat pancreatic islet. Values are mean ± S.E.M (n=5). *, p>0.05, **, p>0.001 as compared with untreated control group.
Fig 3.2. Effect of γ-sitosterol (alone and in combination with other compounds) on glucose–induced insulin release from rat pancreas islets. Values are mean ± S.E.M (n=4). *, p<0.001 as compared with untreated control. +, p<0.001, p<0.05 as compared with compound treated control.
Fig 3.3- Histopathological observation of normal and experimental rat pancreas (H&E 400X).

A- Normal pancreas (Native architectures of pancreatic islets); B- Normal rats treated with $\gamma$-sitosterol 20 mg/kg /b.wt (Native architecture of pancreatic islets); C- Diabetic control (Presence of shrinkage of islets and growth of adipose tissue );D- Diabetic + $\gamma$-sitosterol 20 mg/kg /b.wt) (Mild expansion pancreatic islets shows prominent hyperplastic islets); E- Diabetic + Glibenclamide (600 $\mu$g/kg /b.wt) (Absence of dilation and prominent hyperplastic of islets).
Fig 3.4- Histological examination of normal and STZ induced diabetic rats liver

A- Normal control; B- Normal control + γ-sitosterol (20 mg/kg /b.wt); C- Diabetic control; D- Diabetic+ γ-sitosterol (20/mg/kg /b.wt ); E-Diabetic + glibenclamide (600/µg/kg /b. wt).
Fig 3.5- Histological examination of normal and STZ induced diabetic rats kidney.

A- Normal control; B- Normal control + γ-sitosterol (20 mg/kg /b.wt); C- Diabetic control; D- Diabetic+ γ-sitosterol (20 mg/kg /b.wt ); E-Diabetic + glibenclamide (600 µg/kg /b. wt).
Fig 3.6 – Immunohistochemical analysis of normal and STZ induced diabetic rats pancreas.

A- Immunohistochemical expression of insulin secreting cells in pancreatic islets of a normal rat (X 400); B- Normal rats treated with 20 mg/kg /b. wt of γ-sitosterol for 21 days, Immunohistochemical expression of insulin secreting cells in STZ – induced diabetic rats. Arrow indicates a positively stained insulin secreting cells (X 400). D- Positive insulin secreting cells in a diabetic rat after treatment with the γ-sitosterol 20mg/kg /b.wt (X 400). E- Positive secreting cells in a diabetic rats after treatment with glibemclamide 600µg/kg /b.wt.