Chapter 4: MATERIALS AND METHODS

4.1. Collection and authentication

Aerial parts of *B. monosperma* were collected, and it was authenticated by Dr. Sri Ram murthy, department of botany, Andhra Loyola College, Vijayawada. A voucher specimen (ALS-1) was deposited.

4.2. Extraction and isolation procedure

Leaves were powdered (5 kg) and were soaked sequentially in hexane, ethyl acetate and methanol for 72 h and extracted by percolation. After 72 h the solution was filtered and the filtrate was concentrated under reduced pressure using rotary evaporator. All the filtrates were air dried and yield was 15 g of hexane extract, 22 g of ethyl acetate extract, and 30 g of methanol extract.

Flowers were powdered (5 kg) and were soaked sequentially in hexane, ethyl acetate, and methanol for 72 h and extracted using Soxhlet apparatus with intermittent shaking. After 72 h the solution was filtered and the filtrate was concentrated under reduced pressure using rotary evaporator. All the filtrates were air dried and the yield was 10 g of hexane extract, 15 g of ethyl acetate extract, and 20 g of methanol extract.

4.2.1. Thin Layer chromatography

Thin Layer chromatography (TLC) is the common method used to detect the phytoconstituents (Marston *et al.*, 1997). TLC is the solid-liquid technique in which the two phases are a Stationary phase (solid) and a Mobile phase (liquid). Solids most commonly used in TLC are silica gel (SiO$_2$ x H$_2$O) and alumina (Al$_2$O$_3$ x H$_2$O).

4.2.2. Column chromatography

Ethyl acetate extract (20g) of *B. Monosperma* leaves and flowers was separately subjected to column chromatography over silica gel; 60-120 mesh, 1200 g, column was prepared using hexane, eluent: hexane–ethyl acetate with increasing polarity (90:10–50:50) collecting 42 fractions of 500 ml each for leaves and 36 fractions 500 ml each for flower, which were combined to 12 major fractions for leaves and 8 major fractions for flowers on the basis of TLC analysis. All the obtained fractions were evaluated for anti-diabetic activity; fraction 8 from the
leaf extract and fraction 6 from the floral extract showed some activity; therefore these active fractions were selected for further pharmacological study.

4.2.3. Experimental setup

Adult male albino rats of Wistar strain weighing approximately 180–200 g were obtained from National Institute of Nutrition, Hyderabad, India. All the animals were kept and maintained under laboratory conditions of temperature (22°C±2), humidity (45±5%), and 12 hour day: 12 hour night cycle; and fed with commercial pelleted rat chow (Hindustan Lever Ltd., Bangalore, India) and had free access to water. All the animal experiments were conducted according to the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Committee guidelines (1221-a/08/CPCSEA).

4.3. Experimental induction of diabetes

Diabetes mellitus was induced by single intraperitoneal injection of freshly prepared Streptozotocin (STZ) (30 mg kg\(^{-1}\) b.wt) in 0.1 M citrate buffer (pH – 4.5) in a volume of 1 mL/kg b.wt. Diabetes was developed and stabilized in these STZ treated rats over a period of 3 days. After 3 days of STZ administration, plasma glucose levels of each rat were determined. Rats with fasting plasma glucose (FPG) range of 280–350 mg/dl were considered diabetic and included in the study.

4.3.1. Screening for antihyperglycemic effect of various extracts and dose determination of \(B.\) monosperma leaves and flowers in STZ-induced diabetic rats – a short term 15 day study.

A total of 54 rats were utilized and the animals were randomly divided into 9 groups of six animals each as given below. Different doses (25, 50 and 100 mg/kg b.wt) of ethyl acetate active fractions of \(B.\) monosperma flowers (BMF) were suspended in vehicle solution (Dimethylsulfoxide [DMSO] 0.5%; 1ml/kg b.wt) and administered orally using an intragastric tube for 15 days daily to the respective groups. Reference drug glibenclamide (600 µg/kg b.wt) was suspended in distilled water as vehicle solution and administered orally for 15 days daily. In a separate experiment, ethyl acetate active fractions of \(B.\) monosperma leaves (BML) (12.5, 25 and 50 mg/kg b.wt) was used instead of BMF fraction to determine the effective dose in experimental animals.
Group 1- Normal rats treated with vehicle alone
Group 2- STZ induced diabetic rats treated with vehicle alone
Group 3- STZ induced diabetic rats + BMF fraction (25 mg/kg b.wt)
Group 4- STZ induced diabetic rats + BMF fraction (50 mg/kg b.wt)
Group 5- STZ induced diabetic rats + BMF fraction (100 mg/kg b.wt)
Group 6- STZ induced diabetic rats + BML fraction (12.5 mg/kg b.wt)
Group 7- STZ induced diabetic rats + BML fraction (25 mg/kg b.wt)
Group 8- STZ induced diabetic rats + BML fraction (50 mg/kg b.wt)
Group 9- STZ induced diabetic rats + Glibenclamide (600 µg/kg b.wt)

All 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 50 mg/kg b.wt dose of BML fraction and 100 mg/kg b.wt of BMF fraction for 15 days showed significant plasma glucose lowering effect in STZ-induced diabetic rats. This dose was fixed as effective dose and was selected for further biochemical study.

4.3.2. Screening for antihyperglycemic effect of E.A active fractions of B. monosperma leaves and flowers in normal and STZ-induced diabetic rats – 45 days Study

A total of 42 rats (18 normal; 24 STZ-diabetic surviving rats) were used. The rats were divided into 7 groups of 6 rats each. BML fraction and BMF fraction were suspended in vehicle solution (Dimethylsulfoxide [DMSO] 0.5%; 1ml/kg b.wt) and administered orally using an intragastric tube for 45 days to the respective groups.
Group 1- Normal rats treated with vehicle alone
Group 2- Normal rats + BMF fraction (100 mg/kg b.wt)
Group 3- Normal rats + BML fraction (50 mg/kg b.wt)
Group 4- STZ induced diabetic rats treated with vehicle alone
Group 5- STZ induced diabetic rats + BMF fraction (100 mg/kg b.wt)
Group 6- STZ induced diabetic rats + BML fraction (50 mg/kg b.wt)
Group 7- STZ induced diabetic rats + Glibenclamide (600 µg/kg b.wt)
After 45 days of treatment, the 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
another with anticoagulant-potassium oxalate and sodium fluoride for plasma insulin assay. Tis-
sues (liver, kidney and pancreas) were surgically removed, washed with cold physiological sa-
line, cleared off the adherent lipids and immediately transferred to ice-cold containers and
weighed. Erythrocytes were also prepared for the estimation of various biochemical preparations.

4.4. Body Weight and feed Intake:

Individual body weights of the animals were recorded on weekly basis changes in body
weights of the animals were reported. Individual food consumptions were recorded daily through
out the study and reported.

4.5. Biochemical Parameters

4.5.1. Haematology

Whole blood was used immediately for estimating Haemoglobin (Hb) and Glycosylated
haemoglobin (HbA\textsubscript{1c}).

4.5.2. Plasma

i) Fasting Plasma Glucose and insulin.

ii) **Lipid Per oxidative markers**: Thiobarbituric acid reactive substances (TBARS) and
Lipid hydro peroxides (HP).

iii) **Nonenzymic antioxidants**: Ascorbic acid, \( \alpha \)-tocopherol, Reduced glutathione (GSH)
and ceruloplasmin.

iv) **Lipid profile**: Total cholesterol (TC), free fatty acids (FFA), Triglycerides (TG),
Phospholipids (PL), High density lipoprotein (HDL), Low density lipoprotein (LDL)
and very low density lipoprotein (VLDL) cholesterol.

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

vi) **Glycoprotein component analysis**: Total hexoses, Hexosamine, Sialic acid, and
Fucose.

vii) **Protein profile**: Total protein, Albumin and Globulin
4.5.3. Serum

i) **Carbohydrate metabolizing enzymes:** Glucokinase, Glucose 6-phosphate dehydrogenase, Glucose 6-phosphatase, Fructose 1, 6-bisphosphatase, Glycogen phosphorylase and Glycogen synthase.

ii) **Hepatic marker enzymes:** Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Acid phosphatase (ACP), Alkaline phosphatase (ALP) and \( \gamma \)-glutamyl transferase (GGT).

4.5.4. Erythrocyte

i) Lipid peroxidation markers (as mentioned above).

ii) **Antioxidants:** Enzymic: Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx) and Non-enzymic antioxidants (as mentioned above).

iii) **Membrane bound enzymes:** Total ATPase, Na\(^+\), K\(^+\)-ATPase, Ca\(^{2+}\)-ATPase and Mg\(^{2+}\)-ATPase

4.6. Tissue Parameters

4.6.1. Liver and kidney

i) Carbohydrate metabolizing enzymes (as mentioned above)

ii) Glycoprotein component analysis (as mentioned above)

iii) Membrane bound enzymes (as mentioned above)

iv) Lipid profiles (as mentioned above)

v) Histopathology-lite microscopic studies

4.6.2. Liver, Kidney and Pancreas

Lipid per oxidative markers, Enzymic and Nonenzymic antioxidant markers and (as mentioned above)

4.7. Processing of Blood and Tissue Samples

4.7.1. Preparation of serum and plasma

Blood samples were drawn from each animal into a citrated polypropylene tube. Plasma will be separated by centrifugation and frozen at -20 or -80°C.
4.7.2. Collection of Urine
Rats were deprived of feed but provided with water *ad libitum*. Rats were housed in metabolic cages for 24 hour urine collection. Urine sample was collected in a chilled beaker and centrifuged to remove any impurities.

4.7.3. Erythrocyte preparation
After the separation of plasma, the buffy coat, enriched in white cells, was removed and the remaining erythrocytes were washed three times with physiological saline. A known volume of erythrocyte was lysed with hypotonic phosphate buffer at pH 7.4. The haemolysate was separated by centrifugation at 2500 rpm for 10 min and the supernatant was used for the estimation of enzymic antioxidants.

4.7.4. Tissue homogenate preparation
Liver and kidneys tissues (250 mg) were sliced into pieces and homogenised 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.

4.7.5. Tissue sampling for histopathological study
For histopathological study, three rats from each group were perfused with cold physiological saline, followed by formalin (10% formaldehyde). The liver, kidney and pancreas were excised immediately and fixed in 10% formalin.

4.7.6. Tissue sampling for Immunohistochemical study
At the end of the experiment, the animals were sacrificed by cervical dislocation. Whole pancreas from rats was removed and fixed in 10% buffered formalin for 24 h. Tissues were dehydrated in graded series of alcohol, embedded in paraffin, sectioned at 5µm thickness and used for immuno staining.

4.8. Biochemical determinations

4.8.1. Estimation of haemoglobin
Haemoglobin in the blood was estimated by the method of Drabkin & Austin (1932).
Reagents

1. Drabkin’s reagent: 200 mg of potassium ferricyanide, 50 mg of potassium cyanide and 1.0 g of Na$_2$CO$_3$ were dissolved in water and made up to one litre. 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.

4.8.2. Estimation of glycosylated haemoglobin (HbA1c)
Glycosylated haemoglobin in the blood was estimated by the method of Sudhakar Nayak & Pat-tabiraman, (1981).

Reagents

1. 1 M potassium oxalate in 2 M hydrochloric acid (Oxalate-hydrochloride acid).
2. Phenol: 80%
4. TCA: 40%
5. Saline.
6. Stock solution: Stock standard was prepared by dissolving 100 mg of fructose in 100 mL of water.
7. 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. 0.5 mL of saline washed erythrocytes was lysed with 5 mL of water, mixed and incubated at 37 °C for 15 min. The contents were centrifuged and the supernatant was discarded, then 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 concentrated sulphuric acid 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 concentrated sulphuric acid were added to this. The color developed was read at 480 nm after 30 min.

The concentration of glycosylated Hb was expressed as mg/g of hemoglobin.

4.8.3. Estimation of liver glycogen

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

Reagents

1. Potassium hydroxide: 30%
2. Absolute alcohol.
3. Anthrone reagent: 0.2% of anthrone in concentrated sulphuric acid was prepared just before use.
4. Ammonium acetate: 1 M.
5. Stock standard: 1 mg/mL -100 mg of anhydrous D-glucose was dissolved in 100 mL of distilled water containing 0.01% benzoic acid.
6. 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 1M ammonium acetate was added to precipitate glycogen and left it in a freezer overnight for complete precipitation. Glycogen was collected by centrifuging at 300 rpm for 20 min, then the precipitate was dissolved with the aid of heating and again the glycogen was reprecipitated 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 0.91 and expressed as mg/100 g of tissue.

4.8.4. Estimation of tissue protein

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

Reagents

1. 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.
2. Folin’s phenol reagent: Dilute 1:2 with distilled water.
3. Stock standard: 100 mg of bovine serum albumin/ 100 mL of water.
4. Working standard: 10 mL of the stock standard was diluted to 100 mL to get a working Standard containing 0.1 mg/mL

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 tissue.

4.9. Carbohydrate Metabolic Enzymes

4.9.1. Assay of hexokinase D (glucokinase)

(ATP: D-hexose 6-β-phosphotransferase: (EC 2.7.1.1). Hexokinase D was assayed by the method of Brandstrup et al., (1957).

Reagents

1. Glucose solution: 0.005 M
2. ATP solution: 0.72 M
3. Magnesium chloride solution: 0.05 M
4. Dipotassium hydrogen phosphate solution: 0.0125 M
5. Potassium chloride solution: 0.1 M
6. Sodium fluoride solution: 0.5 M
7. Tris-HCl buffer: 0.01M, 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 5 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 (1991) 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 glucose phosphorylated /h/mg protein.

4.9.2. Assay of glucose 6-phosphatase

(Glucose 6-phosphate phosphohydrolase: (EC 3.1.3.9) Glucose 6-phosphatase was assayed by the method of Koide & Oda, (1959).

Reagents
1. Maleic acid buffer: 0.1 M, pH 6.5
2. Glucose 6-phosphate: 0.01 M 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. TCA: 10%

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 Fiske and Subbarow (1925) method. 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.

4.9.3. Assay of fructose 1, 6-bisphosphatase

(Fructose 1, 6-bisphosphate phosphohydrolase: EC 3.1.3.11). Fructose 1, 6-bisphosphatase was assayed by the method of Gancedo & Gancedo (1971).

Reagents

1. Tris-HCl buffer: 0.1 M, pH 7.0
2. Substrate: Fructose 1,6- bisphosphate, 0.05 M
3. Magnesium chloride: 0.1 M
4. Potassium chloride: 0.1 M
5. EDTA solution: 0.001 M
6. TCA: 10%
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. 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 phosphorus liberated/h/mg protein.
4.9.4. Assay of Hepatic Glycogen synthase and Glycogen phosphorylase

Hepatic Glycogen synthase and Glycogen phosphorylase were assayed by the method of Cornblath et al., 1963.

**Reagents**

1. Tris HCl buffer: 0.05 M, pH 7.5
2. NADP⁺: 0.1 M
3. Magnesium chloride: 0.1 M
4. Potassium chloride: 0.1 M
5. EDTA solution: 0.001 M
6. TCA: 10%
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 catalyzes the transfer of phosphate from phosphoenolpyruvate to UDP and the pyruvate liberated was estimated colorimetrically. The property of synthesizing glycogen from glucose-1-phosphate liberating inorganic phosphate is made use in the assay of glycogen phosphorylase activity.

The activity of glycogen synthase enzyme was expressed as µmoles of UDP formed/h/mg protein and the activity of glycogen phosphorylase enzyme was expressed as µmoles of Pi liberated/h/mg protein.

4.9.5. Glucose 6-phosphate dehydrogenase

(D-glucose 6-phosphate NADP oxidoreductase (EC 1.1.1.49). Glucose 6-phosphate dehydrogenase in the erythrocytes and liver was assayed by the method of Ellis & Kirkman (1984).

**Reagents**

1. Tris HCl buffer: 0.05 M, pH 7.5
2. Magnesium chloride: 0.1 M
3. NADP\(^+\) : 0.1 M
4. 2,6-Dichlorophenol indophenol: 0.01\% in distilled water (freshly prepared)
5. Phenazine methosulphate: 0.005\% in distilled water (freshly prepared)
6. Substrate: 0.02 M glucose 6-phosphate solution.

**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 methosulphate, 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.

Change in OD/min x molar extinction co-efficient x temperature correction factor (Tf). Tf at 37 °C is 0.76 and molar extinction co-efficient of NADPH is 6/17.6.

The activity of enzyme was expressed as mIU/mg of protein for tissue or IU/g of Hb for erythrocytes.

**4.10. Lipid Peroxidation Parameters**

**4.10.1. Estimation of TBARS**

The concentration of TBARS in the plasma, erythrocytes and tissues was estimated by the method of Nichans & Samuelson (1968).

In this method, malondialdehyde and other thiobarbituric acid reactive substances (TBARS) reacts with thiobarbituric acid in an acidic condition to generate a pink color chromophore which was read at 535 nm.

**Reagents**

1. TCA: 15%
2. HCl: 0.25 N
3. TBA: 0.375\% 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 plasma or mmol/100 g of tissues or nmol/mg of protein for erythrocytes.

### 4.10.2. Estimation of lipid hydroperoxides

Lipid hydroperoxide in the plasma, erythrocytes and tissues was estimated by the method of Jiang et al., (1992).

Oxidation of ferrous ion (Fe$^{2+}$) 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 H$_2$SO$_4$ (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 in a Spectronic 20 at 560 nm.

Lipid hydroperoxides were expressed as nmol/mL of plasma or mmol/100 g of tissues or µmol/mg of protein for erythrocytes.

### 4.11. Non Enzymatic Antioxidants

#### 4.11.1. Estimation of reduced glutathione (GSH)

Reduced glutathione in the plasma, erythrocytes 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 sulphhydryl groups.
Reagents
1. Phosphate buffer: 0.1 M, pH 8.0
2. TCA: 5%
3. Ellman’s reagent: 34 mg of DTNB in 10 mL of 0.1% sodium citrate.
4. Disodium hydrogen phosphate: 0.3 M
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
A known weight of tissue was homogenized in phosphate buffer (0.1 M pH 7.0). 0.5 mL of homogenate or plasma 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 in a Spectronic 20 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 plasma or µmol/mg of protein for tissues or mg/g of Hb for erythrocytes.

4.11.2. Estimation of ascorbic acid (vitamin C)
Ascorbic acid in the plasma, erythrocytes and tissues was estimated by the method of Roe & Kuther (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 thiourea, a mild reducing agent. The coupled dinitrophenylhydrazine was converted into a red colored compound when treated with sulphuric acid and read in a Spectronic 20 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: 100 mg of L-ascorbic acid in 100 mL of 6% TCA.
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, stoppered 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 plasma or µg/mg of protein for tissue or µg/mg of Hb for erythrocytes.

**4.11.3. Estimation of α–tocopherol (vitamin E)**

α-Tocopherol in the plasma, erythrocytes 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 in 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 plasma or µg/mg protein for tissue or µg/mg of Hb for erythrocytes.

4.11.4. Estimation of plasma ceruloplasmin

Plasma ceruloplasmin was estimated by the method of Ravin (1961). Ceruloplasmin was measured on the basis of the oxidation of p-phenylenediamine to form a purple colored compound.

Reagents

1. Acetate buffer: 0.4 M, pH 5.5
2. Sodium azide: 0.5% solution
3. p-Phenylenediamine hydrochloride: 0.5% in acetate buffer, freshly prepared prior to use

Procedure

8.0 mL of buffer was added in 2 tubes marked as control and test. To the test, 0.05 mL of plasma and 1.0 mL of sodium azide solution were added. To the control, 1.0 mL of azide solution was added and mixed well. To both the tubes, 1.0 mL of p-phenylenediamine was added, mixed well and kept in a water bath at 37 °C for 1 h. 1 mL of sodium azide was added to the test and all the tubes were kept at 4 °C for 30 min. The color developed was read at 540 nm with control as blank.

Values were expressed as mg/dL of plasma.
4.12. Enzymatic Antioxidants

4.12.1. Assay of superoxide dismutase (SOD, EC 1.15.1.1)

Superoxide dismutase in the erythrocytes and 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 formazan. The reaction was initiated by the addition of NADH and 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. Phenazine methosulphate (PMS): 186 µmol
6. Nitroblue tetrazolium (NBT): 300 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 phenazine 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 at 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; 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 in-
hibit 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/min/mg of protein for tissues or Unit/min/mg of Hb for erythrocytes.

4.12.2. Estimation of Catalase (CAT, EC 1.11.1.6)

The activity of catalase in the erythrocytes and tissues was determined by the method of Sinha (1972).

Dichromate in acetic acid was converted to perchromic acid and then to chromic acetate, when heated in the presence of H₂O₂. The chromic acetate formed was 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 4 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 proceeded as for the test.

The specific activity was expressed as µmol of H₂O₂ consumed / min/ mg of protein for tissues or µmol of H₂O₂ consumed /min/mg of Hb for erythrocytes.
4.12.3. Estimation of glutathione peroxidase (EC 1.11.1.19)

The activity of GPx in the erythrocytes and tissues was measured by the method of Ro-

A known amount of enzyme preparation was allowed to react with H$_2$O$_2$ 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. H$_2$O$_2$ 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 0.1mL of H$_2$O$_2$ 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 µg of GSH utilized/min/mg of protein for tissues or µg of GSH utilized/min/mg of Hb for erythrocytes.

4.13. Analysis of Lipid Profile

4.13.1. 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-ether 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; 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 vol-
ume. Aliquot of this extract was then used for the estimation of cholesterol, free fatty acids, triacylglycerol and phospholipids. Serum was also processed similarly.

4.13.2. Estimation of total cholesterol

Total cholesterol in the plasma, erythrocytes 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 plasma or 10 µL of lipid extract, 1.0 mL of enzyme reagent was added, mixed well and kept at 37 °C for 5 min. 10 µL of cholesterol standard and distilled water (blank) were also processed similarly. The absorbance was measured at 510 nm.

Cholesterol concentration was expressed as mg/dL of plasma or mg/100 g of tissue or µg/mg of protein for erythrocytes.

4.13.3. 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 plasma.

**4.13.4. 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 plasma.

**4.13.5. 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: 1 M
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. Diphenyl carbazide 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 plasma or mg/100 g of tissues.

4.13.6. Estimation of Triacylglycerol
Triacylglycerol in the plasma and tissues were estimated using the diagnostic kit based on the enzymic method described by Mc Gowan 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$_2$O$_2$ reacts with 4-aminoantipyrine and 3, 5-dichloro-2-hydroxybenzene sulfonic acid in the presence of peroxidase to produce red quinonemine 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 plasma 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 plasma or mg/100 g of tissues.

4.13.7. Estimation of phospholipids
Phospholipids in the plasma, erythrocytes and tissues were estimated by the method of Zilversmit & Davis (1950).

Phospholipids were digested with concentrated sulphuric acid to liberate the lipid bound inorganic phosphorus. Then it reacted with ammonium molybdate to form phosphomolybdic acid
which was treated with 1-amino-2-naphthol-4-sulphonic acid to form a stable blue color. The intensity of the color was proportional to the amount of phospholipids in the sample.

**Reagents**

1. Sulphuric acid: 5.0 N
2. Ammonium molybdate: 2.5% in 5.0 N sulphuric acid. 25 g of ammonium molybdate was dissolved, in about 200 mL of water. It was transferred to a one litre volumetric flask containing 500 mL of 10 N sulphuric acid and made up to the mark with water.
3. ANSA reagent: 0.2 g of ANSA was mixed with 1.2 g of sodium bisulphite and 1.2 g of sodium sulphite. 0.25 g was taken from this mixture and dissolved in 10 mL water.
4. Stock standard phosphorus: 35.1 mg of potassium dihydrogen phosphate was dissolved in water. To this, 1 mL of 10 N sulphuric acid was added and made up to 100 mL with water (80 µg/mL phosphorus).
5. Working standard phosphorus: Stock solution was diluted to get a concentration of 8 µg/mL of phosphorus.

**Procedure**

An aliquot of the lipid extract was evaporated to dryness. 1.0 mL of 5.0 N sulphuric acid was added and digested till light brown. Then 2 to 3 drops of concentrated nitric acid was added and the digestion was continued till it became colorless. After cooling, 1 mL of water was added and heated in a boiling water bath for about 5 min. Then, 1.0 mL of ammonium molybdate and 0.1 mL of ANSA were added. The volume was then made up to 10.0 mL with distilled water and the absorbance was measured at 680 nm within 10 min. Standards in the concentration range of 2-8 mg were treated in the similar manner. The values obtained were multiplied with a factor 25 to convert inorganic phosphorus to its phospholipids equivalents.

The amount of phospholipids was expressed as mg/dL of plasma or mg/100 g of tissue or µg/mg of protein for erythrocytes.

**4.14. Estimation of Marker Enzymes**


Serum aspartate aminotransferase was assayed by using the diagnostic kit based on the method of Reitman & Frankel (1957).
AST catalyses the transfer of amino group from L-aspartate to α-ketoglutarate with the formation of oxaloacetate and glutamate. The amount of oxaloacetate was measured by converting it into pyruvate by treating with aniline citrate and then reacting the pyruvate with 2,4-dinitrophenyl hydrazine 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 mM DNPH in 2.0 N hydrochloric acid.
4. Sodium hydroxide: 0.4 N
5. Pyruvate standard: 2.0 mM

**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 treated in the same manner and read at 520 nm after 10 min.

The results were expressed as IU/L of serum.

**4.14.2. Assay of alanine aminotransferase (ALT, EC 2.6.1.2)**

Serum alanine aminotransferase was assayed by using the diagnostic kit based on the method of Reitman & 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-dinitrophenylhydrazone derivative which is brown colored in alkaline medium. The absorbance of this hydrazone 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 same as that used for the assay of aspartate transaminase except the incubation time which was reduced to 30 min (60 min for AST).

   The results were expressed as IU/L of serum.

4.14.3. Estimation of alkaline phosphatase (ALP, EC 3.1.2.3.1)

   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. Colour 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 510 nm.

   The enzyme activity was expressed as IU/L of serum.

4.14.4. Estimation of γ-glutamyl transferase (GGT, EC 2.3.2.2)

   The enzyme activity was assayed according to the method of Rosalki & Rau (1972).

   GGT hydrolyses peptide bonds, in which a terminal glutamic acid residue is linked by its γ-
carboxyl group to an amino group. The enzyme is of low specificity for non-glutamyl moiety, so that synthetic substrates such as \(\gamma\)-glutamyl-p-nitroanilide are acted upon by GGT which catalyses the simultaneous transfer of glutamyl residues to an amino acid or peptide acceptor, glycyl glycine is chosen for this purpose, to form a yellow product paranitroanilide.

**Reagents**

1. Tris-HCl buffer: 0.1 M, pH 8.2
2. Substrate: 30.3 mg of L-\(\gamma\)-glutamyl-p-nitroanilide/10 mL. The substrate was sparingly soluble and was dissolved by warming to 50-60 °C. The substrate solution was used within two hours of its preparation. 13.2 mg of glycyl glycine was dissolved in 10.0 mL of distilled water. This was used as a second substrate.
3. Acetic acid: 10%
4. Standard p-nitro aniline: 13.8 mg of p-nitro aniline/100 mL of distilled water.

**Procedure**

0.5 mL of serum was added to the incubation mixture containing 0.5 mL \(\gamma\)-glutamyl-p-nitroanilide, 2.0 mL glycyl glycine and 1.0 mL buffer. After incubation for 30 min at 37 °C the reaction was arrested by the addition of 1.0 mL of 10% acetic acid. The amount of p-nitro aniline liberated in the supernatant was measured as the difference in optical density at 410 nm, between samples, with and without substrate. The substrate incubated in the absence of serum under the same condition was used as a reference blank. Enzyme activity was expressed as IU/L of serum.

**4.15. Estimation of Nephritic Markers**

**4.15.1. Estimation of urea**

Urea in the plasma and urine was estimated by using the diagnostic kit based on the method of Fawcett & Scott (1960).

Urea is hydrolysed 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 ethylenediaminetetraacetic 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 colour 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 colour developed was read at 600 nm.

The values were expressed as mg/dL of plasma and urine.

4.15.2. Estimation of uric acid
Uric acid in the plasma urine 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 sulfonic acid and 4-amino anti-pyrine to give quinoneimine dye. Intensity of the colour 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 colour developed was read at 510 nm.

The values were expressed as mg/dL of plasma and urine.

4.15.3. Estimation of Creatinine
Creatinine in the serum and urine was estimated using the diagnostic kit based on the method of Tietz (1987) using Jaffe’s (1886) colour 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 colour at a lower rate than does creatinine. The initial rate of colour 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 and urine.

**4.16. Estimation of Protein Profile**

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

Total protein and albumin in the serum were estimated by the method of Reinhold (1953).

Proteins form a purple coloured 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 made 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 mL of water and treated with 5.0 mL of biuret reagent. The purple colour developed was read at 540 nm after 15 min using reagent blank.

Values were expressed as g/dL of serum.

4.16.2. Estimation of globulin

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

4.17. Estimation of Glycoprotein Components

4.17.1. 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 100 mL of water. 1.0 mL of this was mixed with 7.5 mL H$_2$SO$_4$·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.

4.17.2. Estimation of hexosamine

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 concentrated 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 tube graduated at 10 mL, 5 mL of 95% 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 added 2 mL of 3 N HCl and hydrolysed in a boiling water bath with an air condenser for 4 h.

Neutralise the hydrolysate 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. Added 5 mL of 95% ethanol and mixed well. Added 1 mL of Ehrlich reagent 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.

**4.17.3. 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. Placed the test tube in a boiling water bath for exactly 15 min with a glass marble to prevent evaporation, cooled the tubes by immersion in water and filtered. Pipette out 2 mL of clear filtrate in each of tubes, added 4 mL of DPA reagent into one of each pair of tubes and 4 mL of acid-mixture containing without DPA into another.

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.

4.17.4. Estimation of fucose

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

Reagents

1. Sulphuric acid reagent: Conc H$_2$SO$_4$ and distilled H$_2$O 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.

4.18. Membrane Bound Enzymes

4.18.1. Determination of total ATPases
(ATP-phosphohydrolase) (EC: 3.6.1.3)

The activity of the enzyme in the erythrocytes and tissues was estimated according to the method of Evans, (1969).

Reagents

1. Tris-HCl buffer: 0.1 M, pH 7.0
2. Sodium chloride: 0.1 M  
3. Potassium chloride: 0.1 M  
4. Adenosine triphosphate: 0.01 M ATP  
5. TCA: 10%

**Procedure**

2 mL incubation volume of test consisted of 1.5 mL of Tris- HCl buffer, sodium chloride, potassium chloride, adenosine triphosphaste, and suitable aliquot of the enzyme. A control was also run simultaneously without the enzyme. The tubes were incubated at 37 °C for 15 min. The reaction was arrested by 10% trichloroacetic acid. The enzyme was then added to the control. The tubes were centrifuged at 3000 × g for 10 min. The inorganic phosphorus liberated was estimated by Fiske & Subbarow (1925) method as described earlier.

The total ATPases activity was expressed as µmol of Pi liberated/h/mg of protein for erythrocytes and tissues.

**4.18.2. Determination of (Na++ K+) - ATPase [E.C. 3.6.1.3]**

Sodium and potassium dependent ATPase activity in the erythrocytes and tissues was assayed by the method of Bonting (1970).

**Reagents**

1. Tris-HCl buffer: 184 mM, pH 7.5  
2. Magnesium sulphate: 50 mM  
3. Sodium chloride: 160 mM  
4. Potassium chloride: 50 mM  
5. EDTA: 1 mM  
6. ATP: 4 mM  
7. TCA: 10%

**Procedure**

The incubation volume of the test consisted of 1 mL of Tris-HCl buffer and 0.2 mL each of magnesium sulphate, potassium chloride, sodium chloride, EDTA, ATP and suitable aliquot of the enzyme. Control without enzyme was also treated similarly. The tubes were incubated at 37 °C for 15 min. The reaction was arrested by 10% trichloroacetic acid followed by the addition
of the enzyme to the control. The tubes were centrifuged at 3000 x g for 10 min. The inorganic phosphorus was estimated by the method of Fiske & Subbarow (1925) as described earlier.

The enzyme activity was expressed as µmol of Pi liberated/h/mg of protein for erythrocytes and tissues.

4.18.3. Determination of Mg2+- ATPase [E.C. 3.6.1.4]

The activity of the enzyme in the erythrocytes and tissues was estimated by the method of Ohinashi et al., (1982).

Reagents

1. Tris-HCl buffer: 125 mM, pH 7.6
2. Magnesium chloride: 25 mM
3. ATP: 0.01 M.
4. TCA: 10%

Procedure

0.5 mL of incubation volume of the test consisted of 0.1 mL each of Tris-HCl buffer, magnesium chloride, ATP, distilled water and enzyme preparation. Control without enzyme was run simultaneously. The tubes were incubated at 37 °C for 15 min. The reaction was then arrested by 10% trichloroacetic acid followed by 0.1 mL of the enzyme of the control. The tubes were centrifuged at 3000 xg for 10 minutes. The inorganic phosphorus was estimated by the method of Fiske & Subbarow (1925) as described earlier.

The enzyme activity was expressed as µmol of Pi liberated/h/mg of protein for erythrocytes and tissues.

4.18.4. Determination of Ca2+-ATPase [E.C.3.6.1.5]

The activity of Ca2+-ATPase activity in the erythrocytes and tissues was measured according to the method of Hjerken & Pan, (1983).

Reagents

1. Tris-HCl buffer: 125 mM, pH 8.0
2. Calcium chloride: 50 mM
3. ATP: 10 mM
4. TCA: 10%
5. Fiske and Subbarow reagents
Procedure

The incubation mixture contained 0.1 mL of buffer, 0.1 mL of calcium chloride, 0.1 mL of ATP, 0.1 mL of water and 0.1 of enzyme (Tissue homogenate or erythrocytes membrane). The contents were incubated at 37 °C for 15 min. The reaction was then arrested by the addition of 0.5 mL of ice-cold 10% TCA. The amount of phosphorus liberated was estimated by the method of Fiske & Subbarow (1925).

The enzyme activity was expressed as μmol of Pi liberated/h/mg of protein for erythrocytes and tissues.

4.19. Histological Studies

4.19.1. Light microscopic studies–Paraffin method

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

Reagents:
1. Physiological saline (0.9%)
2. Bouin-Hollande fixative
3. Ehrlich’s haematoxylin
4. Eosin

Procedure

The tissues such as, pancreas, liver and kidney 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 haematoxylin with eosin as the counter stain. The slides were mounted using DPX mount.

4.19.2. 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 immuno-chemicals were purchased from Biomeda, CA, USA.

Tissues were dehydrated in graded series of alcohol, embedded in paraffin, sectioned at 5 µm thickness and used for immuno-staining. 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% 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% ethanol. The slides were placed in two changes of 70% 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 diplan microscope (Leica, Germany) and photographed.

ANTIPHARMACOLOGICAL ACTIVITY

4.20. Screening for anti-inflammatory effect of Ethyl acetate active fractions of *B. monosperma* leaves in Wistar rats.

Experimental animals

A total of 42 adult male albino rats of Wistar strain weighing approximately 180–200 g rats (18 normal; 24 STZ-diabetic surviving rats) were used in the present study. The rats were divided into 7 groups of 6 rats each. *BML* fraction was suspended in vehicle solution (Dimethylsulfoxide
[DMSO] 0.5%; 1ml/kg b.wt) and administered orally using an intragastric tube for 21 days to the respective groups.

Group 1: Normal control (vehicle solution)
Group 2: Disease Control (Complete Freund’s adjuvant 0.1ml)
Group 3: BML fraction (50 mg/kg b.wt)
Group 4: BML fraction (100 mg/kg b.wt)
Group 5: BML fraction (250 mg/kg b.wt)
Group 6: Prednisolone (10 mg/kg b.wt)

4.20.1. Experimental induction of Inflammation

On the 0th day, the basal paw volume of left hind paw of each animal was measured using mercury plethysmometer. On the 1st day all the animals except normal group were once anaesthetized, they were injected into the ankle joint of left hind paw with 0.1 ml of Complete Freund’s adjuvant (Sigma Aldrich, USA) containing 0.1 mg of heat killed mycobacterium tuberculosis cells in liquid paraffin and were allowed to recover to serve as control. Dosing with standard drug prednisolone and BML fraction was started on the same day i.e. 1st day and continued for 21st day. Normal and disease control groups rats receives vehicle solution throughout study while the rest experimental groups animals receives respective treatment once daily by oral route. Paw volume of injected paw was measured on 4th, 8th, 14th and 21st day of study period. At the end of day 21st, the animals were anaesthetized with anesthetic ether and blood was isolated from the retro orbital route to all the groups of animals and various haematological parameters such as Hemoglobin content, Total WBC, RBC, and Erythrocyte Sedimentation Rate (ESR) were estimated using routine laboratory methods. The body weight of the animals was measured by digital balance to access the course of the disease at the initial day before induction and at the end of 21st day.

4.21. TOXICITY STUDIES

Acute and Sub-Acute toxicity studies were carried out to determine the toxic effect of active fractions of B.monosperma leaves and flowers using male albino Wistar rats.
4.21.1. Acute Toxicity Study

An experiment was performed to know whether any acute toxicity symptoms were produced by active fractions of *B. monosperma* leaves and flowers. Rats fasted for 12 hours were randomly divided into drug treated as ‘test’ groups and vehicle treated as ‘control’ groups making up to 6 groups of 6 rats in each. Both active fractions of leaves and flowers were dissolved in DMSO (125, 250, 500, 1000, 2000 mg/kg b.wt) and separately administered orally to the rats in each of the test groups. Each of the rats in the control groups was treated with vehicle alone. Then the rats in both the test and control groups were allowed free access to food and water, and behavioural changes were observed over a period of 72 hours for sign of acute toxicity. The number of mortality caused by the compound within this period of time was observed in order to fix the lethal dose \( (LD_{50}) \) of the compound (Lorke, 1983).

4.21.2. Sub-Acute Toxicity Study

Male albino Wistar rats, 6 groups of animals were selected (6 rats each group) separately to study the Sub-Acute toxicity of leaf and floral fractions. The fractions were dissolved in DMSO and administered to fasted rats in a dosage of effective dose \( (ED_{50}) \), 2.5 times of \( ED_{50} \), 5 times of \( ED_{50} \), 10 times of \( ED_{50} \), 15 times of \( ED_{50} \) (50, 125, 250, 500 and 750 mg/kg b.wt) separately orally to the rats in each of group for 28 days. Each of the rats in the control groups was treated with vehicle alone. Subsequently the rats in both the test and control groups were allowed free access to food and water. At the end of the experiment, urine was collected for the qualitative analysis of nephritic markers. All the animals were sacrificed after overnight fasting and the blood samples were collected. Hematological examinations like RBC, WBC, and biochemical parameters like, urea, uric acid, creatinine, alkaline phosphatase, acid phosphatase, aspartate aminotransferase and alanine aminotransferases were carried out. Histopathological examinations of liver and kidney were observed. The experimental design was given below:

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal control</td>
</tr>
<tr>
<td>2</td>
<td>Normal + BMF fraction 50 mg/kg b.wt</td>
</tr>
<tr>
<td>3</td>
<td>Normal + BMF fraction 125 mg/kg b.wt</td>
</tr>
<tr>
<td>4</td>
<td>Normal + BMF fraction 250 mg/kg b.wt</td>
</tr>
<tr>
<td>5</td>
<td>Normal + BMF fraction 500 mg/kg b.wt</td>
</tr>
<tr>
<td>6</td>
<td>Normal + BMF fraction 750 mg/kg b.wt</td>
</tr>
</tbody>
</table>
Group 7  Normal + *BML* fraction 50 mg/kg b.wt
Group 8  Normal + *BML* fraction 125 mg/kg b.wt
Group 9  Normal + *BML* fraction 250 mg/kg b.wt
Group 10 Normal + *BML* fraction 500 mg/kg b.wt
Group 11 Normal + *BML* fraction 750 mg/kg b.wt

4.21.3. Parameters Observed in Sub-Acute Toxicity Study

- Body Weight
- Food Intake
- **Hematological parameters**: Estimation of Haemoglobin, RBC count (millions/mm$^3$ of blood), WBC count (thousands/mm$^3$ of blood).
- **Serum Enzyme markers**: Alanine Transaminase (ALT), Aspartate Transaminase (AST), Alkaline Phosphatase (ALP) and Acid Phosphatase (ACP).
- **Nephritic markers**: Urea, Uric Acid and Creatinine
- Organ weights (Liver & Kidney)

4.22. Statistical Analysis

The experimental results are represented as Mean ±SEM. The data were statistical analysed by one way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. P values< 0.05 were considered as significant.