MATERIALS AND METHODS
5.1 PREPARATION OF PLANT EXTRACT

Plant sources selected for the present study are *Melia composita* Willd., *Gmelina asiatica* Linn., *Sphaeranthus indicus* Linn., *Parmelia perlata* Ach. Plant materials were collected from places in and around Trichy, identified and authenticated with the help of herbarium specimen deposited at RAPINAT herbarium of St.Joseph’s College, Trichy, Tamilnadu, India. All the plants were collected during their blooming period. Fresh plants were collected, cleaned, shade dried and coarsely powered.

200g of coarse powder of specified parts of the selected plants (Table - 1) were taken in 1200 ml of water and boiled. The contents were reduced to one third and filtrate was evaporated to dryness. Paste form of the extract obtained and stored in an air tight container at 4° C. The aqueous extract was then subjected to pre-clinical trials.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Parts used</th>
<th>Percentage yield</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Melia composita</em> Willd</td>
<td>Leaves</td>
<td>32</td>
</tr>
<tr>
<td><em>Gmelina asiatica</em> Linn.</td>
<td>Whole plant (Aerial)</td>
<td>28</td>
</tr>
<tr>
<td><em>Sphaeranthus indicus</em> Linn.</td>
<td>Whole plant (Aerial)</td>
<td>36</td>
</tr>
<tr>
<td><em>Parmelia perlata</em> Ach.</td>
<td>Rosettes of Lichen</td>
<td>46</td>
</tr>
</tbody>
</table>

5.2 PREPARATION OF POLYHERBAL FORMULATION

Freshly prepared aqueous extract of the four selected plants viz. *M. Composita* Willd, *G. asiatica*, *S. indicus* L and *P. perlata* Ach. were mixed in the proportion of 2: 1: 1: 2 respectively and given to the experimental animals at a dose level of 250mg/kg body weight and 500mg/ kg body weight orally for 60 days daily.
5.3 EXPERIMENTAL ANIMALS

Healthy adult wistar strain of albino rats of both sexes, two to three months old and weighing 150g-200g were obtained from Tamilnadu Veterinary and Animal Sciences University, Chennai. The animals were allowed to acclimatize under laboratory conditions for a period of 5 days prior to the experiment. Rats were housed in standard polypropylene cages. Six rats were housed per cage, so as to provide them with sufficient space, and to avoid unnecessary morbidity and mortality. Animals were maintained under standard condition of 12:12- hours light/dark cycle and at an ambient temperature at 23 ± 1°C, with 65 ± 5 % humidity. Animals were fed with standard rat chow pellet obtained from Sai Durga Foods and Feeds, Bangalore, India and water ad libitum. All the studies were conducted according to the ethical guidelines of CPCSEA after obtaining necessary clearance from the committees (Approval No: 790/03/ac/CPCSEA).

5.4 CHEMICALS AND REAGENTS USED

Alloxan monohydrate, a most widely used chemical diabetogen was procured from SD Fine Chemicals Ltd, Mumbai, India and other reagents used in the experiment were analytical grade. Glibenclamide, a standard antidiabetic agent was procured from Aventia Pharma.Ltd., Goa, India.

5.5 TOXICITY STUDIES

The aqueous extracts of selected plants were tested for their acute and short-term toxicity (if any) in albino rats. To determine acute toxicity of a single oral administration of the herbal drug, different doses of the drug (1, 2, 3, 4, 5 g/ Kg body weight) were administered to different group of animals. Two animals were used for each group. Mortality and general behavior of the animals were observed periodically for 48 hrs. Animals were observed continuously for 4hrs and intermittently for the next 6, 12, 24, & 48 hrs. Initial and final body weight and food intake, state of stool and body temperature were observed. Levels of serum enzymes were analyzed after 48h.
Materials & Methods

To study short – term toxicity, 4 groups of animals of 5 each were used for each plant. Group II, III and IV received 200, 300 and 400mg/Kg body weight of aqueous extract of plants. Polyherbal formulation at a dose level of 250 and 500mg/Kg body weight were given for 15 days. Group I received 5% Tween 80 and served as control. The behavior of the animals was observed daily. Initial and final body weight and food intake, state of stool and body temperature were observed. At the end of 15th day, animals were sacrificed and the levels of serum GPT, GOT and ALP were analyzed. Liver tissues were dissected out for histopathological studies.

Toxicity studies were carried out for all the selected plants and the formulation.

5.6 INDUCTION OF DIABETES IN RATS

Diabetes mellitus was induced in a batch of normoglycemic albino rats, starved for 16 hours, 150mg/kg body weight of alloxan monohydrate was dissolved in physiological saline and injected intraperitoneally. This dose of alloxan produced persistent hyperglycemia after 4 days which was determined by estimating blood and urine sugar levels. The diabetes induced rats having blood glucose level >250mg/dl were chosen and grouped for further studies.

5.6.1 ALLOXAN

For experimental studies, diabetogenic chemicals can be used to induce diabetes in animal models. Few such chemicals are streptozotocin, alloxan, dithizone, growth hormone, dexamethasone and insulin antibodies. In the present study alloxan is used to induce diabetes in experimental albino rats.
Materials & Methods

5.6.1.1 ALLOXAN STRUCTURE:

Alloxan or mesoalylurea is an organic compound based on a pyrimidine heterocyclic skeleton. This compound has a high affinity for water and therefore exists as the monohydrate. The compound was discovered by Von Liebig and Fiedrich Wohler following the discovery of urea in 1828 and is one of the oldest named organic compounds.\textsuperscript{106}

STRUCTURE:

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{aloxan_structure.png}
\caption{Figure - 9}
\end{figure}

Chemically alloxan is 2,4,5,6-tetra oxy pyrimidine; 5,6 dioxyuracil. Alloxan is \( \beta \) cell toxic compound which is used very often as diabetogenic agent. It is free soluble in water and slightly acidic with a pKa of 6.63. Its molar mass is 160.09 g/mol.\textsuperscript{107} Alloxan is a hydrophilic and unstable substance. Its half-life at neutral pH and 37°C is about 1.5 min and is longer at lower temperatures.\textsuperscript{108}
5.6.1.2 MECHANISM OF ACTION OF ALLOXAN.

Alloxan diabetes has been commonly utilized as an animal model of insulin dependent diabetic mellitus. Alloxan exerts its diabetogenic action when it is administered parenterally, intravenously, intraperitoneally or subcutaneously. When alloxan is given intraperitoneally or subcutaneously, its effective dose must be 2-3 times higher. The intraperitoneal dose below 150mg/kg b.w may be insufficient for inducing diabetes in the rat. Fasted animals are more susceptible to alloxan, whereas increased blood glucose provides partial protection.\(^{109}\)

Alloxan exhibits high affinity to the SH-containing cellular compounds; reduced glutathione (GSH), cysteine and protein-bound sulphydryl group are very susceptible to its action. Other reducing agents such as ascorbate may also participate in this reduction. The SH-containing compounds essential for proper glucose induced insulin secretion is glucokinase, Which is vulnerable to alloxan. Alloxan reacts with two-SH groups in the sugar binding side of glucokinase resulting in the formation of the disulfide and inactivation of the enzyme.\(^{108}\)

Alloxan and the product of its reduction, dialuric acid establishes a redox cycle with formation of superoxide radicals. These radicals undergo dismutation to hydrogen peroxide. These highly reactive hydroxyl radicals are formed by the Fenton reaction. The action of reactive oxygen species with a simultaneous massive increase in cytosolic calcium concentration cause rapid destruction of \(\beta\) cells.\(^{105}\)
THE MECHANISM OF ALLOXAN - INDUCED REACTIVE OXYGEN SPECIES GENERATION IN β CELLS OF RAT PANCREAS.

Materials & Methods

Gka, Gki – glucokinase active, inactive respectively.

HA* - Alloxan radicals

[Ca^{2+}]_i - intra cellular calcium concentration
Alloxan elevates cytosolic free Ca\(^{2+}\) concentration in pancreatic \(\beta\) cells. Alloxan induced calcium influx from extracellular fluid, exaggerates calcium mobilization from intracellular stores and its limited elimination from the cytoplasm. The calcium influx may result due to the ability of alloxan to depolarize pancreatic \(\beta\) cells. Depolarization of the cell membrane opens calcium channels and enhances calcium entry into the cells. Calcium removal from the cells is restricted due to alloxan-induced inhibition of liver plasma membrane Ca\(^{2+}\)-ATPase. The effects of alloxan on intracellular calcium concentration seem to be mediated at least partially, by \(\text{H}_2\text{O}_2\) since hydrogen peroxide itself exerts a similar effect on calcium concentration in \(\beta\) cells. This causes sudden rise in insulin release from \(\beta\) cells treated with alloxan may be one of the effects of alloxan-induced augmentation in cytosolic Ca\(^{2+}\) concentration. The exaggerated concentration of this ion to insulin release and together with reactive oxygen species, cause damage of pancreatic \(\beta\) cells.

\(\beta\)-cell toxic compound, alloxan is used very often as a diabetogenic agent. The dose of alloxan to produce the desired severity of diabetes is 100-175mg/kg body weight. It is less expensive than STZ. Ca\(^{2+}\) plays an important role in the diabetogenesis of alloxan, since alloxan cause the increase of cytosolic free Ca\(^{2+}\) in rat pancreatic \(\beta\)-cells.

The primary target of reactive oxygen species produced from alloxan is DNA of pancreatic \(\beta\)-cells. Alloxan caused the depletion of cellular ATP and this ATP loss is believed to be a result of the lack of NAD\(^{+}\) available and/or oxidative phosphorylation.\(^{110}\)
5.7 EXPERIMENTAL DESIGN

5.7.1 Experimental design for individual plants

Rats were divided into six groups of six rats each. The experimental design given below has been followed for all the four plants.

Group-I Normal untreated rats

Group-II Disease control - animals treated with alloxan in normal saline at a dose of 150 mg/kg body weight IP.

Group-III Animals were treated as in Group II. After 4 days of alloxan induction, treated with plant extract at a dose level of 200 mg/kg body weight orally for 60 days.

Group-IV Animals were treated as in Group II. After 4 days of alloxan induction, treated with plant extract at a dose level of 300 mg/kg body weight orally for 60 days.

Group-V Animals were treated as in Group II. After 4 days of alloxan induction, and treated with plant extract at a dose level of 400 mg/kg body weight orally for 60 days.

Group-VI Diabetic rats treated with 600 μg/Kg bw of glibenclamide for 60 days daily.
5.7.2 *Experimental design for polyherbal formulation (Selected herbal extract mixture)*

Rats were divided into six groups of six rats each.

- **Group-I** Normal untreated rats
- **Group-II** Disease control - animals treated with alloxan in normal saline at a dose of 150 mg/kg body weight IP
- **Group-III** Animals were treated as in Group II. After 4 days of alloxan induction, and treated with PHF at a dose level of 250mg/kg body weight orally for 60 days daily.
- **Group-IV** Animals were treated as in Group II. After 4 days of alloxan induction, and treated with PHF at a dose level of 500mg/kg body weight orally for 60 days daily.

At the end of the experimental period, the animals were sacrificed by cervical decapitation. Blood was collected and used for various biochemical estimations. Pancreas was excised, weighed and processed for histopathological studies and liver were dissected out and washed in ice-cold saline. Liver tissues were homogenized, in 0.1 M phosphate buffer, pH 7.4 and used for analyzing various biochemical parameters.

5.8 **STATISTICAL ANALYSES**

All the results were expressed as mean ± S.E. The data were statistically analyzed by one – way analysis of variance (ANOVA) followed by Duncan multiple range test. Statistical presentations were organized using the Statistical Package for Social Sciences (SPSS), Windows version 17.0, 2008, SPSS Inc., New York. Inter group comparison were carried out and P values <0.05 were considered significant.
5.9 PHYTOCHEMICAL SCREENING

5.9.1 Preliminary Phytochemical Screening

Preliminary phytochemical screening of the extracts were carried out.\textsuperscript{113}

Test for Saponins:

The substance was shaken well with water.

Test for Tannins:

The substance was mixed with basic lead acetate solution.

Test for Steroids:

Liebermann burchard test:

The sample was taken in a test tube added few drops of glacial acetic acid, acetic anhydride and 1ml of concentrated Sulphuric acid were added along the sides of the test tube.

Test for Terpenoids:

Salkowshi test:

The substance was warmed with tin and thionyl chloride.

Test for Sugars:

The substance was mixed with anthrone and 1 drop of concentrated Sulphuric acid and warmed gently.

Test for Proteins:

The substance was mixed with saturated solution of picric acid.
Test for Flavonoids:

Shinado’s test:

To the substance added alcohol, a few magnesium turnings and few drops of concentrated hydrochloric acid and boiled for 5 minutes.

Test for Coumarin:

A small quantity of substance was mixed with few drops of 10% sodium hydroxide.

Test for Quinones:

The substance was mixed with few drops of concentrated sulphuric acid.

Test for Lignins:

The substance was mixed with Alcoholic solution of Phloroglucinol and added few drops of concentrated Hydrochloric acid.

Test for alkaloid:

1. To the substance few drops acetic acid was added, followed by Dragendorff’s reagent and shaken well.

2. The substance was mixed little amount of dilute hydrochloric acid and Mayer’s reagent.
5.9.2 Thin Layer Chromatography

TLC is an important tool for the separation, identification and different Classes of natural product. This technique allows the separation of different components by the differential migration of solute between two phases, a stationary and mobile Phase. The main principle involved in the technique is adsorption. The stationary phase acts as an absorbent that is silica, polar compounds have less affinity for solute, stick to it and moves slowly up as mobile phases moves. The compounds will comparatively move quickly up the plates, so Rf values will be more, mixture of compounds will separate according to the polarities. 114

Preparation of plates:

100 gms of silica gel G was weighed and made into a homogenous suspension with 200 ml of distilled water to form slurry. The slurry was poured into TLC applicator, which is adjusted to 25 mm thickness on flat glass plate of different dimensions (10X2, 10X5, 30X5, 20X10 cm etc). The coated plates were allowed to dry in air, followed by heating at 100-105° C for 1 hr. Cooled and protected from moisture. Before using the plates were activated by heating at 110° C for 10 minutes.

Preparation of Sample for Thin Layer Chromatography:
The sample was prepared as follows:

1. 1 gm of plant extracts were taken.
2. Weighed samples were added to 10 ml of Methanol and allowed to stand for 18 hours.
3. After 18 hours, the sample was boiled and filtered.
4. The filtrate was concentrated on a water bath and used as a sample to carry out Thin layer chromatography.

Separation of components:
The sample was dissolved in the methanol and spotted using a capillary tube on TLC plates 1-1.5 cm above from the bottom of the plate.
Materials & Methods

5.9.3. ESTIMATION OF PHENOLS

Reagents required:

1. 80% Ethanol.
2. Folin ciocalteau Reagent.
3. 20% Sodium bicarbonate.
4. Stock Standard – 100 mg Catecho in 100 ml Water.

Procedure:

0.5 to 1 gm of the sample was weighed and ground well with 10 times volume of 80% ethanol. The homogenate was centrifuged at 10,000 rpm for 20 min. The supernatant was collected and the residue was re extracted with 5 times the volume of 80% Ethanol. Centrifuged and the supernatant was collected. It was then evaporated to dryness. The residue obtained was dissolved in 5 ml of distilled water. Different aliquots (0.2 – 2.0 ml) were pipetted out in test tubes. The volume was made upto 3 ml with water. 0.5 ml of Folin’s reagent was added to all the tubes after 3 min 2 ml of 20% Sodium bicarbonate solution was added. The content was mixed thoroughly and the test tubes were placed in boiling waterbath for 1 min, cooled and the colour developed was measured at 650 nm.
5.9.4 Estimation of total Alkaloids:

The alcoholic extract of plant sample was treated with 0.1N HCl and aqueous acidified layer thus obtained was partitioned with Chloroform in separating funnel. The Chloroform layer was discarded and the aqueous layer was basified with Ammonium hydroxide to alkaline pH and partitioned with Chloroform in separating funnel. The aqueous layer was discarded and the Chloroform layer was evaporated, the resultant content was treated as total alkaloid and confirmed for alkaloid with dragendorff’s reagent.

5.9.5 Estimation of Total Flavonoids:

Aqueous plant extracts were extracted with ethyl acetate. The extracts were dried over anhydrous sodium sulphate, filtered and concentrated under vacuum upto a concentration of 1g/ml of extract. They are further diluted with ethyl acetate to obtain 0.01g/ml solution and used in the experiments. About 10ml of the solution was transferred into a 25ml volumetric flask, 1ml of 2% aluminium chloride was added and the solution was filled to volume with methanol-acetic acid and was kept aside for 30 min, the absorbance was measured at 390nm. A blank was also maintained. Luteolin was used to construct the calibration curve in the concentration range of 1 to 10 μg/ml.
5.9.6 Estimation of Total Tannins:

Defatted 2g of sample with 25ml petroleum ether for 12 hrs. Boiled the marc for 2 hrs with 300ml of double distilled water. Cooled and diluted upto 500ml and filtered. 25 ml of this infusion was poured into 2-litre porcelain dish; added 20ml of indigo solution and 750 ml double distilled water. Titrated with 0.1N Potassium permanganate solution, 1ml at a time until, blue solution changes to green. Thereafter added dropwise until solution becomes golden yellow in colour.

Similarly, titrated the mixture of 20ml indigo solution and 750ml of double distilled water. Calculated the difference between 2 titrations in ml.

Each ml of 0.1N potassium permanganate solution is equivalent to 0.004157g of total tannins.

5.10. EVALUATION OF BODY WEIGHT

The body weight of the experimental animals was measured by using the rough table top balance. The body weight of the animals were weighed before the induction of diabetes mellitus and during the treatment with plant drug sources at an interval of 15 days till the end of the experimental period.
Materials & Methods

5.11 BIOCHEMICAL STUDIES

5.11.1 ESTIMATION OF BLOOD GLUCOSE

Blood glucose content was estimated by Folin - Wu method.\textsuperscript{119}

Reagents:

1. 10% Sodium tungstate solution.
2. 2/3 N Sulphuric acid.
3. Alkaline copper sulphate solution.
   Added 40 grams of sodium carbonate to 7.5 g of tartaric acid and also added 4.5 g of copper sulphate and made up to 1 litre.
4. Phosphomolybdic acid:
   To 35 g of molybdic acid added 5 g of sodium tungstate to 200 ml of 10% sodium hydroxide, boiled vigorously for 20-30 minutes to remove the whole of ammonia present in molybdic acid. Cooled and diluted to about 350 ml and added 125 ml of orthophosphoric acid and made up to 500 ml.
5. Stock standard glucose solution:
   100 mg of glucose is dissolved in 100 ml of distilled water.
6. Working standard solution:
   1 in 10 dilutions.

Procedure:

To 0.1 ml of the plasma added to 3.4 ml of water, 0.2 ml of 10% sodium tungstate and 0.2 ml of 2/3 N Sulphuric acid in order to precipitate the protein. Mixed well and centrifuged. To 1 ml of the filtrate added 2 ml of alkaline copper sulphate solution and placed in a boiling water bath for 8 minutes, cooled and added 2 ml of phosphomolybdic acid. Various concentrations of standard solution was taken and it was made up to 2 ml of alkaline copper sulphate solution and placed in a boiling water bath for 8 minutes, cooled and added 2 ml of phosphomolybdic acid. A Blank was also maintained. The blue colour, developed was read at 620 nm.
Materials & Methods

5.11.2 ESTIMATION OF SERUM INSULIN

Kit content:

1. Assay buffer – 0.05M phosphosaline pH 7.4 containing 0.025M EDTA, 0.08% sodium azide and 1.1 RIA grade BSA.


3. $^{125}$I – insulin – $^{125}$I insulin label HPLL purified.

4. Label hydrating buffer: assay buffer containing normal guinea pig serum as a carrier used to hydrate $^{125}$I – rat insulin.

5. Standards: purified recombinant rat insulin in standard buffer at the following concentration 0.1, 0.2, 0.5 – 1.0, 2.0, 5.0, 10.0 mg/ml.


7. Precipitating reagent: goat anti guinea pig IgG serum, 3% PEG and 0.05% triton- X- 100 in 0.05M phosphosaline, 0.025M EDTA, 0.08% sodium azide samples processed according to kit protocol.

Insulin levels – μU/ml.
5.11.3.1 ISOLATION OF ERYTHROCYTE MEMBRANE AND PREPARATION OF HEMOLYSATE

Erythrocyte membrane was isolated according to the method of Dodge et al., (1963)\textsuperscript{120} with a change in buffer according to Quist (1980)\textsuperscript{121}.

Reagents required:

1. Tris-HCl buffer (pH-7.2) hypotonic:
   - 5mM Tris and 15mM sodium chloride was dissolved in water.
2. 0.9% sodium chloride: 900mg of sodium chloride was dissolved in 100 ml of distilled water.

Procedure:

Blood was collected with EDTA as anticoagulant. Plasma was separated by centrifugation at 1500Xg for 15 min. The packed cells were washed well with isotonic saline. After washing with saline, the packed cells were lysed by suspending them in hypotonic Tris-HCl buffer for one hour. The lysed cells were centrifuged at 15,000 Xg for 30 minutes. The supernatant was subsequently used as hemolysate, which was subsequently used for the analyses, while the membrane remained at the bottom of the centrifuge tube. This was washed with Tris-HCl buffer, sedimented at 15,000Xg and the washing was repeated twice. The final membrane obtained was colourless or pale yellow which was taken for analysis of glycoprotein component.
5.11.3.2 ESTIMATION OF GLYCOSYLATED HAEMOGLOBIN

Glycosylated hemoglobin content was estimated using the method of Nayak et al., 1981.²²

Reagents required:

1. NaCl 0.9%
2. Oxalic acid 0.3M.
3. TCA 40%
4. Thiobarbituric acid 0.05M.
5. Standard fructose in the range of 10-40μg.

Procedure:

0.2ml of hemolysate was mixed with 1.8 ml of 0.3M oxalic acid and the mixture was hydrolyzed for 2 hours, cooled and added 1.0ml of 40% TCA. After centrifugation at 1400 Xg for 20 minutes. 1.5ml of the supernatant was treated with 0.5ml of 0.05M-thiobarbituric acids. Incubated at 37°C for 40 min. the colour developed was read at 443nm. Standard fructose in the range of 10-40 μg was processed similarly. Values were expressed as % glycosylated Hb.
5.11.4 ESTIMATION OF LIVER GLYCOGEN

Hepatic glycogen was estimated by the method of Morales et al 1973\(^\text{123}\).

Reagents required:

1. Ethanol
2. 30\% KOH
3. 0.2\% Anthrone in H\(_2\)SO\(_4\)
4. Stock Standard Glucose (100 mg/100ml)
5. Working Standard (100 \(\mu\)g/100ml)

Procedure

A weighed amount of the tissue was subjected to alkali digestion in a boiling water bath for 20 min after adding 5 ml of 30\% KOH. The tubes were cooled and 3ml of absolute ethanol and a drop of ammonium acetate were added. The tubes were then placed in a freezer overnight to precipitate glycogen. The precipitated glycogen was collected after centrifugation at 3000 g for 10 min. The precipitate was washed thrice with alcohol and dissolved in 3 ml of water. Aliquotes were taken and made upto 1 ml with water. 4ml of anthrone was added to the tubes kept in an ice bath, mixed and heated in a boiling water bath for 20 min. The green colour developed was read at 640 nm. The factor 0.93 was used to convert glucose to glycogen.

The values were expressed as mg/g tissue.
5.11.5 ASSAY OF GLUCOKINASE (EC 2.7.1.2)

Glucokinase (ATP: D-hexoses-6-phosphotransferases) was assayed by the method of Brandstrup et al. (1957).  

Reagents required:

1. Enzyme solution (Liver extract)
2. Tris buffer (0.2M; pH 7.4)
   4.8gm tris is dissolved in 200ml of distilled water and the pH is adjusted to 7.4 with 0.1N hydrochloric acid.
3. Substrate:
   100 mg glucose in 100ml of distilled water.
4. Alkaline copper sulphate reagent.
5. Phosphomolybdic acid reagent.
6. ATP Solution (0.5%)
   250mg of pure ATP dissolved in 50ml of distilled water.
7. 0.1% Magnesium chloride.
8. Sodium fluoride (0.1%).

Procedure:

1ml and 2ml of tris buffer were pipetted out into test and control test tubes respectively. Then 1ml of substrate was mixed with the buffer taken in the ‘test’ test tubes. To this, 1ml of liver homogenate was mixed with the substrate taken in the ‘test’ test tube. Then 0.5ml 0.1% magnesium chloride, 0.5ml of 0.5% ATP solution and 0.5ml of 0.1% sodium fluoride were added and acclimatized at 37°C for 3 minutes. Then the test tubes were incubated at 37°C for 30 minutes. The enzymatic reaction was arrested by the addition 2ml of alkaline copper sulphate solution and test tubes were heated in the boiling water bath for 8 minutes. Then 2ml of phosphomolybdic acid was added and the developed colour is read at 620nm against control. From the absorbance, the activity of glucokinase was calculated using standard graph. The activity of glucokinase is expressed as micromole of glucose - 6- Phosphate per mg protein per minute.
5.11.6 ASSAY OF FRUCTOSE - 1-6- BISPHOSPHATASE (EC 3.1.3.11)

Fructose-1-6- bisphosphatase was assayed by the method of Gancedo and Gancedo (1971).\textsuperscript{125}

Reagents required:
1. Tris – HCl buffer – 0.1 M, pH 7.0.
2. Substrate: Fructose- 1-6- bisphosphate, 0.05M solution.
3. Magnesium chloride: 0.1 M.
4. Potassium chloride: 0.1 M.
5. EDTA solution: 0.001 M.
6. 10% TCA.

Procedure:

The assay solution consisting of 50 μmoles of buffer, 5 μmoles of substrate, 10 μmoles each of magnesium chloride and potassium chloride, 100nmoles of EDTA and 0.1 ml of the enzyme source were taken. The incubation was carried out at 37° C for15 minutes. The reaction was terminated by the addition of 1.0 ml of 10% TCA. The suspension was centrifuged and the phosphorus content of the supernatant was estimated according to the method described by Fiske and subbarow (1925).\textsuperscript{126}
5.11.7 ASSAY OF GLUCOSE-6-PHOSPHATASE (EC 3.1.3.9)
(King J 1965)^{127}

Reagents required:
1. 0.1 M citrate buffer pH 6.5
2. Substrate glucose-6-phosphate 0.01M in distilled water.
3. 10% TCA

Procedure:
The incubation mixture in a total volume of 1.0 ml containing 0.3ml of buffer, 0.5ml substrate and 0.2ml of enzyme extract. Incubated the mixture at 37° for 1 hour. The reaction was terminated by adding 1.0ml of 10% of TCA. The suspension was centrifuged and the phosphorous content of the supernatant was estimated by Fisk and Subbarow method^{126}. 
ASSAY OF GLUCOSE-6- PHOSPHATASE DEHYDROGENASE (EC 1.1.1.49)
(Gancedo, 1971)\textsuperscript{125}

Reagents required:

1. 0.1M Tris –Hcl (pH:8)
2. 0.1M Magnesium chloride
3. 2mM NADP+
4. 6mM Glucose -6-phosphate

Procedure:

The reaction mixture consisted of 0.1ml each of Tris Hcl, Magnesium chloride, NADP+ and 0.1ml of homogenate. To this 0.5ml of water was added and allowed to stand at room temperature for 10 min. The reaction was initiated by adding of 0.2ml glucose-6-phosphate, the change in OD was read at an interval of 30 sec for 3 min at 340nm. The activity of Glucose –6-phosphate dehydrogenase was expressed as micromole of NADP+ oxidized/min/mg protein.
5.11.9  ESTIMATION OF PHOSPHORUS

Phosphorus was estimated by the method of Fiske and Subbarow (1925)\textsuperscript{126}

Reagents required:

1. Ammonium molybdate
2. ANSA reagent
3. Standard phosphorus: 35.1 mg of Potassium dihydrogen phosphate was dissolved in 100 ml of double distilled water.
4. Working Standard: 1 ml of stock solution was made upto 10 ml to give a concentration of 80 µg phosphorus / ml.

Procedure:

The test sample was made upto 4.3 ml with water and 0.5 ml of ammonium molybdate reagent was added and incubated at room temperature. Then added 0.2 ml of ANSA and the colour developed was read at 640 nm after 20 minutes.
5.11.10.1 Extraction of Lipids

(Folch J et al., 1970)\textsuperscript{128}

A known volume of suspension was mixed with 10ml of chloroform methanol mixture and homogenized. The homogenate was filtered through Whatmann filter paper (No.42) into a separating funnel. The filtrate was mixed with 0.2 ml of physiological saline and the mixture was kept overnight undisturbed. The lower phase containing the lipid extract was drained off into preweighed beakers. The upper phase was re-extracted with more chloroform-methane mixture and extracts were redissolved in 1.0 ml of chloroform-methanol mixture and aliquots were used for estimation of various lipid components.

Chloroform: methanol 2:1 v/v
Saline- 0.89 %
5.11.10.2 ESTIMATION OF SERUM / TISSUE CHOLESTEROL

Cholesterol was estimated in serum and tissue by the method of Parekh and Jung, 1970.\(^{129}\)

Reagents Required:

1. Ferric Chloride – Uranyl Acetate reagent
   - To 500mg of ferric chloride was added 10 ml of water and 3 ml of conc. Ammonia solution. The precipitate formed was washed several times with water and dissolved in acetic acid. 100 mg of uranyl acetate was added and the contents were shaken well and made up to 1 litre with acetic acid. It was left overnight.

2. Sulphuric acid-Ferrous Sulphate reagent
   - 100 mg of anhydrous ferrous sulphate was dissolved in 100 ml of acetic acid. To this added 100 ml of conc sulphuric acid with constant stirring. After cooling to room temperature the volume was made up to 1 litre with sulphuric acid.

3. Standard Cholesterol
   - 100 mg of cholesterol dissolved in 100ml of acetic acid. 10 ml of this stock diluted to 100ml with acetic acid.

Procedure:
   - To 0.1 ml of the lipid extract (tissue / serum), 2.9 ml of uranyl acetate reagent was added. 2 ml of sulphuric acid – ferrous sulphate reagent was added and mixed well. Blank comprised of uranyl acetate reagent and 2 ml of sulphuric acid – ferrous sulphate reagent. A calibration curve was prepared using the standard cholesterol. The optical density was measured after 20 min at 560 nm. The cholesterol content was expressed as mg/dl serum; mg/100g wet tissue.
5.11.10.3 ESTIMATION OF SERUM / TISSUE TRIACYLGLYCEROL

(Foster L B and Dunn RT, 1973)

Reagents required:

1. Isopropanol
2. Aluminium oxide: neutral
3. Saponification reagent: 5g of potassium hydroxide dissolved in 60 ml of distilled water and 440 ml of isopropanol was added to it.
4. Acetyl acetone reagent: 0.75 ml of acetyl acetone was added to 20 ml of isopropanol and mixed.
5. Sodium metaperiodate reagent: 77 g of anhydrous ammonium acetate was dissolved in about 700 ml of distilled water; 60 ml of glacial acetic acid was added to it followed by 650 mg of sodium metaperiodate. The mixture was dissolved and diluted to one litre.
6. Stock Standard Solution: 1.0g of tripalmitin was dissolved in 100 ml of isopropanol.
7. Working Standard Solution: 3 ml of stock standard was dissolved in 10 ml of isopropanol in a 10 ml volumetric flask. (300mg /100 ml).

Procedure:

4.0 ml of isopropanol was added to 0.1 ml of lipid extract (tissue / serum) and mixed well, followed by 0.4 g of alumina and shaken well for 15 min. Centrifuged at 2000 rpm for 10 min and then 2.0 ml of the supernatant was transferred to appropriately labeled tubes. The tubes were placed in a water bath at 65° C for 15 min for saponification after adding 0.6 ml of the saponification reagent. After cooling 1.0 ml of Sodium metaperiodate was added followed by 0.5 ml of acetyl acetone reagent. After mixing, the tubes were kept in a water bath at 65° C for half and hour. The contents were cooled and read at 430 nm. The Triacylglycerol content was expressed as mg/dl serum; mg/100g wet tissue.
5.11.10.4 ESTIMATION OF SERUM / TISSUE PHOSPHOLIPIDS

Phospholipids was estimated by the method of Bartlette (1959)\textsuperscript{131} by digestion with perchloric acid and the phosphorus liberated was estimated by the method of Fisk and Subbarow (1925)\textsuperscript{126}

Reagents required:

1. Perchloric acid
2. Molybdic acid: 2.5% ammonium molybdate in 3N H\textsubscript{2}SO\textsubscript{4}.
3. ANSA
4. Phosphorus stock:
   - 35.1 mg of KH\textsubscript{2}PO\textsubscript{4} dissolved in 100ml of distilled water.

Procedure:

- 0.1ml of lipid extract (tissue / serum) was digested with 0.2ml of perchloric acid over a sand bath. Digestion was continued till it was colorless. The liberated phosphorus was estimated.

- 4.3ml of deionised water was added to the digested sample followed by 0.5ml of ammonium molybdate. After 10min 0.2ml of ANSA was added. Tubes were well shaken and kept aside for 20mins. Blue colour read at 620nm.

The total phospholipids were estimated by multiplying the value of Pi by 25 and phospholipids content was expressed as mg/dl serum; mg/100g wet tissue.
5.11.10.5 ESTIMATION OF SERUM / TISSUE FREE FATTY ACID

Non-esterified fatty acids were estimated by the method of Falholt et al (1973).^132

Reagents required:

1. Extraction solvent: chloroform: methanol (5:1)
2. Stock copper solution (500mM)
3. Trietanolamine-1M
4. Sodium hydroxide-1M
5. Copper reagent
6. Diphenyl carbazide solution-1.5M in ethanol
7. Standard Palmitic acid

Procedure:

0.1ml of lipid extract was evaporated to dryness. To the residue obtained 0.1 ml of phosphate buffer, 6.0ml of extraction solvent and 2.5 ml of copper reagent were added. All the tubes were shaken vigorously and 200mg of activated silicic acid was added and left aside for 30 minutes. The tubes were centrifuged and 3ml of the copper layer was transferred to another tube containing 0.5 ml of diphenyl carbazide and mixed carefully. The absorbance was read at 550nm. The free fatty acid content was expressed as mg/dl serum; mg/100g wet tissue.
5.11.11 ASSAY OF SERUM/ TISSUE HDL CHOLESTEROL
(Friedewald WT et al., 1972)\textsuperscript{133}

Reagents required:

1. Heparin - 20,000 units/ml
2. Manganese chloride – 3.167g of manganese chloride was added to 1.0ml solution of heparin containing 20,000 units. The mixture was made up to 8.0ml with distilled water.

Procedure:

To 1.0 ml of plasma, 0.18 ml of heparin manganese chloride reagent was added and mixed. This was allowed to stand in an ice bath for 30 minutes and then centrifuged in a refrigerated centrifuged at 2500 g for 30 minutes. The supernatant contained HDL fraction. Aliquots of the HDL supernatant were estimated for cholesterol, phospholipids and triacylglycerol. The HDL cholesterol content was expressed as mg/dl serum; mg/100g wet tissue.
5.11.12 AGGREGATION OF VLDL

Sodium dodecyl sulphate – 10% in 0.15 M NaOH (pH – 9)

1 ml of plasma was added to 0.15 ml of SDS solution. The contents were mixed well and incubated at 37°C for 2 hours. The contents were centrifuged in a refrigerated centrifuge at 10,000 g for 30 minutes. VLDL aggregated as a pellicle at the top. The supernatant was a mixture containing HDL and LDL fractions. The fractions of lipoproteins were assayed after heparin manganese chloride and SDS precipitation. The values are expressed in mg/dl plasma. After precipitation the cholesterol levels in supernatant was measured to get HDL cholesterol. SDS precipitated VLDL and the cholesterol content in the supernatant was measured for HDL cholesterol, LDL cholesterol and VLDL cholesterol.

\[
\text{LDL cholesterol} = \frac{\text{Total serum cholesterol} - \text{Total serum TGL} - \text{HDL Cholesterol}}{5}
\]

\[
\text{VLDL} = \frac{\text{Total Serum TGL}}{5}
\]

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5.11.13 ESTIMATION OF PROTEIN IN SERUM AND LIVER TISSUE

Serum protein content was estimated by the method of Lowry et al (1951).³

Reagents required:

1. Alkaline Copper Sulphate Reagent:
   Solution A: 2% Sodium carbonate in 0.1 N sodium hydroxide
   Solution B: 0.5%Coppersulphate, 1 %Sodium potassium tartarate in water.
   Solution A was mixed with B in 50:1 ratio.

2. Folin’s phenol Reagent:
   Into a 1500ml round bottom flask, 100mg sodium tungstate, 25gm of sodium molybdate, 100ml water, and 50 ml of a-phosphoric acid and 100ml of concentrated hydrochloric acid were added and refluxed for 10 hrs. Then 150 gm of lithium sulphate, 50 ml of distilled water and a few drops of bromine were added. The mixture was boiled to remove excess bromine.
   The reagent was diluted 1:2 with distilled water just before use.

3. Standard Bovine Serum Albumin:
   10mg of crystalline Bovine Serum Albumin was dissolved in 100ml of distilled water.

4. Working Standard solution:
   1 in 5 Dilutions

Procedure:

A series of test tubes containing various concentration of standard protein solution were taken and the volume was made unto 1ml with distilled water. For the test, 0.1ml of serum was diluted to 20ml. From this 0.1ml was taken and made to 1ml with distilled water. Added 4.5ml of Lowry’s reagent to all tubes and allowed to stand for 10 minutes.
   After incubation added 0.5ml of Folin’s reagent to all the tubes and again incubated for 20 minutes at room temperature. The blue color developed was read colorimetrically at 620nm. The amount of protein present were expressed as mg/dl of serum; mg/100g of tissue.
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5.11.14 ASSAY OF ASPARTATE TRANSMINASE (AST) [EC2.6.1.1]

The serum AST levels were assayed using the method of King 1965\textsuperscript{127}

Reagents required:

1. 0.1 M Phosphate buffer, pH 7.5
2. Substrate solution (1.33 g of aspartic acid and 15 mg of \( \alpha \) keto glutarate were dissolved in 100 ml of phosphate buffer containing 0.5 mM Na OH)
3. 0.02\% DNPH in 1 N HCl
4. 0.4 N Na OH
5. Pyruvate

Procedure:

The assay mixture containing 1 ml of substrate and 0.2 ml of serum was incubated for 1 hr at 37°C. Added 1 ml of DNPH and kept at room temperature for 30 min. Serum was added to the control tubes after the reaction was arrested by the addition of 1 ml of DNPH. Added 5 ml of Na OH and the colour developed was read at 540 nm.

The activity of AST was expressed as \( \mu \) moles of pyruvate formed /min/mg of protein.
5.11.15 ESTIMATION OF ALANINE TRANSAMINASE (ALT) [EC2.6.1.2]

The serum ALT levels were assayed using the method of King 1965.127

Reagents required:

1. 0.1 M Phosphate buffer ,pH 7.5
2. Substrate solution (1.78 g of Alanine and 30 mg of α keto glutarate were dissolved in 100 ml of phosphate buffer containing 0.5mM Na OH)
3. 0.02%DNPH in 1 N Hcl
4. 0.4N Na OH
5. Pyruvate

Procedure:

The assay mixture containing1ml of substrate and 0.2 ml of serum was incubated for 1 hr at 37°C. Added 1 ml of DNPH and kept at room temperature for 20 min. Serum was added to the control tubes after the reaction was arrested by the addition of 1 ml of DNPH. Added 5 ml of Na OH and the colour developed was read at 540 nm. The activity of ALT was expressed as μmoles of pyruvate formed /min/mg of protein.
5.11.16  ESTIMATION OF SERUM ALKALINE PHOSPHATASE
[EC3.1.3.1]
The serum ALP levels were assayed using the method of King 1965

Reagents required
1 0.1 M carbonate buffer pH 10.0
2 0.1 M Di sodium phenyl phosphate
3 0.1 M Magnesium Chloride
4 15% Sodium Carbonate
5 Folin’s Ciocalteau phenol reagent
6 Phenol

Procedure
The reaction mixture containing 1.5 ml carbonate buffer, 1 ml Di sodium phenyl phosphate, 0.1 ml Magnesium Chloride and 0.1 ml of serum was incubated at 37 °C for 15 min. The reaction was arrested by the addition of Folin’s phenol reagent. Control tubes were also treated similarly but serum was added after the reaction was arrested with Folin’s phenol reagent and than added 1ml of Sodium Carbonate. The colour developed was read after 10 min at 640 nm. The activity of ALP was expressed as μmoles of phenol liberated /min/mg of protein.
5.11.17  ESTIMATION OF SERUM BILIRUBIN

Serum bilirubin was estimated in serum by the method of Malloy, 1937\(^\text{135}\)

**Reagents required**

1. Diazo blank – 15% HCl
2. Diazo reagent - Fresh solution is prepared by taking 10 ml of Solution A + 0.8 ml of Solution B
   Solution A – Contains Sulphanilic acid in Conc. HCl
   Solution B – Sodium Nitrite in water
3. Stock Standard – 10 mg bilirubin in 100 ml of chloroform
4. Working Standard – 2 ml of stock made up to 100 ml.

**Procedure**

For the determination of total bilirubin 0.2 ml of serum was taken and made upto 2 ml with water. Then added 0.5 ml of Diazo reagent, 2.5 ml of methanol. To the blank 0.2 ml serum was added and made up to 2 ml with water and added 0.5 ml of diazo blank and 2.5 ml methanol. The colour developed was read at 540 nm. The values were expressed as mg/dl.
5.11.18 ESTIMATION OF LIPID PEROXIDES

Lipid peroxide content was estimated by the method of Ohkawa, (1979).\textsuperscript{136}

Reagents

1. \( \text{H}_2\text{SO}_4 \) -0.85 N
2. TBA reagent - A mixture of equal volumes of 0.67% TBA aqueous solution and Glacial acetic acid.
3. Phosphotungstic Acid -10%
4. Butanol

Procedure:

0.1 ml of tissue homogenate was mixed with 4 ml of 0.85N \( \text{H}_2\text{SO}_4 \) and mixed gently. 0.5 ml of phosphotungstic acid was added and stirred well. The contents were centrifuged for 10 min. The supernatant was discarded and the sediment mixed with 2.0 ml of N/12 \( \text{H}_2\text{SO}_4 \) and 0.3 ml of 10% phosphotungstic acid. The mixture was centrifuged for 10 min. The sediment was suspended in 4.0 ml of distilled water and 1 ml of TBA reagent. The tubes were kept in a boiling water bath for 1 hr. After cooling 5 ml of butanol was added to each tube and the colour extracted in the butanol phase was read at 532 nm. The lipid peroxide content was expressed as nanomoles of malondialdehyde formed/mg tissue.
5.11.19 ASSAY OF REDUCED GLUTATHIONE

Reduced Glutathione was estimated using the method of Moron et al 1979.137

Reagents required:
1  10% TCA
2  0.6mM 5,5'Dithiobis-2-nitrobenzoic acid(DTNB) in 0.2 M sodium phosphate
3  0.2 M Phosphate buffer pH 8.0

Procedure:

One ml of homogenate/blood was precipitated with 1 ml of TCA and the precipitate was removed by centrifugation. To 5ml of the supernatant added 2ml of DTNB and the total volume was made up to 3 ml with phosphate buffer. The absorbance was read at 412 nm. The concentration of glutathione was expressed as nM /mg prot.
ASSAY OF SUPEROXIDE DISMUTASE (EC 1.15.1.1)

The assay of SOD was carried out using the method of Misra and Fridovich (1972).* 

Reagents Required:
1. Carbonate-Bicarbonate buffer: 0.1 M pH 10.2
2. EDTA solution -0.6 mM
3. Epinephrine -1.8 mM (freshly prepared)
4. Absolute ethanol
5. Chloroform

Procedure:
0.1 ml of tissue homogenate was added to tubes containing 0.75 ml ethanol and 0.15 ml chloroform (chilled in ice) and centrifuged. To 0.5 ml of supernatant added 0.5 ml EDTA solution and 1 ml of buffer. The reaction was initiated by the addition of 0.5 ml of epinephrine and the increase in absorbance was measured at 480 nm. The enzyme activity was expressed as IU/mg protein.
ASSAY OF CATALASE (hydrogen peroxide: hydrogen peroxide oxidoreductase EC 1.11.1.6)

Catalase was assayed by the method of Sinha. et al (1972)\textsuperscript{139}

Reagents Required:
1. Phosphate buffer- pH 7.0, 0.01 M
2. Hydrogen peroxide- 0.2 M solution in phosphate buffer.

Procedure:

0.05 ml of tissue homogenate was added to 1.2 ml of phosphate buffer. To this 1.0 ml hydrogen peroxide was added to start the enzyme reaction. The decrease in the absorbance was measured at 620 nm at 30 seconds interval for 3 minutes. The enzyme blank was run simultaneously with 1.0 ml of distilled water instead of hydrogen peroxide. Activity of catalase was expressed as nm of H$_2$O$_2$ utilized/min/mg/protein.
5.11.22 ESTIMATION OF UREA

Estimation of blood urea was carried out by Diacetyl monoxime method of the Barker, 1944.140

Reagents required:

1. 10% Sodium tungstate
2. 2/3 N H₂SO₄
3. Diacetyl mono oxime reagent.
   2g of Diacetylmonoxime was dissolved in 60ml of distilled water add 2ml of 2% solution of glacial acetic acid was added and the volume was made up to 100 ml with distilled water. The content was shaken with slight warming if necessary.
4. Sulphuric acid Phosphoric acid reagent
   150ml of 85% phosphoric acid was added to 140ml of water. Then 50ml of concentrated H₂SO₄ was added slowly.

Procedure:

To 0.1ml of blood, 3.3ml of distilled water 0.3ml of 10% sodium tungstate, 3.3ml of 2/3 N H₂SO₄ were added and centrifuged for few minutes at 3000 rpm. After centrifugation 2ml of supernatant was taken. To this 2ml of distilled water, 0.4 ml of DAM reagent and 1.6ml of H₂SO₄-H₃PO₄ reagent were added. The test tube was incubated in a boiling water bath for 30 minutes. After incubation, the test tube was cooled and the color was read at 480nm using blank. The values were expressed as mg/dl.
5.11.23 ESTIMATION OF URIC ACID

Reagents Required:
1) 10% sodium tungstate:
   10g of sodium Tungstate dissolved in 100ml of distilled water.
2) 2/3N sulphuric acid:
   1.85ml of sulphuric acid is dissolved and made up to 100ml with distilled water.
3) 10% sodium carbonate:
   10g of sodium carbonate is dissolved in 100ml of distilled water.
4) Phosphotungstic acid reagent:
   Dissolved 5g of sodium tungstate in about 40ml of distilled water and added 4ml of 85% phosphoric acid and refluxed gently for 2 hour, cooled and transferred into a flask and made up to 50ml with distilled water and kept in a brown bottle. This is the stock and before use it is diluted to 10times.
5) Standard uric acid solution:
   Weighed about 100mg of uric acid in a small beaker. Dissolve 60mg of lithium carbonate in 20ml of distilled water in a test tube. The solution was heated to about 60 °C and poured on to uric acid. Mixed the contents and transferred to a standard flask and made up to 100ml.
6) Working standard solution:
   10 ml of stock standard solution is made up to 100ml with distilled water.

PROCEDURE:
To 0.5ml of serum added 2ml of 10% sodium tungstate and 2ml of 2/3 sulphuric acid. Mixed well and centrifuged, 3ml of filtrate was taken. To this added 0.6ml of sodium bicarbonate and 0.6ml of phosphotungstate reagent. A blank was taken consisting of 3ml of water and treated similarity. Tubes were kept in room temperature for 30 minutes. The color is developed read at 700nm. The values were expressed as mg/dl.
5.11.24 ESTIMATION OF CREATININE:

The colorimetric determination of creatinine by the Jaffe reaction of the Bonsness et al., 1945.142

Reagent required:

1. Picric acid:
   Dissolve 10.5 g of picric acid (hydrated) or 3g of anhydrous picric acid in 500ml of hot water and cool to room temperature and then diluted to 1000ml in volumetric flask.
2. 0.75N sodium hydroxide:
   3gm of sodium hydroxide was dissolved in 100ml of distilled water.
3. 10% Sodium tungstate,
4. 2/3N Sulphuric acid.
5. Stock solution:
   100mg of creatinine in 0.1N hydrochloric acid in drops and made up to 100ml with 0.1N hydrochloric acid.
6. Working standard solution:
   1 in 20 dilutions.

Procedure:

Into a series of test tubes various concentration of standard solution was taken and a volume was made up to 3ml with distilled water. For the test 2 ml of serum was added to 2 ml of sulphuric acid and 2ml of sodium tungstate and centrifuged. From that 3ml was taken. Then added 1ml of picric acid and 1ml of sodium hydroxide to all tubes are heated in a boiling water bath for 15 minutes. The colour developed was read at 500nm. The values were expressed as mg/dl.
5.11.25 HISTOPATHOLOGICAL STUDIES*

For histological studies, the tissues were fixed in Bouin's fluid. The classical paraffin sectioning, and haematoxylin eosin staining techniques were used for histological studies. The various steps involved in the preparation of tissues for histological studies were:

1. Fixation
2. Dehydration
3. Clearing
4. Impregnation
5. Embedding
6. Section cutting
7. Staining
8. Mounting

1. FIXATION

In order to avoid tissue autolysis by the autosomal enzymes and to preserve its physical and chemical structure, a bit of tissue from organ was cut and fixed in Bouin's fluid immediately after removal from the animal body. Bouin's fluid which is the commonly used fixative was prepared by mixing the following chemicals.

1. Picric acid (saturated) - 75ml
2. Formaldehyde (40%) - 25ml
3. Glacial acetic acid - 5ml

The tissues were fixed in Bouins fluid for about 24 hrs. The tissues were then taken out and washed in tap water for a day to remove excess of picric acid.
2. DEHYDRATION

The term dehydration means the removal of water from the tissues using alcohol of varying grades. For dehydration ethanol was used. The tissues were kept in the following solutions for one hour.

1) 30% alcohol
2) 40% alcohol
3) 70% alcohol
4) 100% alcohol

Inadequately dehydrated tissues cannot be infiltrated with paraffin, at the same time over dehydration will make tissues brittle. In this condition sectioning will be problem. Hence the tissues were carefully dehydrated.

3. CLEARING

Dealcoholization or replacement of alcohol from the tissues with a clearing agent is called clearing. Xylol was used as the clearing agent. Clearing was carried out as the next step after dehydration which enables tissue spaces to be filled with paraffin. The tissues were kept in the clearing agent till they become transparent and impregnated with xylol.

4. IMPREGNATION

In this process, the clearing agent xylol was replaced by paraffin wax. The tissues were taken out of xylol and were kept in molten paraffin embedding bath, which consisted of metal pots filled with molten wax and maintained at about 50°C. The tissues were given three changes in the molten wax at half an hour intervals.

5. EMBEDDING

The paraffin wax used for embedding was fresh and stable with optimum melting point at about 56°C to 58°C.
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A clear glass plate was smeared with glycerine. L-shape mould was placed on it to form a rectangular cavity. The paraffin wax was powdered and air bubbles removed by using a hot needle. The tissues were placed in the paraffin and oriented with the surface to be sectioned. Then the tissues were pressed gently with a metal pressing rod towards the glass plate to make it settle uniformly and allowed the wax to settle and solidify at room temperature. The paraffin block was kept in coldwater for cooling.

6. SECTION CUTTING

Section cutting was done with a microtome. The excess of paraffin around the tissues were removed through trimming by leaving 0.5cm around the tissues. Then the block was attached to the gently heated object holds. Additional support was given by some extra wax, which was applied at the sides of the block alone. To produce uniform sections, the microtome knife was adjusted to the proper angle in the knife holder with only the cutting edge coming in contact with paraffin block. The tissues were cut at the size ranging from 5 - 7 micron thickness.

FLATTENING AND MOUNTING OF SECTIONS

This was carried out in tissue floatation warm water bath. The sections were spread on a warm water bath after they were detached from the knife with the help of a hair brush. Dust free clean sides were coated with egg albumin over the whole surface. Required section was spread on clean side and kept at room temperature.

7. STAINING

The sections were stained as follows:

Deparaffinization with xylol for five minutes.
Dehydration through descending grades of ethyl alcohol.

- 100% alcohol (absolute) - 2 minutes
- 90% alcohol (absolute) - 1 minute
- 70% alcohol (absolute) - 1 minute
- 50% alcohol (absolute) - 1 minute

Staining with Ehrlich’s Hematoxylin for 15-20 minutes.

Washing in tap water and blowing for 10 minutes.

Rinsing in the distilled water.

Staining with Eosin.

Dehydration again with ascending grades of alcohol.

- 70% alcohol - 2 minutes
- 90% alcohol - 2 minutes
- 100% alcohol - 1 minute

8. MOUNTING

DPX mountant was applied uniformly in a slide and stained sections were placed carefully in the mountant and covered with coverslips and slides were observed in Euromex microscope and photomicrographs were taken.