Chapter 4

EXPERIMENTAL INVESTIGATIONS
## Chapter 4: Experimental Investigations

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of the Sub Title</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Collection and Preparation of Polyherbal formulation</td>
<td>32</td>
</tr>
<tr>
<td>4.2</td>
<td>Standardization of Polyherbal Formulation</td>
<td>32</td>
</tr>
<tr>
<td>4.3</td>
<td>Materials and Methods</td>
<td>41</td>
</tr>
</tbody>
</table>
Chapter 4

EXPERIMENTAL INVESTIGATIONS

4.1. Collection and Preparation of Polyherbal formulation

For preparation of polyherbal formulation the ingredients were purchased from local raw material traders. The powder form of all ingredients (table 1) passed through 80# sieve and then mixed mutually in particular proportions in a geometrical manner to get uniform mixture.

4.2. Standardization of Polyherbal Formulation

Formulation was subjected to the standardization according to WHO guidelines.

Standardization was performed based on organoleptic properties, physical characteristics, physico-chemical evaluation, fluorescence analysis, phytochemical screening, microbial analysis, heavy metal analysis.

4.2.1. Determination of Ash Values of Crude Drugs

After incineration the remaining residue is the ash content of the drug, which purely represents inorganic salts obviously occurring in drug or adhering to it.

4.2.1.1. Determination of Total Ash Value

2g of powder of polyherbal formulation has taken in tarred silica crucible. The powdered drug was incinerated at a temperature not beyond 450°C awaiting free from carbon. The ensuing ash was cooled...
and weighed. The percentage of ash was calculated with reference to the air dried drug.

4.2.2. Determination of Extractive Values:

Extractive values are important tools for assessment of crude drugs and give the nature of chemical constituents present in them. Extracts are obtained by draining the crude drugs with different solvents ranging from non polar to polar are used for measurement of extractive values. All these different solvent extracts subjected for the estimation of petroleum ether soluble extractive value, chloroform soluble extractive value, ethyl acetate soluble extractive value, ethanol soluble extractive value, methanol soluble extractive value, water soluble extractive value.

4.2.2.1. Procedure for Determination of Extractive Values:

5g of the coarsely powdered air dried drug has taken with 100 ml of solvent in a closed flask and macerated for 24 hours, shaken regularly in duration 6 hours and allowed stand for eighteen hours. Filtered speedily and 25 ml of filtrate was evaporated to waterlessness in a tarred flat bottomed shallow dish, and dried at 105\(^{0}\)c to constant weight and weighed. The percentage of soluble extractive (Alcohol, water, ethyl acetate, chloroform, methanol and petroleum ether) was calculated with reference to the air-dried drug.
4.2.3. Phytochemical Screening

4.2.3.1. Test for alkaloids

50mg of the powder + 2 drops of dilute hydrochloric acid, stirred and filtered. The filtrate was tested with various reagents for alkaloids as follows.

**Dragendorff’s Test** (with solution of potassium bismuth iodide)

2 drops of Dragendorff’s reagent was added to 2-3 ml of filtrate, orange brown precipitate was formed, indicates presence of alkaloids.

**Mayer's test** (with solution of potassium mercuric iodide)

2-3 drops of Mayer’s reagent was added along the sides of the test tube which contained 2-3 ml of filtrate. White or creamy precipitate was formed, indicates presence of alkaloids.

**Wagner’s test** (with solution of iodine in potassium iodide)

2-3 drops of Wagner’s reagent was added along the sides of the test tube which contained 2 ml of filtrate. Reddish-brown precipitate was formed, indicates presence of alkaloids.

**Hager’s test** (with saturated picric acid solution)

2-3 drops of hager’s reagent was added along the sides of the test tube which contained 2 ml of filtrate. Yellow precipitate was formed, indicates presence of alkaloids.
4.2.3.2. Test for flavonoids

**Shinoda test** (with magnesium-hydrochloric acid)

2 ml of alcohol was added to the 10 mg of powder. To this, 2 fragments of magnesium turnings and 0.5 ml of conc.HCl were added and observed for the color. Magenta or crimson red color was developed, indicates the presence of flavonoids.

4.2.3.3. Test for steroids

**Salkowski reaction**

2 ml of Chloroform and 2 ml of Concentrated H$_2$SO$_4$ added to 2 ml of test solution, and shaken well. Chloroform layer appeared red and acid layer showed greenish yellow fluorescence.

**Liebermann-Buchard reaction**

Chloroform, 1-2 ml of acetic anhydride and 2 drops of concentrated H$_2$SO$_4$ from the side of test tube were added to 2 ml of test solution. First red, then blue and finally green color appeared.

**Liebermann’s reaction**

3 ml of acetic anhydride added to 3 ml of test solution. Heat and cool. Few drops of concentrated H$_2$SO$_4$ were added. Blue color was appeared.

4.2.3.4. Test for glycosides

Glycoside→Aglycon (genin) +glycon (sugar)
**Test for cardiac glycosides**

**Baljet’s test**

Powder with sodium picrate showed yellow to orange color.

**Killer –killiani test**

Glacial acetic acid, one drop of 5% FeCl₃ and concentrated H₂SO₄ were added to 2 ml of test solution. Reddish brown color was appeared at the junction of the two liquid layers and upper layer appeared in bluish green.

**4.2.3.5. Test for carbohydrates:**

**Molish’s test (general test)**

Few drops of α-naphthol solution in alcohol were added to 2-3 ml test solution, shaken and concentrated H₂SO₄ was added along the sides of the test tube. Violet ring was formed at the junction of two liquids.

**Test for reducing sugars:**

**Fehling’s test**

Equal volume of test solution was added to Fehling’s mixture which one consist of Fehling’s A solution and Fehling’s B solution and boiled for 1 minute. Heated in boiling water bath for 5-10 minutes. First a yellow, then brick red precipitate was observed.
Benedict’s test

In a test tube, equal volumes of Benedict’s reagent and test solution were taken and mixed. Heated in sweltering water bath for 5 min. Solution appears green, yellow or red depending on amount of sugar present in test solution.

Test for monosaccharides

Barfoed’s test

Equal volume of barfoed’s reagent and test solution were mixed, and heated for 1-2 minutes in boiling water and cool. Red precipitate is observed.

Test for hexose sugars:

Selwinoff’s test (for ketohexoses)

3 ml Selwinoff’s reagent was added to 1 ml test solution in boiling water. Heated for 1-2 minutes red color was observed.

Test for non reducing polysaccharides (starch)

Iodine test

A few drops of dilute iodine solution was added to 3 ml test solution & mixed. Blue color appeared, it disappeared on boiling and reappeared on cooling.

Test for tannins and phenolic compounds

5% Fecl₃ solution

Few drops of 5% fecl₃ solution were added to 2-3 ml of test solution. Deep blue color was appeared.
Gelatin solution

Few drops of gelatin solution were added to 2-3 ml of test solution. White precipitate was formed.

Bromine water:

Few drops of acetic acid solution were added to 2-3 ml of test solution. Red color solution is formed.

Dilute iodine solution

Few drops of dilute iodine solution were added to 2-3 ml of test solution. Transient red color was appeared.

4.2.4. Estimation of physical characteristics of polyherbal formulation

4.2.4.1. Estimation of the true density

Principle:

True density is defined as

\[
\text{True density, } \rho_p = \frac{\text{Weight of the powder}}{\text{True volume of the powder}}
\]

Normally, true volume is determined as the volume of liquid it displaces (liquid displacement method). Therefore, true density can be expressed as:

\[
\text{True density, } \rho_p = \frac{\text{Weight of the powder}}{\text{Volume of liquid it displaced by the powder}}
\]
Liquid such as water, ethyl alcohol cannot occupy the pores and crevices. If the powder is nonporous, these liquids can be used. Solids should be insoluble in the solvent and heavier than the liquid.

**Procedure**

A clean and dry specific gravity bottle (25ml) was taken, and the ground –in-stopper was placed. Weighed the empty specific gravity bottle. The weight was \( W_1 \). The bottle was filled with water, and the stopper was kept. The bottle was placed in a water bath (25\(^\circ\)C) for equilibrium (15 minutes). The bottle was removed from water bath. Specific gravity bottle with water was weighed. The weight was \( W_2 \). Water was removed from bottle and the bottle was washed with acetone. The bottle was dried with the help of hot-air dryer. The bottle was filled with benzene and steps 3 and 4 were repeated. Specific gravity bottle with benzene was weighed. The weight was \( W_3 \). Benzene was removed from the bottle and the bottle was washed with water and acetone. Bottle was dried with the help of hot-air dryer. 5 gm of the polyherbal powder was placed in the specific gravity bottle. Specific gravity bottle with polyherbal powder was weighed. The weight was \( W_4 \). Benzene was slowly added to the specific gravity bottle which was containing polyherbal powder with gentle swirling. The bottle was positioned in the thermostat (25\(^\circ\)C) for 15 minutes. The bottle was removed from the water bath, sides were dried. The specific gravity bottle was weighed with powder and benzene. The weight was \( W_5 \).
Calculation:

True density, \( \rho_p = \frac{W_4 - W_1}{V} \)

4.2.4.2. Estimation of the Bulk Density of Powder

Bulk density, \( \rho_p = \frac{\text{Weight of the powder}}{\text{Bulk volume of the powder}} \)

**Procedure**

The polyherbal powder was passed through the sieve No. 20. 5 gm of the powder was taken in a 100 ml measuring cylinder. Fix the measuring cylinder on the table of bulk density apparatus. Adjust the knob for 125 tappings and switch on the apparatus. The volume of the powder was noted at the end of tappings. Adjust the knob for another 50 tapping’s. The volume of the powder was noted at the end of tappings. The two subsequent values were same. Noted the final volume as bulk volume.

4.2.4.3. Carr’s Index\(^7\)

\[ = \frac{\text{Bulk density} - \text{fluff density}}{\text{Fluff density}} \]

4.2.4.4. Hauser Ratio\(^7\)

\[ = \frac{\text{Bulk density}}{\text{Fluff density}} \]

4.2.5. Heavy Metal Analysis of Polyherbal Formulation\(^7\)

**Preparation of Samples by Acid Digestion Method**

Exactly weighed (2gm) of powder was taken in kjeldahl flask. To this HNO\(_3\): HClO\(_4\) (4:1) mixture was added and heated constantly till the solution is colorless. Then transferred to a 25 ml volumetric flask
and the volume was made up with distilled water. Reagent blank was synchronously prepared as for the above procedure. The standards of lead (pb), cadmium (cd), arsenic (As) and mercury (Hg) were prepared as for procedure in the manual and the calibration curve was developed for each of them.

**Detection** Then samples were analysed for the presence of Pb, cd, As and Hg using atomic absorbance spectrophotometer.

4.2.6. **Microbial Analysis of Polyherbal Formulation**

As per the procedures of Indian pharmacopoeia 2010 and WHO guidelines microbial analysis have been carried out for measurement of microbial contamination. The test include total bacterial count, total yeast and mould count, detection of specified organisms such as *salmonella* sp, *Escherichia coli*, *staphylococcus aureus* and *pseudomonas aeruginosa*.

4.3. **Materials and Methods**

4.3.1. **Drugs and Chemicals**

Aminoguanidine hydrogen carbonate was obtained from Spectrochem Pvt. Ltd. Streptozotocin was purchased from Sigma-Aldrich and different commercially available readymade kits made by Bioalab, Span and Tulip diagnostic Pvt. Ltd. For estimation of various biochemical parameters and ELISA kits for cytokines were used.
Table 4.1: Instruments

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semi Autoanalyser</td>
<td>Erba/Mannheim</td>
</tr>
<tr>
<td>UV-Visible spectrophotometer</td>
<td>2200/Systronics</td>
</tr>
<tr>
<td>ELISA reader</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>Remi Rm- 12 C</td>
</tr>
<tr>
<td>Homogenizer</td>
<td>Remi motor/Remi Electro Technik Ltd.</td>
</tr>
</tbody>
</table>

4.3.3. Animals

Male Wistar rats, weighing between 180 and 200g were procured from the Raghavendra enterprises, Bangalore, India. All the animal experiments were conducted according to the protocols approved by the Institutional Animal Ethical Committee (Protocol No: 878/ac/05/CPCSEA/024/2011). All animals were maintained under adequate conditions at an ambient temperature of 21±2°C, and were subjected to 12 h light and dark cycle. They were fed with standard pellet diet and water ad libitum. Animals were kept for 7 days in laboratory for habituation.

4.3.4. Determination of Acute Toxicity Study

The acute toxicity study was conducted based on OECD guideline 423, acute toxicity class (3 animals used). The adult Swiss albino mice (15-25g b.w) were arbitrarily divided into 5 different groups containing 3 animals in each group. The animals were fasted overnight and polyherbal formulation was administered orally of
various dose levels (250, 500, 1000, 2000 and 5000 mg/kg, BW) after dissolving in water. One group was treated as control and administered vehicle only. The animals were observed constantly for 24 hours. Behaviour of the animals and any other toxic symptoms also observed for 72 hours and they were kept under observation up to 14 days.

4.3.5. Selection of Dose

For assessment of protective activity of polyherbal formulation against diabetes induced nephropathy in rats, two dose levels were chosen. One dose was twentieth of the maximum dose during acute toxicity studies and a high dose, which was twice that of twentieth dose (250 mg/kg and 500 mg/kg).

4.3.6. Induction of Diabetes and Experimental Design

Prior to the beginning of the experiment all the animals were not allowed for food for 18 hours but water was allowed without stoppage. Wistar rats received streptozotocin (60 mg/kg, i.p), freshly prepared in 0.1M cold citrate buffer (Ph 4.5). Normal control rats received citrate buffer only. 48 hrs after STZ administration, blood samples were collected from retro orbital plexus and plasma glucose was determined by GOD/POD method. The induction of diabetes mellitus was confirmed by determination of plasma glucose level (≥250 mg/dl). Diabetic rats were kept untreated for four weeks. At the end of 4th week, plasma glucose of diabetic rats ≥250 mg/dl was selected for Diabetic Nephropathy Studies.
Wistar rats were randomly grouped into 5 groups (6 rats/group) and received the following treatment for 16 weeks.

Group 1: Normal control (NC) received normal saline (1ml/100g/day, p.o)

Group 2: DN control (DNC) received vehicle only (1ml/100g/day, p.o)

Group 3: DN rats received PHF 250mg/kg, p.o (DN+TEST 1)

Group 4: DN rats received PHF 500mg/kg, p.o (DN+TEST 2)

Group 5: DN rats received aminoguanidine hydrogen carbonate (1 mg/1ml in drinking water) (DN+AMG)

4.3.7. Blood sample collection

During treatment blood was collected from retro orbital plexus at every 4 week interval and used for determination blood glucose level.

At the end of 16th week before the sacrifice blood was collected from retro orbital plexus for the measurement of various biochemical parameters.

4.3.8. Urine sample collection and tissue preparation

The rats were housed independently in metabolic cages. The urine samples were collected in duration of 24 hrs. After collection, the urinary volume was measured and used for assessment of various renal functional parameters. The rats were sacrificed by cervical dislocation under light ether anaesthesia. The left kidney was quickly removed for homogenate preparation. The right kidney was then
quickly removed and preserved in 10% formalin solution for histopathological examinations. The following parameters were measured.

1. **Physiological Parameters**

   a) Body weight  b) Food intake  c) Water intake  d) Kidney weight  e) Kidney weight/body weight

2. **Haematological Parameters.**

   a) RBC count  b) WBC count  c) Haemoglobin  d) Packed cell volume  
   e) Calcium  f) Phosphorus.

3. **Anti diabetic Parameters**

   a) Blood Glucose  b) HbA1C  c) Plasma insulin  d) Liver glycogen content

4. **Lipid Profile**

   a) Triglycerides  b) Total cholesterol  c) LDL-cholesterol  
   d) VLDL-cholesterol  e) HDL-cholesterol

5. **Renal Function Parameters**

   a) Blood urea nitrogen  b) Protein in urine  c) Urinary urea  d) Serum uric acid  
   e) Urine volume  f) Serum creatinine  g) Urine creatinine  h) Glomerular filtration rate  
   i) Advanced glycation end products  
   j) Urinary albumin excretion rate  k) Type IV collagen excretion  l) Total glycans  
   m) Excretion of glycosaminoglycans
6. Inflammatory Mediators

a) IL-6  
b) TGF-β  
c) TNF-α

7. Kidney Antioxidant Parameters

a) Superoxide dismutase  
b) Catalase  
c) reduced glutathione  
d) glutathione peroxidase  
e) Lipid peroxidation

8. Histopathology of Kidneys

4.3.9. Measurement of Physiological Parameters

4.3.9.1. Measurement of Body Weight, Kidney Weight

The body weight was recorded at 0th week and final day of 16th week and kidney weight of each animal was recorded in grams at the final day of 16th week using digital balance.

4.3.9.2. Measurement of Food Intake and Water Intake

The food intake in grams/rat/day was recorded at 0th week and final day of 16th week. Water intake in ml/rat/day was recorded at 0th week and final day of 16th week.

4.3.10. Measurement of Haematological Parameters

Blood samples were withdrawn from retro-orbital plexus under light ether anaesthesia, collected in heparinized capillary tubes and analyzed for haematological parameters.

4.3.10.1. Measurement of Haemoglobin

Haemoglobin (Hb) content (gm %) of each animal was measured by Sahli’s haemoglobinometer at the end of 16th week. Haemoglobin is converted to brown colored acid hematin when blood is added to 0.1 N
hydrochloric acid. The color developed after dilution is compared with standard brown glass reference blocks of a Sahli haemoglobinometer. 0.1 N hydrochloric acid is added in the tube up to the lowest mark (20% mark) By the help of a Pasteur pipette. Blood was pinched up to 20 μl mark in the Hb-pipette. Blood column was adjusted cautiously without bubbles. Blood was transferred to the acid present in the graduated tube; reaction mixture was mixed and allowed the tube to place for at least 10 minutes. By adding few drops the solution was diluted with distilled water at a time cautiously and by mixing the reaction mixture, until the color matches with the glass plate in the comparator. The matching was done only in opposition to natural light. The level of the fluid was noted at its lower meniscus and the reading corresponding to this level on the scale was recorded in gm % of haemoglobin.

4.3.10.2. Measurement of RBC, WBC, PCV.

Haematological parameters like total red blood cells (RBC), total white blood cells (WBC), packed cell volume (PCV), were estimated on fully automated fluorescence flow cytometry 5-part different analyzers ( Sysmex XS800i, Japan).

4.3.10.3. Measurement of Calcium in Serum

**Principle:** A violet complex with O- cresolphthalein was formed in an alkaline medium in presence of calcium.

**Procedure:** O-Cresolphthalein complexone method
Wavelength: 578 nm

Temperature: 20-30\(^{\circ}\) C

**Table 4.2. Patterns of reagents for the measurement of Calcium**

<table>
<thead>
<tr>
<th></th>
<th>Reagent blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent 1</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Reagent 2</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td></td>
<td>0.02 ml</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>0.02 ml</td>
<td></td>
</tr>
</tbody>
</table>

Serum calcium concentration in mg/100 ml = \(\frac{A \text{ of sample}}{A \text{ of standard}} \times 8\)

\[ A = \text{Absorbance} \]

4.3.10.4. **Measurement of Phosphorus in Serum**\(^{80}\)

**Principle**

In acidic conditions ammonium molybdate reacts with phosphorus to form a compound known as phosphomolybdate, in presence of metol which is converted to blue colored complex. The strength of colour produced is directly relative to the elemental phosphorus concentration.

**Procedure:** Modified metol method

Wave length: 680 nm (red filter)

Incubation temperature: normal room temperature

Linearity: 15 mg%
Reaction type: End point with standard

Standard: 5 mg% elemental phosphorus

Reaction slope: increasing

**Table 4.3. Outline of reagents for the measurement of phosphorus**

<table>
<thead>
<tr>
<th></th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molybdate reagent</td>
<td>1 ml</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Catalyst regent</td>
<td>1 ml</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Deionised water</td>
<td>0.1 ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td>0.1 ml</td>
<td>-</td>
</tr>
<tr>
<td>Standard</td>
<td></td>
<td></td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Metol reagent</td>
<td>1 ml</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

Serum phosphorus in mg% = A of Test/ A of standard x 5

A = Absorbance

**4.3.11. Measurement of Anti Diabetic Parameters**

**4.3.11.1. Estimation of Blood Sugar**

Serum glucose levels were determined by the glucose oxidase – peroxidase (GOD-POD) method.

**Principal:**

The glucose oxidase converts glucose to gluconic acid and hydrogen peroxide when added to serum sample and incubated at 37°C for 15 minutes. The peroxidase catalyses the hydrogen peroxide...
to water and oxygen. 4-aminophenazone, takes up the oxygen and jointly with phenol forms a pink colored chromogen. The optical density (OD) of the solution was observed at 530 nm wavelength. The color formed is directly relative to the glucose level.

Table 4.4. Outline of reagents for the measurement of blood glucose

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Reagent</td>
<td>2 ml</td>
<td>2 ml</td>
<td>2 ml</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>10 μl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td></td>
<td>10 μl</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>10 μl</td>
<td></td>
</tr>
</tbody>
</table>

Mix and read the optical density (OD) after 10 minutes incubation. The final color is stable for at least 1 hour.

Calculations

Serum Glucose (mg/dl) = Abs. of sample/ Abs. of Std. X 100

4.3.11.2. Estimation of Glycosylated haemoglobin

Glycosylated haemoglobin was estimated by photometric method

Principle

The hexose moiety of glycosylated haemoglobin is converted to 5- hydroxymethyl furfural by heating at 100° c in presence of weak
acid, the hydroxymethyl furfural is reacted with 2-Thiobarbituric acid, and the resulting colour is measured photometrically.

**Procedure**

1 ml of diluted haemosylate containing 10 mg of haemoglobin has taken in screw capped tubes. Fructose standard with 1 ml of oxalic acid reagent has taken in screw capped tube. Incubated the tubes for 60 minutes in the autoclave at 124±1° c and 124±3 k p a (18±0.5 lb) in², allowed to cool room temperature, mixed the contents of the tubes by inversion and 1 ml of trichloro acetic acid to each tube, mixed and filtered. To 0.5 ml of the elute 0.5 ml of Thiobarbituric acid was added. All tubes were kept in hot water bath at 40° c for 30 minutes and allowed to cool to room temperature for 50 minutes and measured the absorbance at 443 nm from that of the corresponding of sample.

**4.3.11.3. Estimation of plasma insulin**

Plasma insulin was estimated by RIA kit method.

**Principle**

Radioimmunoassay depends on the ability of an antibody to bind its antigen. To quantitate the antigen, the radioactive form of the antigen competes for binding sites on a specific antibody. As more non radioactive antigen is added, less radioactive antigen remains bound until equilibrium between the free and antibody bound antigen occurs.
**Assay preparations**

Reconstituted the lyophilized insulin I$^{125}$ with distilled water and allowed to sit at room temperature. Brought the all standards, coated tubes and insulin I$^{125}$ to room temperature prior to use. Anti-insulin tubes were placed in the test tube rack.

**Assay**

10 µl of insulin standards, controls and samples were added to their respective tubes. 900 µl of buffer solution was added to all tubes. All tubes were vortex thoroughly and incubated at room temperature for 18 hrs. Aspirated the tubes. Each tube was rinsed with 4 ml of deionised water. Aspirated the tubes. Counted the empty tubes in a gamma counter calibrated with I$^{125}$. The recommended counting time for this assay is 1 minute.

**Calculations**

Determined the average counts per sample. Divided the average counts for each standard and sample by the average counts of the zero standards, and then multiply by 100. This is % B/Bo.

**Formula**

\[
\% \text{ B/Bo} = \frac{\text{CPM (sample)}}{\text{CPM X 100}}
\]

CPM = Average counts of duplicate

Sample = Particular serum or standard being calculated

**4.3.11.4. Estimation of Liver Glycogen Content**

Liver glycogen content was measured by the scheme of van der vies. 200mg of Liver tissue was thinly ground with 20 ml of 5% Trichloroacetic acid in a homogenizer and protein precipitate was
filtered. 2ml of clear supernatant was pipette out into a 20ml capacity calibrated test tube and after that 2ml of 10N KOH was added. This tube was sited in a hot water bath for 1 hr. Later than cooling, to counteract the excess of alkali 1 ml of glacial acetic acid was added and fluid brought equal to the mark with water. Slowly, Test tube containing 4 ml of anthrone reagent, 2ml solution was added from the previous step which was positioned in cold water to stop too much heating. After systematic integration, the tube was to be found in a hot water bath for perfectly 10 min for the improvement of colour and cooled with running tap water. The optical density was interpreted within 2 hr in a spectrophotometer at 650 nm against a blank.

Calculation

$$\frac{DU \times 0.1 \times \text{Volume of extract}}{\text{g of tissue}} \times 0.9 = \text{g of glycogen}$$

Where

$DU =$ Optical density of unknown

$DS =$ Optical density of standard

$0.1 =$ mg of glucose in 2 ml of standard

$0.9 =$ Factor for converting glucose value to glycogen value

4.3.12. Estimation of Lipid Parameters

4.3.12.1. Estimation of triglycerides

Measurement of triglyceride (neutral fat) concentration in serum was done by using enzymatic kit.
**Principle:**

Microbial lipase hydrolyses the triglycerides in the sample to glycerol and free fatty acid (FFA). Glycerol kinase phosphorylates the glycerol to G-3-P by adding phosphate group from ATP. Glycerol phosphate oxidase (GPO) oxidizes the G-3-P to dihydroxy acetone phosphate. In this reaction hydrogen peroxide (H$_2$O$_2$) is formed in equimolar concentration to the level of triglycerides present in the sample. Peroxidase (POD) catalyses the reaction H$_2$O$_2$ reacts with 4-aminoantipyrine and ADPS. The outcome of this oxidative coupling is a quinoneimine purple coloured dye.

The absorbance of this dye in solution is relative to the concentration of triglycerides present in sample.

**Table 4.5. Outline of reagents for the measurement of Triglycerides**

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>20 µl</td>
<td>-</td>
</tr>
<tr>
<td>Specimen</td>
<td>-</td>
<td>-</td>
<td>20 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
</tr>
</tbody>
</table>

A) Mix and incubate at 37ºC for 15 minutes.

Mix and read the absorbance of test and standard against reagent blank at 546 nm.

**Calculations:**

Triglycerides (mg/dl) = \( \frac{\text{Abs. of Test}}{\text{Abs. of Std.}} \times 50 \)
4.3.12.2. Estimation of Total Cholesterol:\textsuperscript{86} (CHOD/POD-Phosphotungstate method)

Measurement of the activity of cholesterol in serum was done using enzymatic kit.

**Principle:**

Cholesterol esterase (CHE) hydrolyses cholesterol ester.

Cholesterol oxidase (CHOD) oxidises the free cholesterol to choleost-4-ene-3-one and hydrogen peroxide. In the presence peroxidase Hydrogen peroxide reacts with 4-aminoantipyrine and phenol developed the pink coloured quinoneimine dye. The intensity of colour formed is relative to cholesterol concentration.

**Procedure**

**Table 4.6. Pipette into 3 test tubes labeled Blank (B), Standard (S) and Total Cholesterol (TC) as shown below**

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Total Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Cholesterol standard</td>
<td>-</td>
<td>20 µl</td>
<td>-</td>
</tr>
<tr>
<td>Specimen</td>
<td>-</td>
<td>-</td>
<td>20 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
</tr>
</tbody>
</table>

A) Mix and incubate at 37°C for 5 minutes (or) RT for 10 minutes.

Absorbance of standard, total cholesterol was read against Blank at 505 nm.
Calculations

Total Cholesterol (mg/dl) = Abs. of Tc / Abs. of S X 200

4.3.12.3. Estimation of HDL-cholesterol:86

(CHOD/POD-Phosphotungstate Method)

Principle

By using PTA the VLDL and LDL fractions of serum sample are precipitated and then HDL in the supernatant is separated by centrifugation and measured for its cholesterol content. The enzyme cholesterol ester hydrolase (CHE) hydrolyses the cholesterol ester. Cholesterol oxidase (CHO) oxidizes the cholesterol to cholest-4-en-3-one and hydrogen peroxide. In presence of enzyme peroxidase (POD) Hydrogen peroxide reacts with 4-aminoantipyrine and phenol to form a red colored complex, whose absorbance is proportional to HDL-cholesterol concentration.

Procedure

**Table 4.7. Pipette into a centrifuge tube**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Precipitating Reagent</td>
<td>0.3 ml</td>
</tr>
</tbody>
</table>

Mix well and allow standing at RT for 5 minutes. Centrifuge at 3000 rpm for 10 minutes to get a clear supernatant.
Table 4.8. Pipette into 3 test tubes labeled Blank (B), Standard (S) and HDL Cholesterol (H) as shown below

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>HDL Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Cholesterol standard</td>
<td>-</td>
<td>20 µl</td>
<td>-</td>
</tr>
<tr>
<td>Supernatant</td>
<td>-</td>
<td>-</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

A) Mix and incubate at 37°C for 5 minutes (or) RT for 10 minutes.

Distilled water 2.0 ml 2.0 ml 2.0 ml

Read the absorbance of standard, total cholesterol against Blank at 505 nm.

**Calculations**

HDL Cholesterol (mg/dl) = Abs. of H / Abs. of S X 50

4.3.12.4. Estimation of LDL-Cholesterol\(^{87}\)

Measurement of LDL-cholesterol was done using the Friedewald Formula.

LDL cholesterol = total cholesterol – (HDL cholesterol + VLDL cholesterol)

4.3.12.5. Estimation of VLDL-Cholesterol\(^{87}\)

Estimation of VLDL-cholesterol was done using the Friedewald formula.

VLDL cholesterol = triglyceride/5
4.3.12.6. Estimation of Atherogenic Index

Atherogenic Index was calculated according to the formula

\[ \text{Atherogenic Index} = \frac{\text{TC} - \text{HDL-C}}{	ext{HDL-C}} \]

4.3.13. Estimation of Renal function parameters

4.3.13.1. Blood urea nitrogen

**Principle**

Urea+2H₂O →2NH₄⁺+CO₂

\[ \text{NH₄⁺+2-oxoglutarate+NADH→L-glutarate+NAD+ H₂O} \]

**Table 4.9. Procedure**

<table>
<thead>
<tr>
<th>Pipette into marked tubes</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1000µl</td>
<td>1000µl</td>
</tr>
<tr>
<td>Standard</td>
<td>20 µl</td>
<td>------</td>
</tr>
<tr>
<td>Sample</td>
<td>------</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

Mix well and aspirate standard followed by samples

**Calculation**

\[ \text{BUN} = \frac{\Delta A \text{ of test}}{\Delta A \text{ of standard}} \times \text{concentration of standard (mg/dl)} \]

4.3.13.2. Estimation of Protein urine (Biuret Method)

Measurement of total protein concentration in urine was done by Biuret method.

**Principle**

In alkaline medium Peptide bonds present in protein react with cupric ion in to form a colored chelate, the absorbance of which is
measured at 578 nm. Absorbance data is proportional to protein concentration.

**Chemicals and reagents**

Biuret reagent: Copper sulphate, Sodium hydroxide, Sodium-potassium tatarate, Surfactant.

Protein Standard: 6.5 mg/dl

**Procedure**

Prepare blank, test and standard solution as follows

Blank solution: 3ml of Biuret reagent

Standard solution: Add 30μl of Total Protein standard solution to 3 ml of Biuret reagent.

Test Solution: Add 30μl urine to 3ml of Biuret reagent.

Mix well and incubate at 37°C or at RT for 5 minutes. Read the absorbance at 578 nm against reagent blank.

Calculations: Total Protein (gm/dl) = Abs. of Test/ Abs. of Std. X 6.5

4.3.13.3. Estimation of Uric Acid

**Principle**

Uric Acid+O₂+ 2H₂O  Weight Allantoine+CO₂+H₂O

2H₂O+ESPAS+4-AAP  Weight Purple quinoneimine+4 H₂O₂
**Procedure**

Pipette out blank (B) Standard (S) and test (T) into clean, dry prelabelled tubes and mix with the reagents in the following order.

**Table 4.10 : Outline of reagents for the measurement of uric acid**

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Reagent</td>
<td>1000µl</td>
<td>1000µl</td>
<td>1000µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000µl</td>
<td>__</td>
<td>__</td>
</tr>
<tr>
<td>Standard</td>
<td>__</td>
<td>100 µl</td>
<td>__</td>
</tr>
<tr>
<td>Serum/plasma</td>
<td>__</td>
<td>__</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

**Calculations**

Uric acid (mg/dl) = Absorbance of test/ Absorbance of standard x 5

**4.3.13.4. Serum Creatinine**

**Principle**

Creatinine reacts with picric acid in presence of alkali to make an orange-yellow coloured compound i.e. alkaline picrate. The absorbance of the orange-yellow color formed is directly proportional to the creatinine concentration and is measured coloremetrically at 500-520nm.
Procedure

**Table 4.11. Outline of reagents for the measurement of serum creatinine**

<table>
<thead>
<tr>
<th>Pipette into marked tubes</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1000µl</td>
<td>1000µl</td>
</tr>
<tr>
<td>Standard</td>
<td>100 µl</td>
<td>------</td>
</tr>
<tr>
<td>Sample</td>
<td>------</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

Mix well and read initial absorbance \((A_1)\) 20 seconds after mixing and final absorbance \((A_2)\) 80 seconds after mixing.

**Calculation**

\[
\Delta A = A_2 - A_1
\]

Creatinine (mg/dl) = \(\frac{\Delta A \text{ of test}}{\Delta A \text{ of standard}}\) \(X\) concentration of standard

**4.3.13.5. Estimation of Creatinine in Urine**

**Principle**

Creatinine reacts with picric acid in the presence of alkali forms the orange-yellow colored compound (the jaffe’s reaction) i.e. alkaline picrate. The intensity of the orange red colour is a measure of creatinine present in urine.

**Procedure**

24 hr urine sample is a preferred specimen. The urine is collected in a clean bottle using chloroform as preservative. Dilute 5 ml of urine to 250 ml in a volumetric Flask. (Dilution is 1 in 10).
Table 4.12. Pattern of reagents for the measurement of urinary creatinine

<table>
<thead>
<tr>
<th>Label three dry test tubes</th>
<th>Blank (B)</th>
<th>TEST (T)</th>
<th>Standard (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>5 ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard creatinine solution (0.1 mg/ml)</td>
<td>-</td>
<td>-</td>
<td>5 ml</td>
</tr>
<tr>
<td>Diluted urine</td>
<td>-</td>
<td>5 ml</td>
<td>-</td>
</tr>
<tr>
<td>0.75 N NaoH</td>
<td>2 ml</td>
<td>2 ml</td>
<td>2 ml</td>
</tr>
<tr>
<td>0.4 M Picric acid</td>
<td>2 ml</td>
<td>2 ml</td>
<td>2 ml</td>
</tr>
</tbody>
</table>

Mix and heat for 15 minutes. Read the optical density with a colorimeter using green filter (520 nm)

**Calculation**

Concentration of creatinine in (mg/dl)

\[
\text{Concentration of creatinine} = \frac{\text{O.D of T}}{\text{O.D of S}} \times \frac{\text{volume of standard}}{\text{O.D of B}}
\]

4.3.13.6 Measurement of Glomerular Filtration Rate

Glomerular filtration rate was calculated using the formula

\[
\text{GFR (ml/min)} = \frac{\text{Urinary creatinine} \times \text{urine volume}}{\text{Serum creatinine} \times 1440}
\]

4.3.13.7. Estimation of Urinary Urea

**Principle**

Under strongly acidic condition and in presence of ferric ions, Urea reacts with diacetyl monoxime and thio-semicarbazide to give a pink coloured complex. Proteins in blood do not interfere as they are precipitated with trichloro acetic acid. The intensity of the pink colour is a measure of the amount of urea present in blood.
Procedure

24 hr urine sample is a preferred specimen. The urine is collected in a clean bottle using chloroform as preservative.

Table 4.13. Outline of reagents for the measurement of urinary urea

<table>
<thead>
<tr>
<th>Label three dry test tubes</th>
<th>Blank (B)</th>
<th>TEST (T)</th>
<th>Standard (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>1 ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard urea solution</td>
<td>-</td>
<td>-</td>
<td>1 ml</td>
</tr>
<tr>
<td>Diluted urine</td>
<td>-</td>
<td>1 ml</td>
<td>-</td>
</tr>
<tr>
<td>Diacetyl monoxime reagent</td>
<td>5 ml</td>
<td>5 ml</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

Mix and keep the tubes in boiling water bath per 15 minutes. Cool and read the optical density using colorimeter with green filter (520 nm).

Calculation

Concentration of urea in (mg/dl) = \( \frac{O.D \text{ of } (T-B) \times \text{Conc. of } S}{O.D \text{ of } (S-B) \times \text{Vol. of } T} \)

4.3.13.8. Estimation of advanced glycation end products

Pulverized kidney tissue was ruptured with methanol and chloroform (1:2) overnight. Tissue was homogenized in 0.1N NaOH. Centrifuged at 8000g for 15 min at 4°C. Amount of AGES in these-soluble samples were measured by measuring the fluorescence at an emission wavelength of 440 nm and an excitation wavelength at 370 nm using a fluorescence spectrophotometer. Bovine serum albumin preparation (1mg/ml of 0.1 N NaOH) was used as a standard. The
fluorescence values of samples were measured at a protein concentration of 1mg/ml and articulated in AU compared with a resident BSA preparation.

4.3.13.9. **Measurement of Urinary Albumin**\(^\text{96}\)

Urinary albumin concentration was quantified by a competitive enzyme linked immunosorbent assay (ELISA).

**Principle**

To the albumin coated well the sample and the horseradish peroxidase-conjugated albumin antibody are added. The antibody binds to albumin immobilized on the stationary phase or to the albumin in the fluid phase. Hence the concept of competitive binding. After washing, only the antibody-conjugate that is bound to the albumin of the stationary phase will remain in the well, and this is detected using a chromogenic reaction. The colour intensity formed is inversely proportional to the logarithm of albumin concentration in the fluid phase.

**Assay procedure**

Urine samples were assayed in duplicate at 1:10 dilution. 90 μl of diluent was added to the remaining columns 3-12, rows A-H. 10 μl of urine was placed from each sample into two adjacent wells. 100 μl nephrat conjugate was added per well to all wells on the plate. The plate was incubated at room temperature for 1 hour. The fluids were detached from the plate by flipping them out into the sink. The plate was flooded with tap water (this procedure was repeated for a total of 6 times). The plate was inverted on absorbant paper to blot off
overload fluids. 100 μl TMB colour developer was added to each well and the plate was incubated for 5 minutes. 100 μl colour stopper was added to each well in the plate. The absorbance was measured at 450 nm against an air blank in a plate reader.

**Standard curve preparation**

Standard rat serum albumin (RSA) is serially diluted in two-fold dilution to produce 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156 and 0 mg/dL in duplicate wells. A standard curve must be run on each plate.

**Calculation:**

The duplicate readings for each standard and sample was averaged and the average zero standard optical density was subtracted. A standard curve was constructed by plotting the mean absorbance of each standard on the y-axis against the log concentration on the x-axis. The concentrations of albumin in samples were calculated from the standard curve.

4.3.13.10. **Estimation of type IV Collagen**

96 Well plates were taken. Add an aliquot of urine sample or standard type IV collagen and left overnight at 4°C. Primary antibody (anti type IV collagen, polyclonal) was added after blocking with 2% bovine serum albumin for 4hr at room temperature. Incubated with alkaline phosphatase conjugated secondary antibody (anti-Ig G). The colour developed by using paranitrophenol phosphate as a substrate and read at 405 nm in ELISA reader. The amount of collagen excreted per day was calculated using the calibration curve generated.
4.3.13.11. Isolation of Glycosaminoglycans (GAGS)

Kidneys were cut into small pieces and defatted in acetone for a week at 4°C and were powdered. Subjected to papain digestion and centrifuged at 3000 rpm for 15 min at 4°C. For the precipitation of proteins ⅓ volume of 40% trichloro acetic acid was added to the supernatant and centrifuged again. The supernatant which contains GAGS was precipitated out by adding 4 volumes of ethanol containing 1.2% potassium acetate. The precipitate was collected, dried using nitrogen gas and reconstituted in minimum volume of water. It was quantitated by DMMB assay (1, 9-dimethyl methylene blue).

4.3.14. Estimation of Inflammatory Cytokines

4.3.14.1. Assay of Interleukin-6 (IL-6)

IL-6 production was measured by using rat IL-6 ELISA 96 well set. Briefly, to every well 100 μl of diluted Capture antibody was added and plate was incubated overnight at 4°C. After that plate was washed 5 times. To every well 200 μl of assay diluent was added and plate was incubated for an hour at room temperature. After that plate was washed 5 times and to every well 100 μl of standard or sample was added, plate was incubated for 2 hrs at room temperature. After that plate was washed 5 times and to every well 100 μl of diluted detection antibody was added. Plate was incubated for an hour at room temperature and washed 5 times. After washing, to every well 100 μl of diluted SAV--HRP was added and plate was incubated for 30 minutes at room temperature. Plate was washed for 7 times and to every well 100 μl of TMB Substrate Solution was added. Plate was incubated for 30 minutes at room temperature in dark. 50 μl of stop
solution was added to every well and plate was read at 450 nm with λ correction 570 nm.

4.3.14.2. Assay of Transforming Growth Factor-β (TGF-β)\textsuperscript{99}

All reagents, working standards, controls and activated samples were prepared. To every well 50 μl of assay diluent RD1-73 was added. To every well 50 μl of standard, control or activated sample were added. The enclosed plates were incubated for 2 hours at room temperature on horizontal orbital microplate shaker set at 500 ± 50 rpm. The solution was aspirated and the wells were washed 4 times using the clean buffer. The plate was upturned and blotted against clean paper towels. To every well 100 μl of TGF-β\textsubscript{1} conjugate was added and the enclosed plates were incubated for 2 hours at room temperature. Aspiration/wash was repeated. To every well 100 μl of substrate solution was added and the wells were incubated for 30 minutes at the room temperature in the dark. To every well 100 μl of stop solution was added and mixed gently. Optical density of every well was determined within 30 minutes, using a microplate reader set to 450 nm.

4.3.14.3. Assay of Tumour Necrosis Factor-α (TNF-α)\textsuperscript{99}

TNF-α production was measured by ELISA. The ELISA was performed by in anti-rat TNF-α precoated 96-well Strip Plate. 50 μl of standard, controls and samples are pipetted into the wells in duplicate. To all wells 50 μl of biotinylated antibody reagent was added. The plate was enclosed and incubated at RT for 2 hours. Later on plate was washed for 5 times with rinse buffer. To every well 100 μl of dilute
Streptavidin- HRP concentrate was added. The plate was enclosed and incubated at RT for 30 minutes. Later on plate was washed for 5 times. To every well 100 μl of premixed TMB substrate solution was added. Dark colour was developed in the Plate at RT for 30 minutes. To every well 100 μl of Stop Solution was added for stopping of reaction. Absorbance was measured on a plate reader at 450 minus 550 nm. The strength of the colour was measured in amount to the amount of TNF-α bound in the initial step. The sample values are then read off the standard curves

4.3.15. Estimation of Kidney Anti-oxidants

4.3.15.1. Tissue Processing

Reagents

1. Phosphate buffered saline (pH - 7.4)

   1.38g of DAEDTA (disodium ethylene diamine tetra acetic acid), 0.19g of potassium dihydrogen phosphate (KH₂PO₄) and 8 g of Nacl were dissolved in 900 ml of distilled water and pH was adjusted using dilute HCL. The volume was made upto 1000 ml.

2. Sucrose solution (0.25M):

   8.85g of sucrose was dissolved in 200 ml of water and diluted to 10000 ml with distilled water.
3. Tris HCl buffer (10 Mm, pH 7.4):

1.21g of tris was dissolved in 900 ml of pure distilled water and the pH was made to 7.4 with 1M HCl. The resultant solution was thinned to 1000 ml using pure distilled water.

**Procedure**

The animals were sacrificed by using euthanasia and the kidneys were isolated and quickly transferred to ice cold phosphate buffered saline (PBS pH 7.4). It was maintained free of blood and other tissue fluids and weighed. The kidneys were nicely chopped with surgical blade into small slices. Then the pieces were placed in ice-cold 0.25M sucrose solution quickly placed on a filter paper. After that crushed and homogenized in ice-cold tris HCl buffer of strength 10Mm of pH 7.4 to a concentration of 10% w/v. The obtained homogenate was centrifuged at 7000 rpm for 25 minutes under normal conditions. The clear supernatant fluid was used for the purpose of different biochemical parameters estimation.

**4.3.15.2. Estimation of Superoxide Dismutase**

SOD was predictable by the Method of Misra and Fridovich (1967)

**Principle**

By raising the pH from 7.8-10.2 rate of self oxidation of epinephrine and the inhibition of sensitivity of self oxidation by Superoxide dismutase was improved. In xanthine oxidase reaction, O₂ generated is accountable for the oxidation of epinephrine to
adrenochrome and the yield of adrenochrome produced per $O_2$ introduced. The self oxidation of epinephrine takings by at least two separate pathways only one of which is free radical chain reaction involving $O_2$ and hence livable by superoxide dismutase.

**Reagents**

1. Carbonate buffer (0.05M, $pH$ 10.2): In 500ml of distilled water, 16.8g of sodium bicarbonate and 22g of sodium carbonate were dissolved and final volume was completed with distilled water.

2. Ethylene diamine tetra acetic acid (EDTA) (0.49 M): in 1000 ml of distilled water, 1.82 g of EDTA was dissolved.

3. Epinephrine (3Mm): In 10 ml of 1 M HCl solution 9.9 mg of epinephrine bitartrate was dissolved.

4. SOD standard: In 100ml of carbonate buffer 1 mg (1000 units/mg) of SOD was dissolved from bovine liver.

**Procedure**

To 0.5ml of distilled water 0.5ml of tissue homogenate was diluted. To this 0.25ml, 0.5ml of chloroform (all chilled reagents) was added. The mixture was shaken for 1 min and centrifuged at 2000 rpm for 20 min. The enzymatic activity of supernatant was determined; to it 0.05 ml of carbonate buffer (0.05M $pH$ 10.2) and 0.5ml EDTA (0.49M) was added. The reaction was initiated by accumulation of 0.4 ml epinephrine and the change in optical
density/mm was measured at 480nm. SOD was expressed as U/mg protein.

\[
\text{SOD} = 0.025 \times \frac{Y_{\text{final}} - Y_{\text{initial}}}{50} \times 100
\]

\(Y=\text{Final reading-initial reading}\)

4.3.15.3. Estimation of Catalase (CAT)\textsuperscript{101}

Catalase was measured by Hugo E. Aebi Method, 1974.

**Principle**

\[\text{H}_2\text{O}_2 \rightarrow \text{H}_2 + \text{O}_2\]

\[\text{RDOH} \rightarrow \text{H}_2\text{O} + \text{ROH} + \text{A}\]

Decomposition of \(\text{H}_2\text{O}_2 = \text{Decrease in absorbance at 240nm}\)

**Reagents**

1. Phosphate buffer (50Mm, pH7.0):

   A. In distilled water 6.81g of \(\text{KH}_2\text{PO}_4\) was dissolved and build upto 1000 ml.

   B. In distilled water 8.9g \(\text{NaH}_2\text{PO}_4.2\text{H}_2\text{O}\) was dissolved and make upto 1000 ml.

   Mix the solution A and B in proportion 1:15 (v/v)

2. Hydrogen peroxide (30Mm/l): 0.34 ml of 30% hydrogen peroxide was diluted with phosphate buffer upto 100 ml.

Kidney homogenate 20 times diluted with phosphate buffer pH 7.0
Table 4.14. Procedure

<table>
<thead>
<tr>
<th>Blank</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 ml of tissue homogenate thinned with 2 ml of phosphate buffer pH 7, and absorbance has taken at 254 nm for 3 minutes with the interval of 30 seconds</td>
<td>2 ml of tissue homogenate thinned with 1 ml of H₂O₂ (8.5 micro lit. in 2.5 ml phosphate buffer (50Mm/l. Ph 7.0) and absorbance has taken at 254 nm for 3 minutes with the interval of 30 seconds (H₂O₂ was added just before taking o.D)</td>
</tr>
</tbody>
</table>

**Calculation**

\[ \text{Log } (A/B) \times 22973 \]

Where,

A: Initial absorbance

B: Final absorbance (after 30 seconds)

Units= μmoles of H₂O₂ consumed/mg protein/min

**4.3.15.4. Estimation of Reduced Glutathione**

Reduced glutathione was estimated by Ellman method

**Principle**

GSH is a major non-protein thiol and endogenous antioxidant that counters balance free radical mediated damage. It is involved in the protection of normal cell structure and function by maintaining
the redox homeostasis, quenching of free radicals and by participating in detoxification reaction.

**Reagents**

1. TCA (10%w/v) solution: In 100 ml of distilled water 10 g of TCA was dissolved.

2. Phosphate buffer (0.2M, pH 8)

3. DTNB reagent (0.6 M): In 100 ml of 0.2M sodium phosphate (pH 8), 60 mg of 5, 5'-dithio bis (2- nitrobenzoic acid) was dissolved.

4. Standard glutathione: In 100 ml of distilled water 10 mg of reduced glutathione was dissolved.

**Procedure**

1 ml of 10% TCA was added to 1 ml of kidney tissue homogenate. The precipitated fraction was centrifuged and 2ml DTNB was added to 0.5 ml supernatant. The final volume was ended up to 3 ml with phosphate buffer. The colour formed was read at 412 nm. The amount of glutathione was expressed as µg/g tissue. Reduced glutathione was used as standard (100µg/ml).

\[ X = \frac{Y - 0.0046}{0.0034} \]

\[ Y = \text{Absorbance of test sample} \]
4.3.15.5. Estimation of Glutathione Peroxidase\textsuperscript{103}

**Principle**

Glutathione peroxidase of enzymes plays important role in the protection of organisms from oxidative damage. Glutathione peroxidase converts reduced glutathione (GSH) to oxidized glutathione (GSSG) while reducing lipid hydroperoxides to their corresponding alcohols or free hydrogen peroxide to water. Low levels of Glutathione peroxidase have been correlated with free radical related disorders.

**Reagents**

1. 2.1 Mm reduced glutathione

2. 0.5 Units/ml glutathione reductase

3. 300 µM t-Bu-OOH

**Procedure**

1 ml of optimized assay reagents were mixed with 0.4 ml diluted whole blood, 10µl substrate added in 2 ml cuvette. The absorbance was read with UV/Vis spectrophotometer at 340 nm at 25\textdegree C against the reagent blank. In each measurement of the enzyme activity, the corresponding decrease of $A_{340}$ nm was standardized against Hb concentrations, and the activity was expressed as oxidized NADPH in mmol/ml.
4.3.15.6. Estimation of lipid peroxidation

Principle

Oxidative stress is associated with peroxidation of cellular lipids, which is determined by measurement of Thiobarbituric acid reacting substances (TBARS). The concentration of LPO products may reflect the degree of oxidative stress. The increased level of TBARS results increased oxygen free radicals, which attacks the polyunsaturated fatty acids in cell membrane and cause LPO. The malondialdehyde (MDA) content, a measure of lipid peroxidation was assayed in the form of TBARS.

Reagents

1. Thiobarbituric acid: In 100 ml of distilled water.0.67% w/v in 1 M tris hydrochloride pH-7, 0.67 g of Thiobarbituric acid was dissolved.

2. Trichloroacetic acid (20% w/v): In 100 ml of distilled water 20g of TCA was dissolved.

3. Standard malondialdehyde (0-25 n.mol)

Procedure

In 2ml of 20% TCA 1 ml of tissue homogenate was mixed and kept in ice for 15 minutes. The precipitate was separated by centrifugation and 2 ml clear supernatant solution was mixed with 2 ml aqueous 0.67% TBA solution. This mixture was heated on a hot water bath for 10 min. It was cooled in ice for 5 minutes and
absorbance read at 535 nm. The values were expressed as μmol of MDA formed/mg of protein.

\[ X = \frac{(y+0.002)}{0.0026086} \]

### 4.3.16. Histopathology of Kidneys

#### 4.3.16.1. Processing of Isolated Kidneys

At the end of the protocol the animals were sacrificed and their kidneys were isolated. The isolated kidneys were made into small pieces and after removal of extra tissues preserved in 10% formalin solution. Then the isolated kidneys were washed in running water for about 10 minutes. By increasing concentrations of isopropyl alcohol ranging about 70%, 80%, and 90% for about 12 hrs each was used for the dehydration of isolated tissue and the final step of dehydration was done by way of absolute alcohol.

By using chloroform with two changes at about 15 to 20 minutes each, clearing of the tissue was performed. After clearing the organ pieces were located in L moulds followed by paraffin infiltration in self- tissue- processing- unit.

To the L-shaped moulds hard paraffin which was premelted must be poured. The pieces of kidney were then placed into the molten paraffin preparation carefully, quickly and the whole set up was allowed to cool.
**Sectioning:**

By using microtome the tissue which was present inside the square blocks cut into tiny sections. The sections obtained will be of thickness of 5µ. The sections were taken on a micro glass slide preapplied egg albumin (sticking substance). The sections were located in a boiling air oven maintained at 60°C for about an hour. As a result paraffin melts and egg albumin solidified or denatured, which fixes the tissues to the slide on which they are placed.

**Staining**

Eosin which is an acid stain stained all the cellular machinery to pink colour which is basic naturally. Whereas, haematoxylin which is a basic stain stained all the cellular machinery to blue colour which are acidic naturally.