Materials & Methods
4.1. PREPARATION AND PURIFICATION OF HERBOMINERAL PREPARATION (The Selected Traditional Metal Based Preparation)

The main ingredient is zinc metal, which is purified with various agents like sesame oil, buttermilk, cow urine, rice gruel, horse gram extract, Achyranthes aspera Linn. powder and Aloe vera Burm.f. juice. The stages used to process and purify the zinc metal were as follows -

Stage I - (Process of Purification)

Stage II - (Process of Stirring)

Stage III - (Process of Powdering)

4.1.1. STAGE-I (PROCESS OF PURIFICATION)

Step 1

125g of Zinc metal (granulated) was taken in an iron pan and was melted at 170°C using Gas stove. The melted Zinc was poured into 200ml of Gingelly oil (Taila) for solidification. The solidified metal was again melted and poured into oil. This process was repeated for 3 times.

Step 2

The oil treated metal was heated and the molten metal was poured into 200ml of Takra. The solidified metal was again melted and poured into takra. It was repeated for 3 times. Takra is a liquid obtained by adding equal quantity of water to curd and decanting the same by churning.

Step 3

The Takra treated metal was heated and the molten metal was poured into 200ml of cow urine (Gomutra). The solidified metal was again melted and poured into cow urine. It was repeated for 3 times.

Step 4

The cow urine treated metal was heated and the molten metal was poured into 200ml of rice gruel (Kanjika). The solidified metal was again melted and poured into rice gruel. It was repeated for 3 times.
Step 5

The rice gruel treated metal was heated and the molten metal was poured into 200ml of horse gram extract (Kulatta). The solidified metal was again melted and poured into the extract. The process was repeated for 3 times.

4.1.2. STAGE II (PROCESS OF TRITURATION)

The purified zinc was kept in an Iron pan and heated. While it was melting, powder of Achyranthes aspera Linn. was sprinkled in small quantities (2g) and stirred with Iron spatula. This process was continued till the melted zinc was reduced to powder form.

4.1.3. STAGE III (PROCESS OF CALCINATION)

The Jarita Yasada (triturated zinc) was ground with 10ml of Kumari Swarasa (Aloe vera Burm.f. juice) for 6 hours. Flat round cakes (Cakrikas) were prepared and dried well for a day. The Cakrikas (round pellets) were kept in a shallow earthen container. The container was covered with another shallow earthen container. It was sealed with clay and subjected to Gajaputa.

4.1.4. GAJA PUTA

Gaja puta is an arrangement of pyramid shape of dried cow dungs in a pit of 90cm in length, breadth and depth. Half of the pit was filled with Cow dung flakes and Sarava Samputa was kept on it. The empty space above was covered again with cow dung flakes and ignited.

The process of Gajaputa was repeated for 7 times till the medicated calx obtained a faint reddish yellow colour.
4.2. PHYSICAL STANDARDS DETERMINED ARE

1. Colour

2. Odour

3. Taste

4. Test for Effervescence
   A pinch of the sample was mixed with lemon juice and the reaction was observed.

5. Floating Test
   A pinch of the sample was added on the surface of the water and the floating tendency was noted.

6. Silver Metal Test
   A pinch of the sample was ignited with a known weight of silver metal piece and the change in the weight of the silver metal was noted.

7. Test for Fineness
   A pinch of sample was spread between the index finger and thump to test the entry of the Bhasma into the crevices of the fingers.

8. Total Ash
   250 mg of HMP was taken in a silica crucible and it was heated in a muffle furnace for 6 hours at 450-600°C. The crucible was taken out and cooled at room temperature. The change in the weight of the HMP was noted and the percentage of total ash was calculated.
9. Acid insoluble ash

The incinerated ash of HMP was dissolved in 25 ml of dil. hydrochloric acid. The volume was made up to 50 ml and boiled in waterbath. Then it was filtered using Whatmann filter paper. The residue was taken in a silica crucible and heated in a muffle furnace for 6 hours at 450-600°C. The crucible was taken out and cooled at room temperature. The change in the weight of the HMP was noted and the percentage of acid insoluble ash was calculated.

10. Water soluble ash

The incinerated ash of HMP was dissolved in 25 ml of distilled water. The volume was made up to 50 ml and boiled in waterbath. Then it was filtered using Whatmann filter paper. The filtrate was taken in a beaker and kept in a boiling water bath for evaporation. Then the beaker was taken and cooled at room temperature. The change in the weight of the HMP was noted and the percentage of water-soluble ash was calculated.

11. Bulk Density

1 gm of the HMP was taken in a measuring cylinder and it was tightly packed with gentle tapping of the measuring cylinder. The volume occupied by the HMP was noted and from which the bulk density was calculated.
4.3. QUALITATIVE ANALYSIS OF ELEMENTS PRESENT IN VARIOUS STAGES OF PURIFICATION OF HERBOMINERAL PREPARATION

Tests for Sulphate
1. 1 ml of test solution was mixed with 1 ml of BaCl₂ solution.
2. 1 ml of test solution was treated with 1 ml of lead nitrate.

Tests for Nitrate
1. A little of the substance was taken in a test-tube and heated.
2. A little of the substance was treated with Conc. H₂SO₄
3. A little of the substance was mixed with Cu turnings and Conc. H₂SO₄
4. 1 ml of test solution was mixed with equal volume of freshly prepared ferrous sulphate solution. Conc. H₂SO₄ was added along the sides of the test-tube carefully.

Tests for Bromide
1. To a little of the substance, 1 ml of Conc. H₂SO₄ was added and warmed.
2. A pinch of MnO₂ was mixed with a little of the substance. About 1 ml of Conc. H₂SO₄ was added and gently warmed
3. 1 ml of test solution was acidified with dil. HNO₃. A few drops AgNO₃ were added.
4. 1 ml of test solution was mixed with 1 ml of lead nitrate solution.

Tests for Iodide
1. To a little of the substance, 1 ml of Conc. H₂SO₄ was added and warmed.
2. 0.5 gm of the substance was mixed with a pinch of MnO₂ and 1 ml of Conc. H₂SO₄ was added and gently warmed
3. 1 ml of test solution was acidified with dil. HNO₃. A few drops of AgNO₃ were added
4. 1 ml of test solution of iodide was mixed with 1 ml of lead nitrate solution.

Tests for Fluoride
1. (a) To a little of the substance, a few drops of Conc. H₂SO₄ were added and warmed.
   (b) A moistened glass rod was introduced into the test solution.
2. (a) Action of CaCl₂ Solution: 1.0 ml of test solution was taken in a test tube,
   1 ml of CaCl₂ solution and 5 drops of acetic acid were added.
Tests for Phosphate
1. 1ml of test solution was treated with equal volume of AgNO₃
2. 1ml of test solution was treated with BaCl₂ solution.
3. 1ml of test solution was treated with 1ml of NH₄Cl. NH₄OH solution was added in excess and then MgSO₄ solution was added.
4. 1ml of test solution was mixed with ferric chloride solution.
5. 1ml of acidified test solution was treated with ammonium molybdate.

Tests for Arsenite
1. 1ml of an aqueous solution of sample was treated with a few drops of ammonia solution and acidified with acetic acid. Few drops of AgNO₃ solution was added.
2. Scheels’ Green Test:
   To 0.5 ml of test solution, a few drops of CuSO₄ solution was added
3. 1ml of test solution was treated with 3ml of ammonium molybdate and gently heated.
4. To 1 ml of acidified test solution, H₂S gas was passed.
5. 1ml of test solution was treated with 1ml of I₂ solution.

Tests for Arsenate
1. 1ml of test solution was treated with 1ml of AgNO₃ solution.
2. 1ml of test solution was precipitated with CuSO₄.
3. 1ml of test solution was treated with 1ml NH₄Cl. NH₄OH was added in excess and then MgSO₄ solution was added.
4. 1ml of acidified test solution was treated with excess of ammonium molybdate.

Tests for Chromate
1. A little of the substance was mixed with a few drops of Conc. H₂SO₄ and gently warmed.
2. A little of the substance was mixed with a few drops of Conc. HCl and heated.
3. 1ml of test solution was treated with 1ml of BaCl₂ solution.
4. 1ml test solution was treated with 1ml of AgNO₃ solution and 1ml of acetic acid.
Tests for Oxalate

1. Action of heat:
   A little of the substance was heated in a dry test tube.

2. A little test solution was warmed gently with a few drops of Conc. H₂SO₄

3. A little test solution was mixed with a pinch of MnO₂ and warmed gently with a few drops of Conc H₂SO₄

4. 1ml of solution was warmed with 1ml of acidified KMnO₄ solution.

5. 1ml of oxalate solution was treated with 1ml of CaCl₂ solution.

Tests for Group--1 Cations

Tests for Lead (ii) ion

1. To a little test solution, 1ml of dilute hydrochloric acid was added.

2. To a little test solution, a few drops of K₂CrO₄ solution were added.

3. To a little test solution, a few drops of potassium iodide solution were added.

4. To a little solution dil. H₂SO₄ was added.

Tests for Nickel (ii) ion

1. To a little test solution, few drops of NaOH solution was added.

2. To a little test solution, NH₄OH was added in excess.

3. To a little test solution, potassium cyanide solution was added drop by drop to excess.

4. To a little test solution, NH₄Cl and NH₄OH solution were added followed by the dimethyl glyoxime

Tests for Barium (ii) ion

1. To a little test solution, NH₄Cl and NH₄OH solutions were added and then ammonium carbonate solution was added.

2. To a little test solution, a few drops of dil. H₂SO₄ were added.

3. To a little test solution, few drops of potassium chromate solution were added.

4. To a little test solution, ammonium oxalate solution was added.
Tests for Calcium (ii) ion
1. To a little test solution, NH₄Cl, NH₄OH and (NH₄)₂CO₃ solution were added.
2. To a little test solution, dil. H₂SO₄ was added.
3. To a little test solution, few drops of acetic acid was added followed by K₂CrO₄ solution.
4. A few drops of ammonium oxalate was added to the test solution.

Tests for Strontium (ii) ion
1. To a little test solution, NH₄OH, and (NH₄)₂CO₃ solutions were added.
2. To a little test solution, a few drops of dilute sulphuric acid were added.
3. To a little test solution, a few drops of K₂CrO₄ solution was added.
4. To a little test solution, a few drops of ammonium oxalate solution was added.

Tests for Magnesium (ii) ion
1. To a little test solution, NH₄OH solution was added.
2. To a little test solution, NH₄Cl and NH₄OH were added followed by disodium hydrogen phosphate (Na₂HPO₄) solution.
3. To a little test solution, few drops of NaOH solution was added in excess.
4. To a little test solution, a solution of Na₂CO₃ was added.

Tests for Ammonium ion (NH₄⁺)
1. Action of Heat:
   A little amount of the substance was heated in a dry test tube.
2. To a little test solution, 1 ml of NaOH solution was added and heated strongly.
3. To a little test solution, 1 ml of Nessler's reagent was added.

Tests for Antimony (iii) ion
1. To the test solution, an excess of water was added.
2. To the test solution, an excess of NaOH solution was added.
3. To the test solution, KI solution was added.
4. To the test solution, NH₄OH solution was added.
Tests for Aluminum (iii) ion
1. To the test solution, NH₄Cl and NH₄OH solutions were added.
2. To the test solution, few drops of sodium hydroxide solution was added.

Tests for Ferrous (ii) ion
1. To the test solution, NH₄Cl and NH₄OH solutions were added.
2. To the test solution, K₃[Fe(CN)₆] solution was added.
3. To the test solution, dil. H₂SO₄ was added followed by KMnO₄ solution.

Tests for Chromium (iii) ion
1. To the test solution, NH₄Cl and NH₄OH solutions were added.
2. To the test solution, few drops NaOH solution was added.
3. To the test solution, 1 ml of di-sodium hydrogen phosphate solution was added.

Tests for Mercury
1. To the test solution, a few drops of dil. HCl were added
2. To the test solution, potassium iodide solution was added in excess.
3. To the test solution 1 ml K₂CrO₄ solution was added.

Tests for Copper
1. To a part of the precipitate, potassium cyanide solution was added.
2. To another part of the precipitate, KI solution was added.
3. To the test solution, few drops of NH₄OH solution was added.
4. To the test solution, a few drops of potassium ferrocyanide solution was added.

Tests for Cadmium
1. To the test solution, KI solution was added.
2. To the test solution, few drops of NH₄OH solution were added.

Tests for Zinc
1. To a little of test solution, few drops of NH₄OH were added.
2. To a little of test solution, a few drops of potassium ferrocyanide solution was added.
 Materials And Methods

4.4. ATOMIC ABSORPTION SPECTROSCOPIC ANALYSIS

PRINCIPLE

The AAS is based on the principle that atoms of metallic elements (Fe, Mn, Cu and Zn etc.), which normally remain in ground state under flame conditions, absorb energy when subjected to radiations of specific wavelength. The absorption of radiation is proportional to the concentration of atoms of that element. The absorption of radiation by the atoms is independent of the wavelength of absorption and temperature of the atoms. These two features provide AAS a distinct advantage over flame emission spectroscopy. It also has greater sensitivity and accuracy.

INSTRUMENTATION

A double beam atomic absorption spectrophotometer is required. The two most common oxidant / fuel combinations used in atomic absorption spectroscopy are air-acetylene and nitrous – oxide acetylene. Other flames that can be used are air hydrogen and argon – hydrogen and argon – hydrogen entrained air.

Air acetylene – Air acetylene is the preferred flame for the determination of approximately 35 elements by atomic absorption. The temperatures range from 2125° to 2400° C and is used for the determination of elements, which form refractory oxides. It is also used to overcome chemical interferences that may be present in flames of lower temperature. However, light emission from the nitrous oxide acetylene flames is very strong at certain wavelengths. This may cause fluctuations in the analytical results for determination performed at this wavelength, particularly if the lamp emission for the element of interest is weak. Only the nitrous oxide burner head can be used with the nitrous oxide acetylene flame. The AAS technique is very specific because only the ten atoms of a particular element can be absorb radiation of their own characteristic wavelength.
4.5. FUNCTIONAL GROUPS ANALYSIS OF INTERMEDIATES AND HMP BY FTIR

Infrared (IR) spectroscopy is one of the most common spectroscopic techniques that are characterized by the absorption measurement of different IR frequencies by a sample positioned in the path of an IR beam. The main goal of IR spectroscopic analysis is to determine the chemical functional groups in the sample. Different functional groups absorb characteristic frequencies of IR radiation. Using various sampling accessories, IR spectrometers can accept a wide range of sample types such as gases, liquids and solids.

There are three basic spectrometer components in an FT system: radiation source, interferometer and detector. The same types of radiation sources are used for both dispersive and Fourier transforms spectrometers. However, the source is more often water-cooled in FTIR instruments to provide better power and stability.

The most commonly used interferometer is a Michelson interferometer. It consists of three active components: a moving mirror, a fixed mirror, and a beam splitter. The two mirrors are perpendicular to each other. The beam splitter is a semi reflecting device and is often made by depositing a thin film of germanium onto a flat KBr substrate. Radiation from the broadband IR source is collimated and directed into the interferometer, and impinges on the beam splitter. At the beam splitter, half the IR beam is transmitted to the fixed mirror and the remaining half is reflected to the moving mirror. After the divided beams are reflected from the two mirrors, they are recombined at the beam splitter. Due to Infrared Spectroscopy 255 changes in the relative position of the moving mirror to the...
Materials And Methods

fixed mirror, an interference pattern is generated. The resulting beam then passes through the sample and is eventually focused on the detector.

The two most popular detectors for a FTIR spectrometer are deuterated triglycine sulfate (DTGS) and mercury cadmium telluride (MCT).

SAMPLES
State
Almost any solid, liquid or gas sample can be analyzed. Many sampling accessories were available.

Amount
A solid 50 to 200 mg was desirable, but 10 µg was ground with KBr. This was the minimum quantity required for qualitative determinations; 1 to 10 µg solid was taken in a suitable solvent.

Preparation
Solid was ground well into KBr matrix.
4.6. SEM, TEM, UV-SPECTRAL & X-RAY DIFRACTION ANALYSIS

Scanning electron microscopy studies were performed using cold field emission scanning electron microscope (FE-SEM) model JSM-6701F.

The TEM experiments were performed on HITACHI H-600 at an accelerating voltage of 100 KV. The thin sections required for the TEM experiments were made by mixing the sample in a suitable medium and it was incubated at room temperature for 6 hrs.

The UV-Visible spectrum of the product dispersed in distilled water was recorded in a UV-Visible Spectrophotometer (UV-1601PC).

Powder X-Ray diffraction studies were carried out using second differential Rigaku Rotaflex diffractometer with CuKα radiation source (λ = 0.154178 nm). The typical width maintained in diffractometer was about 0.140 degree. The d spacing of the sample was calculated using the Bragg's equation

\[ n\lambda = 2d\sin\theta \]

Where,
\( \lambda = \) Wave length of the X-Ray
\( d = \) Interspaced distance and
\( \theta = \) Angle of incident radiation
FIGURE 10: X-RAY DIFFRACTION
FIGURE 11: ANALYTICAL SCANNING ELECTRON MICROSCOPE
FIGURE 12: ANALYTICAL TRANSMISSION ELECTRON MICROSCOPE
4.7. TOXICOLOGICAL STUDIES

Toxicological studies were carried out for periods mentioned below

a) Acute toxicity for 3 days
b) Sub-acute toxicity for 15 days
c) Chronic toxicity for 45 days

Wistar albino rats of initial body weight ranging from 100 to 200 gm were used. The animals had free access to standard laboratory rat food pellets obtained from Sai Durga Foods & Feeds, Bangalore, India and water ad libitum. This study was conducted according to the Ethical guidelines of CPCSEA after obtaining necessary clearance from the committee (Approval No: 790/03/ac/CPCSEA).

In these studies, different doses of Herbomineral preparation (Yasada Bhasma) at various dose levels such as 100, 150 and 200mg/kgbw were orally given to rats for 3, 15 and 45 days. In all the experiments, animals were sacrificed after the treatment and biological samples such as blood and tissues were collected for analyzing the biochemical parameters which include serum enzymes such as SGOT, SGPT, Alkaline Phosphatase, LDH, Serum Urea and Serum Creatinine. Histopathological observations of vital organs such as liver and kidney were carried out using microtome sections stained with hematoxylin and eosin. Inverse relation in the dose and duration of drug level of administration was maintained as in normal course of treatment. All data were expressed as mean ± SE.

Experimental Design

A total number of 24 albino rats were equally divided into four groups.

- **Group I**: Normal animals received saline
- **Group II**: HMP treated animals at a dose level of 100mg/kgbw.
- **Group III**: HMP treated animals at a dose level of 150mg/kgbw.
- **Group IV**: HMP treated animals at a dose level of 200mg/kgbw.
4.8. SCIENTIFIC VALIDATION STUDIES OF HMP

4.8.1. ANTI DIABETIC ACTIVITY

Wistar albino rats of initial body weight ranging from 140 to 160 gm were used and the animals were divided as normal, disease control, HMP treated and standard drug treated. The animals had free access to standard laboratory rat food pellets obtained from Sai Durga Foods & Feeds, Bangalore, India and water *ad libitum*. This study was conducted according to the Ethical guidelines of CPCSEA after obtaining necessary clearance from the committee (Approval No : 790/03/ac/CPCSEA). The control animals received intraperitoneal injection of alloxan at concentration of 150mg/kg body weight. The normal animals received only saline. The drug treated diabetic animals received the Herbomineral preparation under study at three different doses. Standard drug used was Glibenclamide. Significant differences among the groups were determined by One way Analysis of Variance. All data were expressed as mean ± SE. The level of significance was set at less then 0.05.

**EXPERIMENTAL DESIGN**

A total number of 36 albino rats were equally divided into six groups.

- **Group I**: Normal animals received saline
- **Group II**: Disease control animals received intraperitoneal injection of alloxan at a dose level of 150mg/kgbw.
- **Group III**: Alloxan induced diabetic animals received HMP at a dose level of 100mg/kgbw.
- **Group IV**: Alloxan induced diabetic animals received HMP at a dose level of 150mg/kgbw.
- **Group V**: Alloxan induced diabetic animals received HMP at a dose level of 200mg/kgbw.
- **Group VI**: Alloxan induced diabetic animals received Glibenclamide at a dose level of 200mg/kgbw.
4.8.2. HEPATOPROTECTIVE ACTIVITY

ANIMALS AND TREATMENT

Wistar albino rats of initial body weight ranging from 140 to 160 gm were used and the animals were divided as normal, disease control, HMP treated and standard drug treated. The animals had free access to standard laboratory rat food pellets obtained from Sai Durga Foods & Feeds, Bangalore, India and water *ad libitum*. This study was conducted according to the Ethical guidelines of CPCSEA after obtaining necessary clearance from the committee (Approval No: 790/03/ac/CPCSEA). The control animals received intraperitoneal injection of CCl₄ at concentration of 0.5ml/kg body weight in olive oil. The normal animals received only saline. The drug treated hepatotoxic animals received Herbomineral preparation at three different doses and silymarin. Significant differences among the groups were determined by One way Analysis of Variance. All data were expressed as mean ± SE. The level of significance was set at less then 0.05.

EXPERIMENTAL DESIGN

A total number of 36 albino rats were equally divided into six groups.

Group I : Control animals received saline

Group II : Disease Control animals received intraperitoneal injection of CCl₄ at a dose level of 0.5ml in olive oil /kgbw.

Group III : CCl₄ induced hepatotoxic animals received HMP at a dose level of 100mg/kgbw.

Group IV : CCl₄ induced hepatotoxic animals received HMP at a dose level of 150mg/kgbw.

Group V : CCl₄ induced hepatotoxic animals received HMP at a dose level of 200mg/kgbw.

Group VI : CCl₄ induced hepatotoxic animals received Silymarin at a dose of 200mg/kgbw
4.8.3. NEPHROPROTECTIVE ACTIVITY

Wistar albino rats of initial body weight ranging from 140 to 160 gm were used and the animals were divided as normal, disease control, HMP treated and standard drug treated. The animals had free access to standard laboratory rat food pellets obtained from Sai Durga Foods & Feeds, Bangalore, India and water ad libitum. This study was conducted according to the Ethical guidelines of CPCSEA after obtaining necessary clearance from the committee (Approval No : 790/03/ac/CPCSEA). The control animals received oral administration of Sodium Phosphate at concentration of 4.1g/kg body weight. The normal animals received only saline. The drug treated nephrotoxic animals received Herbomineral preparation at three different doses and Norfloxacin. Significant differences among the groups were determined by One way Analysis of Variance. All data were expressed as mean ± SE. The level of significance was set at less than 0.05.

EXPERIMENTAL DESIGN

A total number of 36 albino rats were equally divided into six groups.

Group I : Control animals received saline

Group II : Disease control animals received oral administration of Sodium Phosphate at a dose level of 4.1g/kg bw.

Group III : Sodium Phosphate induced nephrotoxic animals received HMP at a dose level of 100mg/kgbw.

Group IV : Sodium Phosphate induced nephrotoxic animals received HMP at a dose level of 150mg/kgbw.

Group V : Sodium Phosphate induced nephrotoxic animals received HMP at a dose level of 200mg/kgbw.

Group VI : Sodium Phosphate induced nephrotoxic animals received Norfloxacin at a dose level of 200mg/kgbw.
4.9. PARAMETERS STUDIED FOR SCIENTIFIC VALIDATION STUDIES

4.9.1. ESTIMATION OF ASPARTATE TRANSAMINASE (AST)

Reagents

1. 0.1 M Phosphate buffer pH 7.5
2. Substrate solution:
   1.33 g aspartic acid and 15mg α-keto glutarate were dissolved in 100 ml of phosphate buffer.
3. 0.02% DNPH in 1 N HCl
4. 0.4N NaOH
5. Working Standard: Sodium Pyruvate (10mg/100ml)

Method

1 ml of substrate was taken in a reaction tube. To this, 1 ml of phosphate buffer was added and the tube was acclimatized at 37°C for 3 minutes. Then 0.2 ml of serum was added and incubated at 37°C for 1 hr. the reaction was arrested by adding 1 ml of DNPH. To the control tube, serum was added after the reaction was arrested by DNPH. The tubes were kept at room temperature for 30 min. Then 0.5 ml of NaOH was added and the colour developed was read at 540 nm.

Different volumes such as 0.1, 0.2, 0.3, 0.4 and 0.5 ml of working standards were pipetted out into S1, S2, S3, S4 and S5 test tubes respectively. The volume was made upto 2.2 ml with distilled water. Then 1 ml of DNPH was added and incubated at room temperature for 30 min. Finally 0.5 ml of NaOH was added and the colour developed was read at 540 nm.

From the standard optical density values, the activity of AST was calculated and expressed as IU/L.
4.9.2. ESTIMATION OF ALANINE TRANSAMINASE (ALT)\textsuperscript{101}

**Reagents**

1. 0.1 M Phosphate buffer, pH 7.5
2. Substrate solution
   
   1.78 g alanine and 30 mg of α - keto glutarate were dissolved in 100 ml of phosphate buffer.
3. 0.02%DNPH in 1 N HCl
4. 0.4N NaOH
5. Working Standard: Sodium Pyruvate (10mg/100ml)

**Method**

1 ml of substrate was taken in a reaction tube. To this, 1 ml of phosphate buffer was added and the tube was acclimatized at 37°C for 3 minutes. Then 0.2 ml of serum was added and incubated at 37°C for 1 hr. the reaction was arrested by adding 1 ml of DNPH. To the control tube, serum was added after the reaction was arrested by DNPH. The tubes were kept at room temperature for 30 min. Then 0.5 ml of NaOH was added and the colour developed was read at 540 nm.

Different volumes such as 0.1, 0.2, 0.3, 0.4 and 0.5 ml of working standards were pipetted out into S1, S2, S3, S4 and S5 test tubes respectively. The volume was made upto 2.2 ml with distilled water then 1 ml of DNPH was added and incubated at room temperature for 30 min. Finally 0.5 ml of NaOH was added and the colour developed was read at 540 nm.

From the standard optical density values, the activity of ALT was calculated and expressed as IU/L.
4.9.3. ESTIMATION OF ALKALINE PHOSPHATASE (ALP)

Reagents
1. 0.1 M carbonate buffer pH 10.0
2. Substrate: (0.1 M Disodium phenyl phosphate)
3. 0.1 M Magnesium Chloride
4. 15% Sodium Carbonate
5. Folin’s Ciocalteau phenol reagent
6. Working Standard: (Phenol – 94mg/100ml)

Procedure
1.5 ml carbonate buffer, 1 ml Disodium phenyl phosphate and 0.1 ml Magnesium Chloride were taken in the reaction tube. The tube was acclimatized at 37 °C for 3 min. Then 0.2 ml of serum/tissue homogenate was added and incubated at 37 °C for 15 min. For standard, different volumes such as 0.1, 0.2, 0.3, 0.4 and 0.5 ml of working standards were pipetted out into S1, S2, S3, S4 and S5 test tubes respectively and the volume was made upto 2.8 ml with distilled water. The reaction was arrested by the addition of 2ml of 15% sodium carbonate followed by 0.5ml of Folin’s phenol reagent. The control tube was treated similarly but serum was added after the reaction was arrested with sodium carbonate and Folin’s phenol reagent. The colour developed was read after 10 min at 640 nm.

From the standard optical density values, the activity of ALP was calculated and expressed as IU/L.
4.9.4. ESTIMATION OF ACID PHOSPHATASE (ACP)\textsuperscript{101}

Reagents

1. 0.1 M Citrate buffer pH 4.5
2. Substrate: (0.1 M p- nitro phenyl phosphate)
3. 0.4 N Sodium hydroxide
4. Working Standard: (p- nitro phenol – 136mg/100ml)

Procedure

1.5 ml citrate buffer and 1 ml substrate were taken in the reaction tube. The tube was acclimatized at 37 °C for 3 min. Then 0.2 ml of serum or tissue homogenate was added and incubated at 37 °C for 15 min. For standard, different volumes (0.1, 0.2, 0.3, 0.4 and 0.5 ml) of working standards were pipetted out into S1, S2, S3, S4 and S5 test tubes respectively and the volume was made upto 2.7 ml with distilled water. The reaction was arrested by the addition of 2ml of 0.4 N sodium hydroxide. The control tube was treated similarly but serum was added after the reaction was arrested with sodium hydroxide. The colour developed was read after 10 min at 420 nm.

From the standard optical density values, the activity of ACP was calculated and expressed as IU/L.
4.9.5. ESTIMATION OF LACTATE DEHYDROGENASE (LDH)

Materials:

1. Stock sodium pyruvate solution (1mM/100ml)
2. Working standard (0.1mM/100ml)
3. DNPH solution (1%)
4. Sodium hydroxide (0.4N)
5. Tissue homogenate
6. Phosphate buffer (pH 7; 0.1M)

Procedure

1ml of each of phosphate buffer and substrate were taken in “test” reaction tube, while 2.1ml phosphate buffer was taken in “control” reaction tube. The tubes were acclimatized at 37°C for 3 minutes. Then 0.1ml of serum was added to test reaction tube. 0.1ml to 0.5ml of working standards were pipetted out into S1, S2, S3, S4 & S5 test tubes respectively. Then 2ml of DNPH and 4ml of 0.1N sodium hydroxide were added and incubated at room temperature for 10 minutes. Then, the colour developed was read at 420nm against blank which containing distilled water and other reagents.
4.9.6. DETERMINATION OF HEXOKINASE

Reagents

1. Enzyme solution (Liver extract)

2. Tris buffer (0.2M; pH 7.4)
   4.8gm Tris was dissolved in 200ml of distilled water and the pH was adjusted to 7.4 with 0.1N Hydrochloric acid.

3. Substrate
   100mg Glucose was dissolved in distilled water and made up to 100ml.

4. Alkaline copper sulphate solution.
   40 grams of sodium carbonate, 7.5 g of tartaric acid and 4.5 g of copper sulphate were mixed with distilled water and the volume was made up to 1000ml.

5. Phosphomolybdic acid
   35 g of molybdic acid and 5 g of sodium tungstate were dissolved in 200 ml of 10 % sodium hydroxide and boiled vigorously for 20-30 minutes to remove the ammonia present in molybdic acid. The content was cooled and diluted to 350 ml with distilled water. Then 125ml of orthophosphoric acid was added and the final volume was made up to 500 ml with distilled water.

6. Adenosine Tri Phosphate (ATP) Solution (0.5%)
   250mg of pure ATP dissolved in 50ml of distilled water.

7. 0.1% Magnesium chloride (100mg in 100ml)

8. 0.1% Sodium fluoride (100mg in 100ml).
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’ reaction tube. To this, 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 1ml of tissue homogenate was added and incubated at 37°C for 30 minutes. For standard, different volumes such as 0.1, 0.2, 0.3, 0.4 and 0.5 ml of working standards were pipetted out into S1, S2, S3, S4 and S5 test tubes respectively and the volume was made up to 4.8 ml with distilled water. The enzymatic reaction was arrested by the addition of 2ml of alkaline copper sulphate solution and test tubes were heated in the boiling water bath for 10 minutes. Then 2ml of Phosphomolybdic acid reagent was added and mixed well. The developed color was read at 620nm against control.

From the standard optical density values, the activity of Hexokinase was calculated and expressed as micromole of glucose phosphorylated per ml per minute.
4.9.7. DETERMINATION OF GLUCOSE-6-PHOSPHATASE

**Reagents**

1. 0.1 M citrate buffer pH 6.5
2. Substrate (0.01M Glucose-6-phosphate in distilled water).
3. 10% TCA
4. Test sample (Serum/Homogenate)
5. Working Standard: (Glucose – 10mg/100ml)

**Procedure**

0.3ml of citrate buffer and 0.5ml substrate were taken in a reaction tube and the tube was acclimatized at 37°C for 3 minutes. Then 0.2 ml of serum/tissue homogenate was added and incubated at 37°C for 1 hour. For standard, different volumes such as 0.1, 0.2, 0.3, 0.4 and 0.5 ml of working standards were pipetted out into S1, S2, S3, S4 and S5 test tubes respectively and the volume was made upto 1 ml with distilled water. The reaction was arrested by the addition of 1.0ml of 10% TCA. The control tube was treated similarly but serum was added after the reaction was arrested with TCA. The reaction mixture was centrifuged and the phosphorous content of the supernatant was estimated.

From the standard optical density values, the activity of Glucose-6-Phosphatase was calculated and expressed as micromole of glucose produced per ml per minute.
4.9.8. ESTIMATION OF REDUCED GLUTATHIONE

Reagents
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
1 ml of homogenate was precipitated with 1ml of TCA and the precipitate was removed by centrifugation. To 1ml of the supernatant 2ml of DTNB was added and the total volume was made upto 3ml with phosphate buffer. The absorbance was read at 412 nm.

The concentration of glutathione was expressed as μg/mg protein.
4.9.9. ASSAY OF SUPEROXIDE DISMUTASE (SOD)\textsuperscript{105}

**Reagents**

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 mixed with 0.75 ml ethanol and 0.15 ml ice cold chloroform. The reaction mixture was mixed well and centrifuged at 3000rpm for 10 minutes. To 0.5 ml of supernatant, 0.5 ml EDTA solution and 1 ml carbonate buffer were added. The reaction was initiated by the addition of 0.5 ml of epinephrine and the increase in absorbance was measured at 480 nm.
4.9.10. ASSAY OF CATALASE (Hydrogen Peroxide Oxido Reductase)\textsuperscript{106}

Reagents

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

Procedure

To 0.05ml of tissue extract 1.05ml of phosphate buffer was added and mixed well. To this, 1.0 ml of hydrogen peroxide was added to start the enzyme reaction. The decrease in absorbance was measured at 240 nm at 30 seconds intervals for 3 minutes. The enzyme blank was run simultaneously with 1.0ml of distilled water instead of hydrogen peroxide.

Activity of Catalase was expressed as nano moles of H\textsubscript{2}O\textsubscript{2} utilized /min/mg protein.
4.9.11. ESTIMATION OF LIPIDPER OXIDE

**Reagents**

1. H$_2$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**

4.1 ml of tissue homogenate was mixed with 4 ml of 0.85N H$_2$SO$_4$ and 0.5 ml of phosphotungstic acid and stirred well. The contents were centrifuged at 3000rpm for 10 minutes. The supernatant was discarded and the sediment was mixed with 2.0ml of N/12 H$_2$SO$_4$ and 0.3 ml of 10% phosphotungstic acid. The mixture was centrifuged again at 3000rpm 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 TBA reactants/mg of protein.
4.9.12. ESTIMATION OF TRIGLYCERIDE

**Reagents**

1. Isopropanol
2. Aluminium oxide
3. Saponification reagent- 5 g of KOH 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 1 lt.
6. Stock Std- 1 g of Tripalmitin /100 ml of isopropanol
7. Working standard- 300 mg/100 ml was prepared by diluting 3 ml of stock with isopropanol to 10 ml.

**Procedure**

1.0 ml of isopropanol was mixed with 0.1 ml of sample and 0.1, 0.2, 0.3, 0.4 and 0.5 ml of standard solution. To this 0.4g of alumina was added and mixed well for 15 min. The content was centrifuged at 3000 rpm for 10 minutes and 0.5 ml of the supernatant was transferred to reaction tubes and heated at 65 °C for 15 min. Then 0.6 ml of the saponification reagent was added and cooled. Then 1.0 ml of sodium metaperiodate and 0.5 ml of acetyl acetone reagent were added and heated at 65 °C for 30 minutes. The contents were cooled and the colour developed was read at 430nm.

From the standard optical density values, the amount of triglyceride in the sample was calculated and expressed as mg/dl.
4.9.13. ESTIMATION OF TOTAL CHOLESTEROL

Reagents
1. Stock Ferric Chloride
   840mg of FeCl₃ in 100ml of glacial acetic acid.
2. Ferric Chloride precipitating reagent
   10 ml of stock was diluted to 100ml in glacial acetic acid
3. Ferric chloride diluting reagent
   85 ml of stock was made up to 100ml with glacial acetic acid.
4. Working standard
   To 5mg of cholesterol, 5 ml of FeCl₃ precipitating reagent was added and the total volume was made up to 50ml with glacial acetic acid.

Procedure
For test
0.1ml of the serum was added to 4.9ml of FeCl₃ precipitating reagent and mixed well. The content was centrifuged at 3000 rpm for 15 minutes. To 2ml of supernatant, 2.5ml of diluting reagent and 4ml of concentrated sulphuric acid were added and mixed well. The intensity of color was read at 560 nm.

For Standard
Different volumes (0.2, 0.4, 0.6, 0.8 and 1.0ml) of working standard were taken and the volume was made up to 2.5 ml with FeCl₃ diluting reagent. Then 5 ml of concentrated sulphuric acid was added and the intensity of color was read at 560 nm.

From the standard optical density values, the amount of Cholesterol in the sample was calculated and expressed as mg/dl.
4.9.14. ESTIMATION OF PROTEIN

Reagents

1. Alkaline copper sulphate reagent
   Solution A - 2% sodium carbonate in 0.1 N sodium hydroxide
   Solution B - 0.5% copper sulphate in 1% sodium potassium tartarate
2. Folin's Phenol reagent
3. Working Standard (Bovine Serum Albumin 10mg/100ml)
4. Test samples

Procedure

0.1 ml of serum/tissue homogenate was taken in a reaction tube and the volume was made up to 1 ml with distilled water. The blank was treated by taking 1 ml distilled water. Then 4.5 ml of alkaline copper reagent was added and incubated at room temperature for 10 minutes. Then 0.5 ml of Folin's Phenol reagent was added to all the tubes and incubated at room temperature for 20 min. The blue colour developed was read at 640nm.

Different volumes (0.2, 0.4, 0.6, 0.8 and 1.0ml) of working standard were taken and the volume was made up to 2.5 ml with distilled water. Then 4.5 ml of alkaline copper reagent was added and incubated at room temperature for 10 minutes. Then 0.5 ml of Folin's Phenol reagent was added to all the tubes and incubated at room temperature for 20 min. The blue colour developed was read at 640nm.

From the standard optical density values, the amount of Protein in the sample was calculated and expressed as mg/dl.
4.9.15. ESTIMATION OF GLUCOSE

Reagents

1. 10% Sodium tungstate solution.
2. 2/3 N Sulphuric acid.
3. Alkaline copper sulphate solution.
   40 grams of sodium carbonate, 7.5 g of tartaric acid and 4.5 g of copper sulphate were mixed with distilled water and the volume was made up to 1000ml.
4. Phosphomolybdic acid
   To 35 g of molybdic acid, 5 g of sodium tungstate in 200 ml of 10% sodium hydroxide was added and boiled vigorously for 20-30 minutes to remove the ammonia present in molybdic acid. The content was cooled and diluted to 350ml with distilled water. Then 125ml of orthophosphoric acid was added and made up to 500 ml with distilled water.
5. Stock standard glucose solution
   100 mg of glucose was dissolved in distilled water and made up to 100 ml.
6. Working standard solution:
   1 in 10 dilutions

Procedure

0.1 ml of plasma was mixed with 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. The content was centrifuged at 3000rpm for 10 minutes. To 1ml of the supernatant 2 ml of alkaline copper sulphate solution was added and heated in a boiling water bath for 10 minutes. Then it was cooled and 2 ml of Phosphomolybdic acid was added and mixed well. The colour developed was read at 620 nm.

Different volume (0.1, 0.2, 0.3, 0.4 and 0.5) of working standard solution was taken and the volume was made up to 1ml with distilled water. To this, 2 ml of alkaline copper sulphate solution was added and the tubes were heated in a boiling water bath for 10 minutes. Then it was cooled and 2 ml of Phosphomolybdic acid was added and mixed well. A Blank was treated by taking 1ml of distilled water and other reagents. The colour developed was read at 620 nm.
4.9.16. ESTIMATION OF BLOOD UREA

Reagents

1. 10% Sodium tungstate
2. 2/3 N H₂SO₄
3. Diacetyl monoxime reagent.
   2g of DAM was dissolved in 60ml of distilled water. To this, 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 and shaken well.

Procedure

For Test
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 tubes were cooled and the color developed was read at 480nm.

For Standard
Different volumes of working standards (0.2, 0.4, 0.6, 0.8 and 1.0ml) were taken. The volume was made up to 2 ml with distilled water. Then 0.4ml of DAM reagent and 1.6ml of sulphuric acid – Phosphoric acid reagent were added to all the test tubes and heated in a boiling water bath for 30 minutes. A Blank was treated by taking 2ml of distilled water and other reagents. Then test tubes were cooled and intensity of the colour was read at 480 nm.
4.9.17. ESTIMATION OF URIC ACID

Reagents Required
1. 10% Sodium Carbonate
2. 10% Sodium tungstate
3. Uric acid reagent
4. Stock Uric acid Solution- 100mg/100ml

Procedure

Preparation of Supernatant
The supernatant was prepared by centrifuging 0.1 ml serum with 3 ml of 10% sodium tungstate at 3000 rpm for 10 minutes.

Quantification of Uric Acid
2 ml supernatant was taken in reaction tube. To this, 0.6 ml of 10% sodium carbonate and 0.6 ml uric acid reagent were added and incubated at 37 °C for 30 minutes. The intensity of color was read at 700nm.

For Standard
Different volumes of working standards (0.2, 0.4, 0.6, 0.8 and 1.0ml) were taken. The volume was made up to 2 ml with distilled water. Then 0.6 ml of 10% sodium carbonate and 0.6 ml uric acid reagent were added and incubated at 37 °C for 30 minutes. A Blank was treated by taking 2ml of distilled water and other reagents. The intensity of color was read at 700nm.

From the standard optical density values, the amount of uric acid in the sample was calculated and expressed as mg/dl.
4.9.18. ESTIMATION OF CREATININE

Reagents Required

1. 10% Sodium tungstate
2. 2/3 N Sulphuric acid
3. 0.04M Picric acid
4. 0.75N Sodium hydroxide
5. Stock standard creatinine

100mg of pure creatinine was dissolved in 0.1N HCl and the volume was made up to 100ml with the same.

6. Working Standard

5 ml of the stock solution was diluted to 100ml with distilled water.

Procedure

Preparation of supernatant

The supernatant was prepared by centrifuging the 0.1ml serum with 2ml of 5% sodium tungstate and 2ml of 2/3 N H₂SO₄ at 3000 rpm for 10 minutes.

Quantification Of Creatinine

2ml supernatant was taken in reaction tube. To this, 1ml of picric acid and 1 ml of sodium hydroxide were added and incubated at 37°C for 15 minutes. The intensity of colour was measured at 550nm.

For Standard

Different volumes of working standards (0.2, 0.4, 0.6, 0.8 and 1.0ml) were taken. The volume was made up to 2 ml with distilled water. Then 1ml of picric acid and 1 ml of sodium hydroxide were added and incubated at 37°C for 15 minutes. A Blank was treated by taking 2ml of distilled water and other reagents. The intensity of colour was measured at 550nm.

From the standard optical density values, the amount of creatinine in the sample was calculated and expressed as mg/dl.
4.9.19. ESTIMATION OF BLOOD UREA NITROGEN

Reagents

α - Ketoglutarate - 6.4 mmol/L
NADH - >0.3 mmol/L
Urease - >6000 U/L
GLDH - >500 U/L
Tris buffer - 120mmol/L (pH 8.2)

Activators and Stabilizers

Procedure

10μl of sample, standard and distilled water was pipetted out into test, standard and blank reaction tubes respectively. Then 1ml of working reagent was added and incubated at 37°C for 30 seconds. The absorbance was recorded and marked as A₁. Then exactly 60 seconds after the first reading, the absorbance was recorded again and marked now as A₂. The actual absorbance was calculated by substituting the A₁ with A₂.
4.9.20. ESTIMATION OF PHOSPHORUS

Reagents
1. 10% TCA
2. 10N Sulphuric acid
3. Molybdic reagent
4. 20% Sodium sulphite
5. 15% Sodium sulphite
6. 0.25% ANSA

Procedure

To 2ml of sample 8ml of 10% Trichloroacetic acid was mixed and centrifuged. To
5ml of filtrate, 1 ml of molybdate reagent II and 0.4ml of ANSA were added and mixed
well. The volume was made upto 8ml with distilled water.

For standard, different volumes of working standard were taken and the volume
was made up to 8ml with distilled water. Then 1ml of molybdate reagent I and 0.4ml of
ANSA were added and mixed well. Blank was also maintained by taking deionised
water instead of standard. The colour developed was read at 680nm.
4.9.21. ESTIMATION OF CALCIUM

**Reagents**

1. 4% Ammonium oxalate
2. 0.01N Potassium Permanganate
3. 2% Ammonia
4. 1N Sulphuric acid

**Procedure**

To 2 ml of sample, 2ml of distilled water and 2ml of ammonium oxalate were added and allowed to stand for overnight. After precipitation of calcium, the content was centrifuged and the supernatant was discarded. The precipitate was washed with 5ml of 2% ammonia and centrifuged again. The supernatant was discarded and the precipitate was dissolved in 2ml of 1N sulphuric acid using glass rod.

The above mixture was incubated at 70 - 72°C in boiling water bath, titrated against 0.01N potassium permanganate. End point was the appearance of pale pink colour. 2ml of sulphuric acid was taken as a blank and titrated against 0.01N potassium permanganate. The difference gives the volume of potassium permanganate required to neutralize the oxalic acid liberated from calcium oxalate.
4.9.22. ESTIMATION OF BILIRUBIN BY MALLOY AND EVELYN METHOD

Reagents

1. Diazo blank : 1.5% Hydrochloric acid.
2. Diazo reagent A : 1 gm of Sulphanilic acid was dissolved in 15 ml of Con.HCl and the final volume was made upto 1 litre with distilled water.
3. Diazo reagent B : 0.5 gm of Sodium nitrate was dissolved in water and the final volume was made upto 100ml.
4. Diazo reagent was prepared freshly by adding 0.1ml of Diazo B and 3.3ml of Diazo A.
5. Bilirubin standard : 10mg of Bilirubin was dissolved in 100 ml of Chloroform.
6. Methanol.

Procedure

Four test tubes were taken and marked as total Bilirubin test (T_t), Total Bilirubin blank (T_b), Direct Bilirubin test (D_t) and Direct Bilirubin blank (D_b). 0.2 ml of serum was taken in all the four test tubes. The volume in all four test tubes were made upto 2ml with distilled water, 0.5ml of Diazo blank and 2.5ml of Methanol were added to Total Bilirubin blank and 0.5ml of Diazo reagent, 2.5ml of Methanol were added to Total Bilirubin test. 0.5ml of Diazo blank and 2.5ml of water were added to Direct Bilirubin blank, 0.5ml of Diazo reagent and 2.5ml of water were added to Direct test.

0.1 to 0.5 ml of Bilirubin standard were taken and made upto 2ml with Methanol. 0.5ml of Diazo reagent and 2.5 ml of Methanol were added to all the standard tubes. The Colour developed was read at 540 nm against a blank containing 2ml of Methanol, 0.5ml of Diazo blank and 2.5ml of distilled water.
4.9.23. ENUMERATION OF RED BLOOD CELLS

Materials
1. Hemocytometer
2. Red blood cell pipette
3. Red blood cell diluting fluid
   It contains 3.13gm of trisodium citrate, 1ml of Formalin and 100ml of distilled water.

Procedure

20μl of blood was taken in the RBC pipette. It was diluted up to the mark with RBC diluting fluid. The dilution of blood was 200 times. The Hemocytometer was cleaned with cotton and were observed the counting chambers under microscope. Then the counting chamber was filled with diluted blood and placed on the stage of the microscope. The number of Red blood cells were counted and recorded. The counted cells were calculated and the result was expressed as millions of cells/mm³.
4.9.24. ENUMERATION OF WHITE BLOOD CELLS

Materials
1. Hemocytometer
2. White blood cell pipette
3. White blood cell diluting fluid

Procedure
20µl of blood was taken in the RBC pipette. It was diluted up to the mark with RBC diluting fluid. The dilution of blood was 20 times. The Hemocytometer was cleaned with cotton and the counting chambers were observed under microscope. Then the counting chamber was filled with diluted blood and placed on the stage of the microscope. The number of Red blood cells were counted and recorded. The counted cells were calculated and the result was expressed as millions of cells/mm³.
4.9.25. DETERMINATION OF HEMOGLOBIN

Materials
1. 0.1N Hydrochloric acid
2. Sahli’s Hemoglobinometer
3. Sahli’s Pipette

Procedure

The Sahli’s pipette was filled upto 20 mark with 0.01N hydrochloric acid. Then 20μl of blood was added and mixed well. The mixture was allowed to stand at room temperature for 10 minutes. The colour of diluted blood was compared with that of standard. If the colour did not match, it was diluted with distilled water until the colour matches with the standard.
4.10. HISTOPATHOLOGICAL STUDIES

For histopathological study, the tissues were fixed in Bouin's fluid. The classical paraffin sectioning, and haematoxylin eosin staining techniques were used for histopathological studies. The various steps involved in the preparation of tissues for histopathological 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 it's 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 filtered with paraffin, at the same time over dehydration will make tissues brittle. In this condition sectioning will be a problem. Hence the tissues must be 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. Some of the clearing agents are miscible with both dehydration and embedding agents and they permit paraffin to infiltrate the tissues. So the 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 consists of metal posts filled with molten 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 should be fresh and stable in its optimum melting point at about 56°C to 58°C.

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 towards the glass plate to make it settle uniformly with a metal pressing rod 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 was 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 cover slips and micro glass cover slides were observed in Euromex microscope and photomicrographs were taken.