Materials and Methods
3.1 Animals

Male adult Wistar strain albino rats (90-100 day old; 100-150 g) were used for the test. The animals were obtained from Tamilnadu Veterinary and Animal Science University, Chennai, India. The rats were maintained at the local animal house conditions (temperature 33±3°C; humidity 55±5 %; day length 12.75±0.50 h). The animals were fed with a standard feed (Sai Durga Feeds and Foods, Bangalore, India) and water ad libitum. The animals described as fasted were deprived of food for 16 h but were allowed free access to water (Subramoniam et al., 1996). After randomization into various groups, the rats were acclimated to the laboratory conditions of temperature and photoperiod for a period of 1-2 weeks before initiation of the experiments (Pushparaj et al., 2000).

3.2 Collection of plant materials

Elephantopus scaber (root/leaf) (ESR/ESL) (Fig.1, 2) was collected from Palode, Thiruvananthapuram district, Kerala, India. Eugenia jambolana (seed/bark) (EJS/EJB) (Fig.3,4), Clitoria ternatea (leaf/flower) (CTL/CTF) (Fig.5,6) and fruits of Phyllanthus acidus (Fig.7) and Phyllanthus emblica (Fig.8) were collected from Thirumayam, Pudukkottai District, Tamilnadu, India. The plant parts that were chosen for the study were air-dried.

3.3 Preparation of extracts

3.3.1 Aqueous extract of the plants – Air-dried parts of each plant were powdered and boiled in water (100 g/L distilled water). The decoction was filtered through nitrocellulose filter and the filtrates were evaporated to dryness under reduced pressure and at a lower temperature in a rotary evaporator (Prince and Menon, 2000). The dried residues were stored in air-
Photograph of

Fig. 1. *Elephantopus scaber* root
Fig. 2. *Elephantopus scaber* leaf
Fig. 3. *Eugenia jambolana* seed
Fig. 4. *Eugenia jambolana* bark
Fig. 5. *Clitoria ternatea* leaf
Fig. 6. *Clitoria ternatea* flower
Fig. 7. *Phyllanthus acidus* fruit
Fig. 8. *Phyllanthus emblica* fruit
Fig. 9. Photograph of Soxhlet apparatus
tight containers for further use. The residue was dissolved in distilled water before use.

3.3.2 Methanolic extract of the plants: 100 g of each plant part was extracted in Soxhlet apparatus (Fig.9) separately using 1 L of methanol for 6 h and then filtered (Akhtar and Iqbal, 1991). The filtrate was collected and evaporated to dryness under reduced pressure and at a lower temperature in a rotary evaporator. The residues obtained for each plant part was dissolved in distilled water before use.

3.4 Induction of diabetes

Alloxan monohydrate was obtained from Sigma Chemical Company (MO, USA). Diabetes mellitus was induced in a batch of normoglycemic albino rats starved for 16 h, by injecting intraperitoneally 150 mg/kg body weight of alloxan monohydrate dissolved in physiological saline (Prince et al., 1998). Since alloxan is capable of producing fatal hypoglycemia, as a result of massive pancreatic insulin release, rats were treated with 20% glucose solution intraperitoneally after 6 h. For the next 24 h, the rats were kept on 5% glucose solution in their cages to prevent hypoglycemia (Al-Shamaony et al., 1994). This dose of alloxan produced persistent hyperglycemia, as revealed by the determination of blood glucose levels. After 7 days of alloxan injection, rats with blood glucose>300 mg/dl were considered as diabetic and included in the study (Lamela et al., 1986).
3.5 Experimental design

3.5.1 Preliminary study

One week after the induction of diabetes, rats with blood glucose >300 mg/dl were subjected to fasting for 16 h. They were divided into different groups, with 10 rats in each group. Aqueous and methanolic extracts of each plant part ranging from 50 mg/kg body weight to 500 mg/kg body weight at an interval of 50 mg/kg body weight were administered to the animals and blood glucose was estimated at the end of 5 h after the oral administration of the extract (Karunanayake et al., 1984). The lowest dose that brought about the maximum antihyperglycemic effect for each plant part was given through oral intubations for the repeated administration. It was observed that the dose was the same for both aqueous and methanolic extracts; for ESR and ESL the dose was found to be 300 mg/kg body weight. It was 400 mg/kg body weight for the extracts of EJS, EJB, CTL and CTF. The dose for PEF and PAF was 350 mg/kg body weight.

3.5.2 Repeated oral administration of plant extracts

One week after induction of diabetes in albino rats, the fasting blood glucose levels of fasted rats were measured (Pushparaj et al., 2000). Rats with blood glucose >300 mg/dl were included in the study. They were divided into different groups with ten rats in each group. The selected dose of the plant extracts (the dosage arrived at after preliminary study for aqueous and methanolic extracts) were given everyday till completion of the experiment (i.e. 84 days), whereas untreated and diabetic control group was given distilled water everyday through oral intubations (Grover et al., 2000).
At the end of the experiments, five animals from each group were sacrificed by cervical dislocation for biochemical studies. Blood was collected from the heart and allowed to clot and the serum was separated by centrifugation at 3500 r.p.m. for 10 min. Serum was assayed either immediately or stored at -20°C (Mathur et al., 1996). Tissues like liver, skeletal muscle and pancreas were also collected. Liver and skeletal muscle was collected for biochemical estimations and pancreas was used for routine histological studies and immunocytochemical analysis. The remaining five animals in each group were used for perfusion fixation of tissues for TEM.

3.6 Biochemical estimations

3.6.1 Estimation of glucose (Diagnostic Kit–Reddy’s Laboratories, Bachupally, Hyderabad, India)

Principle

Glucose was oxidized by the enzyme glucose oxidase to give D-gluconic acid and hydrogen peroxide. Hydrogen peroxide, in the presence of the enzyme peroxidase oxidized phenol, combined with 4-aminoantipyrine to produce a pink-coloured quinoneimine dye. The intensity of the colour produced was proportional to glucose concentration in the sample.

\[
\begin{align*}
\text{D–glucose} + \text{H}_2\text{O} + \text{O}_2 & \xrightarrow{\text{Glucose oxidase}} \text{D-gluconic acid} + \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + 4\text{–Aminoantipyrine} + \text{Phenol} & \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + \text{Phenol} + \text{H}_2\text{O}
\end{align*}
\]

Reagents

1. Glucose reagent
2. Standard (100 mg/dl)
**Procedure**

For the estimation of glucose, 10 µl each of serum and working standard were incubated with 1 ml of the reagent for 15 min at 37°C and the absorbance at 505 nm was measured against a reagent blank. For reagent blank, 10 µl of distilled water was added to 1 ml of the reagent. The concentration of glucose in serum samples was calculated as:

\[
\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 100 = \text{mg/dl}
\]

**3.6.2 Estimation of glycosylated hemoglobin** (Diagnostic Kit–Bio Systems, Costa Brava, Spain)

**Principle**

The hemolysate was prepared first, in which the labile fraction was eliminated, hemoglobins were retained by a cationic exchange resin, HbA\text{1a+b} fraction was washed away and later, HbA\text{1c} was specifically eluted and quantified by direct photometric reading at 415 nm.

**Reagents**

1. Reagent 1 : Potassium biphtalate 50mM/L, detergent, pH 5.
2. Reagent 2 : Phosphate buffer 48mM/L, pH 6.5, Sodium azide 0.95g/L.
3. Reagent 3 : Phosphate buffer 72mM/L, pH 6.4, Sodium azide 0.95g/L.
4. Microcolumns

**Procedure**

Whole blood was collected in a test tube that contained EDTA. Columns and reagent were brought to room temperature for a few min. Into a test tube, 50 µl of blood and 200 µl of reagent were pipetted out. The
contents in the test tube was mixed well and allowed to stand at room temperature for 10-15 min, and this formed the hemolysate. The column for the separation was prepared by removing the upper cap from the column first and then the lower cap, the rounded end of a pipette was used to push the upper disc down to the resin surface; care was taken to avoid compressing it. The column was allowed to drain completely.

50 µl of the hemolysate was pipetted on the upper filter and the column was allowed to drain to waste. The sample residue left above the upper disc was removed by adding 200 µl of reagent 2 and the column was allowed to drain to waste. Again 2 ml of reagent 2 was added and the column was allowed to drain to waste. Next, the column was placed over a test tube and 4 ml of reagent 3 was added to the column, the eluate HbA1c was collected and the absorbance was read at 415 nm against distilled water. In order to read the absorbance of the total hemoglobin (AHbTOTAL), 12 ml of reagent 3 was mixed with 50 µl of hemolysate and the absorbance was read at 415 nm against distilled water. HbA1c was calculated as:

\[
\frac{A_{HbA1c}}{3 \times A_{Hbtotal}} \times 100 = \% \text{ HbA1c}
\]

3.6.3 Estimation of liver and skeletal muscle glycogen (Plummer, 1987)

Principle

Glycogen in liver and skeletal muscle was liberated when heated with strong alkali. Released glycogen was precipitated by the addition of ethanol and sodium sulphate (coprecipitant) to give a quantitative yield of glycogen. The polysaccharide was then hydrolysed in acid and the glucose released was estimated.
Reagents

1. Potassium hydroxide (30%)
2. Saturated Na₂SO₄
3. Ethanol (95 percent v/v)
4. HCl (1.2 M/L)
5. Phenol red indicator
6. NaOH (0.5 M/L)
7. Reagents for the estimation of glucose

Procedure

1 g each of liver and skeletal muscle was weighed into a calibrated centrifuge tube that contained 2 ml of KOH (30%) and heated in a boiling water bath for 20 min with occasional shaking. The tubes were cooled on ice and 0.2 ml of saturated Na₂SO₄ was added. Glycogen was precipitated by adding 5 ml of ethanol (95% v/v). The precipitate was separated by centrifugation and dissolved in 5 ml of water with gentle warming, and then diluted with distilled water to 10 ml.

1 ml samples of the glycogen solution were pipetted out into a test tube, 1 ml of HCl (1.2 M/L) was added to it, a marble was placed inside the test tube and the test tube was heated on a boiling water bath for 2 h. At the end of this period, 1 drop of phenol red indicator was added and neutralized carefully with NaOH (0.5 M/L) till the indicator changed from pink through orange to a yellow colour. Finally, it was diluted to 5 ml with distilled water and proceeded as for the estimation of glucose. Duplicates were maintained.
3.6.4 *Estimation of serum insulin* (Radioimmunoassay kit–Diasorin, Italy).

**Principle**

The principle of the assay was based on the competition between labelled insulin and insulin contained in standards or specimens to be assayed for a fixed and limited number of antibody binding sites. After the incubation, the amount of labelled insulin bound to the antibody was inversely related to the amount of unlabelled insulin present in the sample. The method adopted for separation was based on the use of a precipitating reagent, in which a second antibody is pre-precipitated and in excess.

**Sensitivity and Cross Reactivity**

The assay sensitivity is below 4 μU/mL at 95% confidence limit. The specificity for rat insulin is 100%.

**Reagents**

1. ^125^I-labelled insulin: The vial contains labelled porcine insulin, phosphate buffer, bovine albumin, preservatives and an inert red dye.
2. Insulin standards: The vials contain increasing amounts of human insulin, human serum and preservatives.
3. Insulin antiserum: The vial contains antiserum raised in guinea-pigs, phosphate buffer, bovine albumin, preservatives and an inert blue dye.
4. Precipitating reagent: Each bottle contains buffer, polyethylene glycol, antibody to guinea-pig IgG and preservatives.

**Procedure**

Standards: 100 μl of the standard from the respective standard bottles (0-5) was pipetted out into different vials and 100 μl of tracer and 100 μl of antiserum were added to it.

Sample: 100 μl of serum sample was pipetted out into a vial, 100 μl of tracer and 100 μl of antiserum were added to it.

Total activity: To measure the total activity, 100 μl of tracer was pipetted out into a vial.
The contents of the above vials were mixed with a vortex and incubated for 1.5 h at room temperature. The bottle of precipitating reagent was allowed to reach the room temperature and mixed well by repeated tilting. 1 ml of precipitating reagent was dispensed into all vials (except total activity vial). The contents of all the vials were again vortexed and the vials were allowed to stand for 15 min at room temperature. The vials were centrifuged at 1500 r.p.m. for 15 min. The supernatant was discarded. The radioactivity of the precipitate was measured. The mean net counts for each group of tubes were computed.

The binding ability was evaluated as follows:

\[
(B / T)_{0\%} = \frac{\text{Zero standard mean counts}}{\text{Total activity mean counts}} \times 100
\]

The mean counts for each standard and unknown sample was expressed as a percentage of zero standard mean counts.

\[
B / B_{0\%} = \frac{\text{Standard or sample mean counts}}{\text{Zero standard mean counts}} \times 100
\]

The percent values of each standard versus the insulin amount expressed as μU/ml was plotted in linear-linear or semilog coordinates to obtain a calibration curve. By interpolation of the calibration curve, the insulin level of the samples was obtained.

3.6.5 Assay of glucokinase (Brandstrup et al., 1957)

Principle

Glucokinase was assayed by the estimation of glucose-6-phosphate formed from glucose.
Reagents

1. Tris HCl buffer – 0.1 M, pH 7.4
2. Glucose – 0.005 M
3. Adenosine triphosphate (ATP) – 0.072 M
4. Magnesium chloride – 0.05 M
5. Potassium dihydrogen phosphate – 0.0125 M
6. Potassium chloride – 0.1 M
7. Sodium fluoride – 0.5 M
8. TCA-10%
9. Tris-HCl buffer -0.01 M, pH 8.0
10. Reagents for the estimation of glucose

Procedure

1g of liver was homogenized in 0.1M Tris HCl buffer, pH 7.4, at 4°C in a potter-Elvehjem homogenizer for 3 min. The homogenate was centrifuged at 2500 r.p.m. for 10 min.

The reaction mixture in a total volume of 5.3 ml contained 1 ml of glucose solution, 0.5 ml of ATP solution, 0.1 ml of magnesium chloride solution, 0.4 ml of potassium dihydrogen phosphate solution, 0.4 ml of potassium chloride solution, 0.4 ml of sodium fluoride solution and 2.5 ml of Tris-HCl buffer. The mixture was pre-incubated at 37°C for 5 min. The reaction was initiated by the addition of 2 ml of tissue homogenate. 1 ml of the reaction mixture was immediately removed to the tubes containing 1 ml of 10% TCA which was considered as zero time. A second aliquot was removed after 30 min incubation at 37°C. The protein precipitate was
removed by centrifugation and the residual glucose in the supernatant was estimated. Protein content of the samples was estimated by the method of Lowry et al., (1951). The enzyme activity is expressed as μ moles of glucose phosphorylated / min / g protein.

3.6.6 Assay of glucose–6-phosphatase (Baginsky et al., 1992)

Principle
Glucose–6–phosphatase was assayed by estimation of inorganic phosphate liberated from glucose–6–phosphate.

Reagents
1. Sucrose (250 mM)
2. Sucrose/EDTA buffer (0.25 M/mM, pH 7)
3. Glucose–6–phosphate (100 mM)
4. Imidazol buffer (100 mM, pH 6.5)
5. Na₂HPO₄ (1.5 mM)
6. TCA (10%)
7. Ascorbate (2%)
8. Ammonium molybdate (1%)
9. Sodium arsenite (2%)
10. Sodium citrate (2%)

Procedure
On a precooled watch glass, 1 g of liver was chopped into small pieces and homogenized in a Potter-Elvehjem homogenizer for 2 min with ice-cold
sucrose (15 ml, 250 mM). The homogenate was centrifuged at 2°C for 30 min at 9000 r.p.m.

Four tubes labeled as sample, control, blank and standard were taken. To each tube was added 0.1 ml of sucrose/EDTA buffer (0.25 M/mM, pH 7), 0.1 ml of glucose-6-phosphate (100 mM) and 0.1 ml of imidazol buffer (100 mM, pH 6.5). This was followed by the addition of 0.1 ml of liver homogenate supernatant to the sample tube, 0.1 ml of distilled water to the blank and 0.1 ml of Na₂HPO₄ (1.5 mM) to the standard tube. All the tubes were incubated at 37°C for 1 min and the enzyme activity was terminated by adding 2 ml TCA/ascorbate (10%/2% : w/v). Liver homogenate supernatant (0.1 ml) was added to the control tube only. It was followed by centrifugation of all tubes at 3,000 r.p.m. for 10 min. To 1 ml of the clear supernatant were added 0.5 ml ammonium molybdate (1%) and 1 ml of sodium arsenite/sodium citrate (2%/2%: w/v). The tubes were then allowed to stand for a min at room temperature and absorbance was read at 700 nm. The amount of inorganic phosphate liberated by the enzyme was calculated by comparing with the absorbance values of the standard. Enzyme activity is expressed as μmole of Pᵢ liberated/min/mg protein. Protein content of the samples was estimated by the method of Lowry et al., (1951).

3.6.7 Estimation of total cholesterol (Diagnostic kit-Beacon Diagnostics, Kabilpore, Navsari, India)

Principle

Cholesterol esters were hydrolysed to free cholesterol by cholesterol ester hydrolase. The free cholesterol produced was oxidized by cholesterol oxidase to cholest-4-en-3-one with the simultaneous production of hydrogen
peroxide, which oxidatively coupled with 4-aminoantipyrine and phenol in the presence of peroxidase to yield a chromogen with maximum absorption at 500 nm.

\[
\text{Cholesterol ester} + \text{H}_2\text{O} \xrightarrow{\text{Cholesterol ester hydrolase}} \text{Cholesterol} + \text{Fatty acids}
\]

\[
\text{Cholesterol} + \text{O}_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholest-4-en-3-one} + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + \text{Phenol} + 4\text{-Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4\text{H}_2\text{O}
\]

**Reagents**

1. Working reagent
2. Standard (200 mg/dl)

**Procedure**

In the assay of total cholesterol, 10 µl each of serum and working standard were incubated with 1 ml of reagent for 5 min at 37°C and the absorbance at 500 nm was measured against a reagent blank. For reagent blank, 10 µl of distilled water was added to 1 ml of working reagent. Concentration of cholesterol in serum samples was calculated as:

\[
\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 200 = \text{mg/dl}
\]

**3.6.8 Estimation of triglycerides** (Diagnostic kit-Bio Systems, Costa Brava, Spain)

**Principle**

Serum triglycerides were hydrolysed using lipase, and the released glycerol was assayed in a reaction catalysed by glycerol kinase and L-α-
glycerol-phosphate oxidase in a system that generated hydrogen peroxide. The hydrogen peroxide generated was monitored in the presence of horseradish peroxidase with 3, 5-dichloro-2-hydroxybenzenesulfonic acid/4-aminophenazone as the chromogenic system. The high absorbance of the chromogen system at 500 nm gave the results.

\[
\text{Triglycerides} + \text{H}_2\text{O} \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{Free fatty acids.}
\]

\[
\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol Kinase}} \text{L-\(\alpha\)-Glycerol-phosphate} + \text{ADP}
\]

\[
\text{L-\(\alpha\)-Glycerol-Phosphate} + \text{O}_2 \xrightarrow{\text{L-\(\alpha\)-Glycerol-Phosphate Oxidase}} \text{H}_2\text{O}_2 + \text{Dihydroxyacetonephosphate}
\]

\[
\text{H}_2\text{O}_2 + 3, 5\text{-Dichloro-2-hydroxybenzenesulfonic acid} + 4\text{-Aminophenazone} \xrightarrow{\text{Peroxidase}} \text{Quinonemonoimine dye} + 2\text{H}_2\text{O}.
\]

**Reagents**

1. Working reagent
2. Standard (200 mg/dl)

**Procedure**

For the estimation of triglycerides, 10 \(\mu\)l each of serum and working standard were mixed with 1 ml of working reagent and kept at room temperature for 15 min and the absorbance was read at 500 nm against the reagent blank. For reagent blank, 10 \(\mu\)l of distilled water was added to 1 ml of working reagent. Concentration of triglycerides in serum samples was calculated as:

\[
\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 200 = \text{mg/dl}
\]
3.6.9 Estimation of serum HDL-cholesterol (Diagnostic kit-Beacon Diagnostics, Kabilpore, Navsari, India)

**Principle**

In the presence of precipitating reagent, all lipoproteins of the serum, except HDL fraction, were precipitated. After centrifugation, the precipitate was discarded and the HDL-cholesterol content of the supernatant was determined.

**Reagents**

1. Working reagent
   
   Reagent 1 : Phosphate buffer 50 mM/L, pH 6.9, phenol 24 mM/L, sodium cholate 0.5 mM/L.
   
   Reagent 2 : Cholesterol esterase 200 U/L, cholesterol oxidase 250 U/L, peroxidase 1000 U/L, 4-aminoantipyrine 0.5 mM/L, Reagents 1 and 2, 1:1.

2. Precipitating reagent

3. Standard (200 mg/dl)

**Procedure**

In the assay of HDL-cholesterol, 500 µl of serum was added to 50 µl of precipitating reagent and kept at room temperature for 10 min. Later, it was centrifuged at 3000 r.p.m. for 10 min. The clear supernatant was used for HDL-cholesterol estimation. 20 µl of the supernatant and 10 µl of standard were incubated with 1 ml of the reagent for 5 min at 37°C and the absorbance at 510 nm was measured against the reagent blank. For reagent blank, 10 µl of distilled water was added to 1 ml of the reagent. The amount of HDL-cholesterol in the samples was calculated as:

\[
\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 100 \times 1.1 = \text{mg/dl}
\]
3.6.10 Estimation of protein (Lowry et al., 1951)

**Principle**

The aromatic amino acids such as tyrosine and tryptophan present in protein reacted with Folin-Ciocalteu reagent to give a dark blue colour. The intensity of the colour obtained was directly proportional to the amount of protein present in the sample.

**Reagents**

1. 5% Trichloroacetic acid
2. Reagent A – 2 g of sodium carbonate in 100 ml of 0.1 N sodium hydroxide
3. Reagent B – 1 g of copper sulphate in 100 ml of distilled water
4. Reagent C – 2 g of sodium potassium tartarate in 100 ml of distilled water
5. Reagent D – Reagents B and C mixed in 1:1 ratio
6. Reagent mixture – Reagents A and D in 50:1 ratio
7. Folin–Ciocalteu’s reagent – Folin phenol reagent diluted 1:1 with distilled water

**Procedure**

0.5 ml of the serum was mixed with 1 ml of 5% trichloroacetic acid, and centrifuged to precipitate the protein. The precipitate was dissolved in 1 N sodium hydroxide and made upto 10 ml. To 1 ml of the sample, 5 ml of reagent D was added, and after 10 min incubation, 0.5 ml of Folin–Ciocalteu’s reagent was added and mixed. After 30 min, the intensity of the blue colour
was read at 620 nm against a reagent blank. Protein content of serum sample was determined from a standard curve.

Standard curve was prepared using bovine serum albumin prepared at a stock concentration of 1 mg/ml and diluted to obtain serial dilutions at 50, 100, 150, 200 and 250 µg/ml.

3.6.11 Estimation of urea (Fawcett and Scott, 1960)

Principle

The ammonia formed from urease action reacted with phenol in the presence of hypochlorite to form an indophenol, which with alkali gave a blue coloured compound. The nitroprusside acted as a catalyst, increasing the rate of reaction. The intensity of the colour obtained was directly proportional to the amount of urea present in the sample.

Reagents

1. Buffered urease solution
2. Phenol–sodium nitroprusside solution
3. Sodium hydroxide–sodium hypochlorite
4. Urea solution – 1 mg per ml

Procedure

20 µl each of serum and working standard were incubated with 200 µl of urease–buffer solution at 37°C for 15 min, 5 ml of the phenol nitroprusside solution was added to it, followed by 5 ml of the hypochlorite reagent. It was placed in a water bath at 37°C for 15 min and the absorbance was measured against reagent blank at 630 nm. For reagent blank, 20 µl of distilled water
was added to 200 μl of urease buffer. Urea content in the sample was calculated as:

\[
\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 100 = \text{mg/dl}
\]

3.6.12 *Estimation of creatinine* (Diagnostic kit—Dr. Reddys' Laboratories, Bachupally, Hyderabad, India)

**Principle**

Creatinine reacted with alkaline picrate to produce a red coloured complex; the rate of red coloured complex formed was directly proportional to the creatinine content in the sample.

**Reagents**

1. Working reagent: Buffer solution 50 ml, picric acid reagent 50 ml.
2. Standard (2 mg/dl)

**Procedure**

For the estimation of creatinine, to 1 ml of working reagent, 100 μl of serum or working standard was added and a stopwatch was started. Initial absorbance, \( A_0 \), was read exactly after 30 seconds of adding test and standard. Absorbance, \( A_1 \), for test and standard was read exactly after 60 seconds of adding test and standard to the working reagent. Creatinine content in serum was calculated as:

\[
\frac{\Delta A_T}{\Delta A_S} \times 2 = \text{mg/dl}
\]

Where, \( \Delta A_T = A_1T - A_0T \)
\( \Delta A_S = A_1S - A_0S \)
3.7 Histological studies

3.7.1 Light microscopic studies—Paraffin method (Humason, 1979)

Following solutions were used:

1. Physiological saline (0.9%)
2. Bouin-Hollande fixative
3. Ehrlich's hematoxylin
4. Eosin

Procedure

The pancreas from untreated 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 for 72 h. 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 cleared 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. 3-5 μm thick sections of all the tissues were obtained using a rotary microtome (Leica, Germany) and stained in Ehrlich's hematoxylin with eosin as the counterstain. The slides were mounted using DPX mountant.

3.7.2 Light microscopic as well as transmission electron microscopic studies adopting resin embedding

Plastic embedding was done according to Hayat (1981), using Karnovsky's fluid (Karnovsky, 1965) as the perfusate.
Reagents

1. Perfusion fluid

The perfusate (Karnovsky's fluid) was prepared with the following composition: glutaraldehyde 1% and paraformaldehyde 1% in phosphate buffer 0.1 M

2. Phosphate buffer (0.2 M)

3. Washing buffer

Prepared by dissolving 5 g of sucrose in 100 ml of 0.1 M phosphate buffer.

4. Osmium tetroxide

A stock solution of 2% osmium tetroxide was prepared

5. Spurr's mixture

Prepared by adding 8 ml of 2-nonen-1-ylsuccinic anhydride (NSA), 1 ml of 4-vinylcyclohexane dioxide, 10 ml of resin and 0.1 ml of 2-dimethylaminoethanol (DMAE).

6. Toluidine blue (TBO)

Aqueous toluidine blue O solution was prepared by dissolving 0.5 g toluidine blue in benzoate buffer at pH 4.4

7. Uranyl acetate

Uranyl acetate was prepared by adding 10 ml of 50% ethanol to a saturated solution of uranyl acetate. After two min of centrifugation, the excess uranyl acetate was allowed to settle and the clear supernatant was used as the stain (Hess and Thurston, 1977).
8. **Lead Citrate**

One half pellet of sodium hydroxide was added to 12 ml of double distilled water and centrifuged. 50 mg of lead citrate (Anala R) was dissolved in it by thorough shaking followed by centrifugation.

**Procedure**

**Perfusion fixation and embedding**

For perfusion fixation, the procedure adopted was according to Hess and Moore (1993). The rat (5 animals in each group) were anesthetized using ether and laid on its back in a shallow tray. A vacuum system was used to remove the fluids accumulating into a holding container. Vascular perfusion is generally carried out intracardially and offers a good preservation of organs (Hess and Thurston, 1977). In the present study, the transfusion set consisted of a bottle with polythene catheter containing the perfusate suspended at about 150 cm above the animal. The ascending aorta was cannulated with the polythene catheter through an incision in the left ventricle. Care was taken not to let in air during this process. The right atrium was incised for the outflow of the fixative. Initially the fixative was allowed to flow rapidly and then the rate of flow was reduced to minimum. Following perfusion, pancreas was cut into 1 mm cubes and immersed in 2.5% glutaraldehyde (primary fixative) overnight. Then the tissues were rinsed in washing buffer and post-fixed in 1% osmium tetroxide (OsO₄) (secondary fixative) for 2-3 h. Subsequently, the tissues were washed thoroughly in washing buffer to remove excess OsO₄. Then the tissues were dehydrated gradually in ethyl alcohol and dealcoholized using propylene oxide. Infiltration was carried out with propylene oxide and Spurr's mixture (Sigma, USA) at increasing
concentrations at room temperature, using a slow speed rotary shaker. Embedding was done in a flat embedding mold with tissues oriented to get cross sections. Semithin sections were obtained with Reichert Jung (Austria) ultramicrotome and stained in TBO. Area was chosen to obtain ultra-thin sections (silver to gray, 60 nm–90 nm) using LKB-Bromma ultracut (Germany). Sections were picked in copper grids and stained with uranyl acetate and lead citrate.

Leitz diaplan microscope (Leica, Germany) was used to obtain light micrographs of TBO-stained semithin sections, while Philips 200 (Holland) transmission electron microscope (TEM) was used to obtain electron micrographs at 2000 to 70,000 X magnifications.

3.8 Electrophoretic separation of serum proteins

The proteins of the serum from untreated, diabetic control and extract-treated diabetic rats were separated adopting SDS–polyacrylamide gel electrophoresis according to Laemmli (1970), using acrylamide gel (10%).

The various solutions were prepared as follows:

Acrylamide stock solution for separating gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>4.4 g</td>
</tr>
<tr>
<td>Bisacrylamide</td>
<td>80 mg</td>
</tr>
<tr>
<td>Water to make up</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

Buffer for separating gel (pH 8.8)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>3.655 g</td>
</tr>
<tr>
<td>HCl</td>
<td>0.413 ml</td>
</tr>
<tr>
<td>Water to make up</td>
<td>10 ml</td>
</tr>
</tbody>
</table>
the stacking gel. The glass slabs were fixed in the slab gel chamber after removing the lower strip.

**Solublizing the samples**

The samples containing 100 µg of protein were mixed separately with solublizing buffer in 1:1 ratio, heated at 90°C for 2 min., and loaded on the gel. The upper and lower chambers of the slab gel apparatus were carefully filled with the running buffer. The electrophoresis was carried out at 1.5 mA current per slot, until the dye reached the bottom of the stacking gel.

**Visualizing the protein fractions**

After electrophoresis, the gels were stained with Coomassie blue R-250, using a solution of 0.2% Coomassie blue R-250 in 50% methanol and 7% acetic acid for overnight. Then, the gels were destained with destaining solution containing 30% methanol and 7% acetic acid. Subsequently, the gels were stored in 7% acetic acid for the purpose of photography.

**Molecular Weight Markers**

Recombinant SDS-PAGE Protein Marker ranging from 14-80 K Da viz., Protein 1 (80,000), Protein 2 (66,000), Protein 3 (56,000), Protein 4 (40,000), Protein 5 (20,000), Protein 6 (17,000) and Protein 7 (14,000) was run on the gel for finding the molecular weight of the protein fraction in the gels. The gels were scanned densitometrically by subjecting the electropherogram to chromoscan. The approximate molecular weights were calculated, comparing the motilities of the fractions from the serum with those of the markers.
3.9 Immunocytochemistry (Biomeda, Foster City, CA, USA)

Reagents

1. Buffered formalin (10%)
2. Tris buffer (pH 7.6)
3. Primary antibody (Primary polyclonal anti-guinea pig antibody to insulin)
4. Secondary antibody (anti-rabbit polyclonal antibodies)
5. Peroxidase solution
6. AEC (3-amino, 9-ethyl carbazole chromogen substrate)
7. Blocking reagent
8. Tissue conditioner

All immunochemicals were purchased from Biomeda, (CA), USA.

Slices of pancreas from rats, as mentioned vide Supra (p 39), were fixed in 10% buffered formalin for 24 h. Tissues were dehydrated in graded series of alcohol, embedded in paraffin, sectioned at 5 μm thickness and used for immunostaining. All sections were de-paraffinized in xylene bath. The slides were placed in two changes of absolute alcohol for 3 min each. The same procedure was repeated with 90 percent alcohol. The slides were placed in blocking reagent in order to block the endogenous peroxidase activity for five min which was pre-diluted with 5 volumes of 100 percent ethanol. The slides were placed in two changes of 70 percent alcohol for three min each. The excess alcohol around the sections was removed and the slides were quickly immersed in Tris buffer (pH 7.6), for 5 min. Two drops of tissue conditioner were added and the sections were incubated for 5 min and then rinsed in
buffer solution. Pre-diluted primary polyclonal anti-guinea pig antibody to insulin (1:1,000), raised against human insulin, were added to the sections and incubated for one h. The secondary antibody for insulin was anti-rabbit polyclonal antibody. After incubation for half an hour, the sections were rinsed with Tris buffer and peroxidase solution was added, incubated for 30 min and later rinsed with the buffer. AEC (3-amino, 9-ethyl carbazole) chromogen substrate was added to the sections and incubated for 15 min and rinsed with distilled water. The sections were observed under a Leitz diaplan microscope (Leica, Germany) and photographed.

3.10 Preliminary Phytochemical Screening

Preliminary Phytochemical Screening of the plant were carried out as per the methods and tests given by Dey and Raman (1957).

Test for Terpenoids

Noller's Test: The substance was warmed with tin and thionyl chloride. Pink colouration indicates the presence of triterpenoids.

Test for flavonoids

Shinado's Test: To the substance in alcohol, a few magnesium turnings and few drops of concentrated hydrochloric acid were added and boiled for five minutes. Red coloration shows the presence of flavonoids.

Test for Steroids

Liebermann Burchard Test: 1mg of the test substance was dissolved in a few drops of chloroform, 3ml of glacial acetic were added warmed and cooled under the tap and drops of concentrated sulphuric acid were added along the
### Separating gel (10%)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide stock solution</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>Separating gel buffer</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>Water</td>
<td>13.6 ml</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate (SDS)</td>
<td>20 mg</td>
</tr>
<tr>
<td>Ammonium persulphate (APS)</td>
<td>14 mg</td>
</tr>
<tr>
<td>Tetramethyl ethylenediamine (TEMED)</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

### Acrylamide stock solution for stacking gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>3.8 g</td>
</tr>
<tr>
<td>Bisacrylamide</td>
<td>80 mg</td>
</tr>
<tr>
<td>Water to make up</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

### Buffer for stacking gel (pH 6.8)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>0.6 g</td>
</tr>
<tr>
<td>HCl</td>
<td>0.413 ml</td>
</tr>
<tr>
<td>Water</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

### Preparation of the stacking gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide stock</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>3.75 ml</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate (SDS)</td>
<td>10 mg</td>
</tr>
<tr>
<td>Water</td>
<td>8.9 ml</td>
</tr>
<tr>
<td>Ammonium persulphate</td>
<td>10 mg</td>
</tr>
<tr>
<td>Tetramethyl ethylenediamine</td>
<td>5 μl</td>
</tr>
</tbody>
</table>
Running buffer (pH 8.3)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>3 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>14.4 g</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Water to make up</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Solublizing buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>3.62 ml</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>0.062 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.25 ml</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate</td>
<td>75 mg</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>500 mg</td>
</tr>
</tbody>
</table>

All the solutions were stored at 4°C. The running buffer was prepared fresh every time.

Polymerization of the gels

Three sides of two rectangular glass plates were closed by sandwiching three perspex strips (1.5 mm thick) tightly between them. The fourth side was left open. The separating gel mixture was poured between the glass plates upto 12 cm height. A thin layer of isobutanol was layered on top of the separating gel. The gel was allowed to polymerise for 45 min. Isobutanol was removed using a piece of filter paper, and a comb (1.5 mm thick) was fixed up into the open space. The stacking gel was then poured on top of the separating gel. The comb was removed after polymerization of
the stacking gel. The glass slabs were fixed in the slab gel chamber after removing the lower strip.

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Test for Steroids

Liebermann Burchard Test: 1mg of the test substance was dissolved in a few drops of chloroform, 3ml of glacial acetic were added warmed and cooled under the tap and drops of concentrated sulphuric acid were added along the
sides of the test tube. Appearance of bluish green colour shows the presence of steroid.

**Test for Glycosides**

The substance was mixed with a little anthrone on a watch glass. One drop of concentrated sulphuric acid was added made into a paste and warmed gently over waterbath. Dark green coloration is the indication of the presence of glycosides.

**Test for Reducing Sugars**

The substance was mixed with the Fehling's solution II and I. Formation of red coloration is the indication of the presence of reducing sugars.

**Test of Alkaloid**

To the test substance a few drops of acetic acid were added, followed by Dragendorff’s reagent and shaken well. Formation of orange red precipitate indicates the presence of alkaloid.

**Test for Quinones**

To the test substance, sodium hydroxide was added. Blue green or red indicates the presence of quinone.

**Test for Phenols**

To the substance a few drops of alcohol and ferric chloride solution were added. Bluish green or red indicates the presence of phenol.

**Test for Tannins**

The substance is mixed with basic lead acetate solution. Formation of white precipitate indicates the presence of tannins.
Test for Saponins

The substance shaken with water, copious lather formation indicates the presence of saponins.

3.11 Statistical analysis

The data were subjected to the following statistical analyses as per the standard procedures.

1. Standard deviation

2. To bring out the significance of variation between different groups, one way analysis of variance (ANOVA) was used and further subjected to Duncan's post-hoc test to group them into different categories based on their mean values.