Appendices
APPENDICES

APPENDIX 1
IDENTIFICATION OF RHEUMATOID ARTHRITIC FACTOR

PRINCIPLE

The RA test antigen consists of polystyrene latex particles coated with specially purified human $\gamma$-globulin. The suspension of coated latex particle agglutinate visibly when mixed with dilute serum containing rheumatoid factor in concentration equal to greater than the sensitivity ie, 20.00 Iu/ml.

REAGENTS
1. Latex- $\gamma$globulin reagent
2. Accessories :- test slide, disposable applicator sticks, disposable plastic droppers
3. Serum.

PROCEDURE

Using a disposable plastic dropper a drop of the test serum was placed in a circle area on the slide respectively. A drop of latex $\gamma$-globulin reagent was added and rocked gently for 2 minutes. Formation of agglutination indicates the sample to be positive.

The highest dilution for which there is a visible agglutination is known as titre. From the observed titre, the concentration of rheumatoid factor can be determined. RF in Iu/ml is equal to sensitivity Iu/ml/titre.

APPENDIX 2
ESTIMATION OF COLLAGEN
Necomann and Logan (1958)

PRINCIPLE

Collagen was estimated by Necomann and Logan (1958) method.
REAGENTS

1. CuSO₄ - 0.05 M
2. NaOH - 2.5N
3. H₂O₂ - 6%
4. H₂SO₄ - (3N)
5. DMAB - (5%) (Dimethyl amino benzaldehyde)
6. Standard Hydroxyproline was prepared with the concentration of 100µg/ml in water.

PROCEDURE

Weighed known amount of cartilage, hydrolysed with 3.0ml of 6N HCL in sealed tube for 24 hours at 110°C. After hydrolysis, evaporated to dryness, decolorised with activated charcoal, made upto 3.0ml with distilled water.

Standard solution containing 25-100µg of hydroxyproline were used. Volume made up to 1.0 ml. The substance was mixed with 1.0 ml of CuSO₄ and 1.0 ml NaOH heated at 40°C for 5 min. The temperature was raised up to 80°C. Cooled an ice. 4.0 ml of 3N H₂SO₄ was added, stirred and added 2.0 ml of DMAB was added, boiled in a water bath for 16 min. Read against reagent blank at 540 nm.

APPENDIX 3

ENUMERATION OF WHITE BLOOD CELLS
Chesbrough and McArthur (1972)

PRINCIPLE

Blood is diluted with acid solution which removes the red cells by haemology and also accentuates the nuclei of the white cells; thus the counting of total white cells become easy. Counting is done with a microscope under low power (100x magnification) and knowing the volume of fluid examined and dilution of the blood, the number of white cells per cu mm (or µL) undiluted whole blood is calculated.
REAGENTS

1. WBC Diluting Fluid, Truck’s:
2. Acetic acid, glacial 3ml.
3. Distilled water 97ml.
4. Add Gention violet to give a pale violet colour.

PROCEDURE

Draw the blood upto the 0.5 mark in WBC pipette marked 11 and diluted upto the mark 11 with WBC fluid as described in RBC counting and fill the counting chamber in the same manner. Allow 3 minutes for the cells to settle. If the neubauer counting chamber is used, count the cells in the four corner blocks. Each of these 4 square millimeter area is sub-divided into 16 squares, by using the low power objective and a medium ocular. In counting the cells include those cells touching on the inner line on the top, but do not count the cells touching the lines on the left and bottom. The difference between the two square millimeter areas should not be more than 10 WBC’S.

APPENDIX 4

ENUMERATION OF RED BLOOD CELLS
Chesbrough and Mc Arthur (1972)

PRINCIPLE

The blood specimen is diluted (usually 200 times) with red cell diluting fluid which does not remove the white cells but allows the red cells to be counted under 400x magnification in a known volume of fluid. Finally, the number of red cells/μL of whole blood.

PROCEDURE

The Hemocytometer Neubaurer counting chamber has a total ruled of 9 sq.mm. It consists of a centrally heavy ruled area of 1sq.mm in size of four others of the same size in each corner. The central area is divided into 25 squares and each square is sub-divided into 16 squares. For total RBC counts, count one each at the
corner and the center (that is 80 small squares.). The four outer 1-sq.mm area are divided into 16 squares. The squares are used for total WBC counts.

**Red Blood Cells (Erythrocytes)**

The mature Erythrocyte is a biconcave disk, circular in shape, centrally unstained and periphery stained, pink in colour Size: 7.2 microns in average diameter. It contains Haemoglobin.

**RBC Diluting Fluid:**

a) Formal Citrate Solution:

- Trisodium Citrate -- 3g.
- Distilled water -- 99 ml.
- Formalin, commercial -- 1 ml.

Or

b) Hayem’s Fluid:

- Sodium Chloride -- 0.5 g.
- Sodium sulphate -- 2.5 g.
- Mercuric Chloride -- 0.25 g.
- Distilled water -- 100 ml.

Draw the blood from the finger directly into RBC pipette or from a sample of oxalated blood exactly to the 0.5 mark (Thoma pipette mark 101) and immediately draw up the diluting fluid (Formal Citrate solution or Hayem’s fluid) to the mark 101, and rotate the pipette between the thumb and forefinger. This will give a dilution of 1:200.

Clean the counting chamber and cover glass thoroughly. Place the cover glass in position over the ruled area, using gentle pressure. Mix the suspension thoroughly by rotating the pipette for about a minute, holding it in horizontal position, and finally shake sidewise. Expel the fluid from the stem of the pipette and without loss of time, fill the chamber by holding the pipette at an angle of
45 degrees and lightly touching the tip against the edge of the cover glass. Care should be taken to ensure that the suspension does not flow into the moats on either side, nor should any bubble form under cover glass. Allow two to three minutes for the red corpuscles to settle.

Count the number of RBC'S in 180 small squares (4 squares of 16 at therefore corners and one of 16 at center). Do not count the cells touching the lower and right hand lines, but count the cells touching the upper and left hand lines.

The cells counted are expressed as thousands/mm$^3$ of blood.

APPENDIX 5
PACKED CELL VOLUME
(Wintrobe, 1933)

PRINCIPLE

When anticoagulated blood (whole blood) is centrifuged at a standard speed, erythrocytes which are heavier than white cells, platelets and plasma, sediments at the bottom. This red cell column is called haematocrit or packed red cell volume which is expressed as fraction of the whole blood (level of plasma). Two methods are applied for the determination of haematocrit-macrohaematocrit method and microhaematocrit method.

Haematocrit is defined as the volume of Erythrocytes expressed as percentage of the volume of whole blood.

PROCEDURE

The whole blood after adequate mixing was filled in the Wintrobe Macrohaematocrit tube. The level of the blood was noted and the tubes were centrifuged at 2500 g for 30 min. The result was calculated using the formula:
Haematocrit (%) = 100 x L1/L2

Where,

L1 = height of the red cell volume in mm
L2 = height of the whole blood specimen in mm

The greyish white layer of Leucocytes and platelets above the Erythrocyte was not included in L1.

APPENDIX 6
ERYTHROCYTE SEDIMENTATION RATE
Westergren’s (1988)

PRINCIPLE

Anticoagulated blood is taken in a tube and left undisturbed in a vertical position. The level of the column of red cells is noted in the beginning (0 hr) and after 1 hr. The distance (mm) the column has moved is the ESR (mm/hr). Temperature and slanting of the tube affect the ESR. Hence, always work in a quiet area of the laboratory, and away from the window in order to avoid temperature variation. Also make sure that the tubes are vertical during the ESR measurement.

Westergren’s method

Take 0.4ml of 3.8% Sodium Citrate in a tube. With draw 2 ml of venous blood in a dry sterile syringe and place exactly 1.6 ml of blood in the tube containing Sodium Citrate solution. Invert the tube 2 to 3 times to mix the blood with the Citrate solution. Fill the westergren’s E.S.R. tube to exactly the 0 mark and place it in the stand. The tube must be held firmly at an exactly vertical position. Reading may be made at 5 minutes interval over a period of 1 hour, or one reading may be made at the end of half-an-hour and another at the end of one hour and two hours.
APPENDIX 7

ESTIMATION OF HAEMOGLOBIN COUNT
(Drabkin and Austin, 1932)

PRINCIPLE

Cyanmethaemoglobin method is a colorimetric procedure for determining haemoglobin concentration. An aliquot of well-mixed whole blood is taken and reacted with a solution of potassium cyanide and potassium ferricyanide (called Drabkin’s solution). The chemical reaction yields a product of stable colour—the cyanmethaemoglobin. The intensity of the colour is proportional to the haemoglobin concentration and obeys Beer’s Law.

RE AGENTS

1. Ferricyanide – Cyanide reagent: this was prepared by dissolving 200 mg Potassium Ferricyanide, 50 mg Potassium Cyanide and 140 mg. Potassium Dihydrogen Phosphate in a litre of water.

2. Cyanmethaemoglobin standard: Purchased from span diagnostics, Surat, India. This was kept in the dark at 4°C. It has an equivalent Haemoglobin concentration of 60 mg%.

PROCEDURE

20 µl of blood was added to 4.0 ml of the Ferricyanide-Cyanide reagent. They allowed to stand for 15 min and was read against a reagent blank at 540 nm. The standards were diluted in Ferricyanide-Cyanide solution to obtain a range of concentrations in the same manner.

Blood Haemoglobin value was expressed as g/dl.
APPENDIX 8

ESTIMATION OF PROTEIN
(Lowry et al., 1951)

PRINCIPLE

Protein was estimated by the method of Lowry et al., (1951). The colour which developed in this reaction is due to the reaction of protein with copper sulphate in alkaline medium and the reaction of tyrosine and tryptophan with Folin’s phenol reagent and was measured at 660 nm.

REAGENTS

1. Solution A: 1% Copper sulphate
2. Solution B: 2% Sodium potassium tartrate
3. Solution C: 2% Sodium carbonate in 0.1N NaOH
4. Solution D: Mixed just before use, 1ml of solution A, 1ml of solution B and 100 ml of solution C.
5. Solution E: 1N Folin-ciocalteau reagent (Mixed equal volumes of commercially available reagent and distilled water just prior to use).
6. Standard Bovine Serum Albumin (BSA): 50mg BSA in 50ml of 0.1N NaOH (stock). Diluted 1:10 for working standard.
7. 10% TCA

PROCEDURE

Pipetted out 0.2ml to 1.0ml working standard solution. 0.1ml of the sample was taken. The volume in all the tubes was made up to 1.0ml with distilled water. Added 5.0ml of solution D to each tube. Mixed well and incubated at 10 minutes, then added 0.5ml Folinciocalteau reagent. Mixed well and incubated at room temperature for 30 minutes. A reagent blank was also prepared. After 30 minutes, the blue colour developed was read at 660nm.
APPENDIX 9

ESTIMATION OF UREA
(Natelson et al., 1951)

PRINCIPLE

Urea reacts directly with Diacetyl Monoxime in the presence of thiosemi carbazide to form a pink coloured product which is measured Colorimetrically at 540 nm.

REAGENTS

1. Diacetyl monoxime : 1.56g of Diacetyl monoxime was dissolved in 250ml of distilled water.

2. Thiosemicarbazide : 41 mg of Thiosemicarbazide was dissolved in 250ml of distilled water and stored in a brown bottle.

3. Ferric Chloride reagent : 324 mg of Ferric chloride was dissolved in 10ml of 56% Orthophosphoric acid and stored in a brown bottle.

4. 20% Sulphuric acid

5. Acid reagent : To 1 litre of 20% sulphuric acid added 1 ml of Ferric Chloride reagent.

6. Stock standard : 100mg of Urea /100ml

7. Working standard : 2.0ml of Stock standard was diluted to 100ml. 1 ml of this solution contains 20μg/ml.

PROCEDURE

To 0.5 ml of supernatant, 1.0, ml of Diacetyl Monoxime and 1.0ml of Thiosemicarbazide and 3.0 ml of acid reagent was added. Kept in a boiling water bath for 30 minutes. A blank was also set up with water. A series of standard were put up simultaneously and treated as for test. Cooled and read at 540 nm. The values are expressed in mg/dl.
APPENDIX 10

ESTIMATION OF URIC ACID
(Caraway, 1963)

PRINCIPLE

Uric acid reduced sodium phospho tungstate in the given alkaline medium to give a blue colour which is measured colorimetrically at 610nm.

REAGENTS

1. Phosphotungstic acid : Dissolved 5 gm of sodium tungstate in 400 ml of distilled water. Added 40 ml of 84% phosphoric acid refluxed gently for 2 hours. Cooled and transferred to a 50 ml standard flask and made up to the mark with distilled water. Store the reagent in a brown bottle. Diluted 1 to 10ml before use.

2. 10% Sodium carbonate

3. Stock standard : 100 mg of Uric acid and 60mg of lithium carbonate were taken in a breaker and about 50ml of water was added. Heated to about 60°C to dissolve the uric acid completely. After cooling, the solution was finally made up to 100 ml with water.

4. Working standard : Dilute 2.0 ml of the stock standard to 100 ml with water. 1 ml of this solution contains 20 µg of uric acid.

PROCEDURE

2 ml of the supernatant was taken and to this 1.0 ml of phosphotungstic acid and 1 ml of 10% sodium carbonate was added. A blank was set up with water. Standard with graded volume were also set up for the test. After 10 minutes the color was read at 610 nm. The values are expressed in mg/dl.
APPENDIX 11

ESTIMATION OF CREATININE
(Jaffe’s method - Owen et al., 1954)

PRINCIPLE

This method make use of the Jaffe’s reaction. The production of mahogony red colour with an alkaline picrate solution. The intensity of the colour developed was read at 420nm.

REAGENTS

1. Picric acid : 0.05 M
2. Sodium hydroxide : 0.75 N
3. Stock standard : Dissolved 100 mg of Creatinine in N/10 Hydrochloric acid and made upto 100 ml with the same.
4. Working standard : Diluted 2.0 ml of stock solution to 100 ml with water. This contains 20µg of Creatinine / ml.

PROCEDURE

To 4.0 ml of the supernatant, 1.0 ml of 0.15 N sodium hydroxide and 1.0ml of Picric acid was added Standard graded volumes and a reagent blank was treated in a similar manner. The colour development was read at 470nm.

The values are expressed as mg of creatinine / dl.

ESTIMATION OF PROTEIN BOUND CARBOHYDRATES

A known amount of de-lipidated residues of liver, kidney and spleen were hydrolysed with 2.0 ml of 3N HCl at 80°C for 4h. The hydrolysed materials were neutralized and used for the estimation of hexoses, hexosamine, hexouronic acid and sialic acid.
APPENDIX 12

ESTIMATION OF HEXOSE
Niebes (1972)

REAGENTS
1. Orcinol- H$_2$SO$_4$ reagent:
   Solution A: 60 ml of concentrated H$_2$SO$_4$ was mixed with 40 ml of water.
   Solution B: 1.6 g of orcinol (recrystallised from benzene) was dissolved in 100 ml of water. 7.5 volumes of solution A was mixed with 1 volume of solution B before use.

2. Standard : 5 mg each of galactose and mannose were dissolved in 100ml of water (100 µl/ml).

PROCEDURE

0.5 ml of the neutralized solution was made up to 1.0 ml with water and 8.5 ml of cold orcinol reagent was added. The mixture was heated at 80 °C for 15 min. Cooled and left in the dark for 25 min for colour development. Then the absorbance was read at 540 nm against a reagent blank. Standard solution containing 25-100µg of hexose were treated in a similar manner to obtain a standard curve for comparisons. The hexose content was expressed as mg/100mg de-fatted tissue.

APPENDIX 13

ESTIMATION OF HEXOSAMINE
(Wagner 1979)

REAGENTS
1. Acetyl Acetone reagent:
   ReagentA: 1N Tri sodium phosphate
   ReagentB: 0.5N potassium tetra borate.
   3.5ml of acetyl acetone was added to mixture of reagents A and B in the ratio of 98:2 (v/v).
2. Ehrlich’s reagent: 320 mg of P-dimethyl amino benzaldehyde was dissolved in 21 ml of isopropanol and 3.0 ml of concentrated Hcl.

3. Standard galactosamine was prepared with the concentration of 100 μg/ml in water.

PROCEDURE

0.5 ml of the neutralized sample was made up to 1.0 ml with water. Standard galactosamine in the range of 10-40μg was also made up to 1.0ml. Blank comprised of 1.0ml water. 0.6ml of acetylacetone reagent was added to all the tubes and heated in a boiling water bath for 30 minutes. After cooling, 2.0ml of Ehrlich’s reagent was added and the contents were shaken well. The pink colour developed was measured at 540nm against the blank. Hexosamine content was expressed as mg/100mg de-fatted tissue.

APPENDIX 14

ESTIMATION OF HEXURONIC ACID
Bitter and Muir (1962)

REAGENTS

1. Sulphuric acid reagent: 0.025 M Sodium borate prepared in concentrated H₂SO₄
2. Carbazole regent :0. 125% Carbazole was prepared in ethanol and stored at 40°C in the dark.
3. Standard glucuronolactone was prepared by dissolving 10 mg in 100 ml distilled water (100μg/ml).

PROCEDURE

5.0 ml of H₂SO₄ reagent was placed in tubes and cooled to 4°C. 0.5 ml of the neutralized sample and standards were layered carefully into the acid. The tubes were shaken, first gently and then vigorously and heated for 10min in a water bath. After cooling, 0.2ml of carbazole reagent was added and the tubes were once again heated for further 15min and cooled. The colour developed was read at 530nm. The values were expressed as mg/100mg de-fatted tissue.
APPENDIX 15

ESTIMATION OF SIALIC ACID
Niebes (1972)

PRINCIPLE

A known amount (50mg) of delipidated residue of liver was hydrolysed with 1 ml of 0.1N H₂SO₄ at 80°C for 60 min. The hydrolysed material was neutralized and used for the estimation of sialic acid.

REAGENTS

1. 0.1 N H₂SO₄
2. 0.25 M peroxidate in 0.1 N HCl
3. 4% Sodium meta arsenite in 0.5 N HCl
4. 0.1 N Thiobarbituric acid: 144mg of thiobarbituric acid was dissolved in 10 ml of water. The pH of the solution was adjusted to 9.0 ml with 6 N NaOH. This reagent was prepared freshly.
5. Acidified butanol: 5% HCl in n- butanol
6. Standard sialic acid: 10 mg of N- acetyl neuraminic acid in 100 ml of water.

PROCEDURE

0.5 ml of the neutralized sample was taken along with standards (in the range of 10-40µg). Blank contained 0.5 ml of 0.1 N H₂SO₄. 0.25 ml of periodate was added to all tubes at 37°C. After 30 min, 0.25 ml of arsenite solution was added to inhibit the reaction. Contents were mixed and 2 ml of thiobarbituric acid was added and the tubes were heated in a boiling water bath for 6 min. After cooling, the pink colour developed was extracted into 5 ml of acidified butanol phase and was measured at 540nm against a reagent blank. The values are expressed as mg/100mg defatted tissue.
APPENDIX 16

ESTIMATION OF ACID PHOSPHATASE
(King et al., 1956a)

PRINCIPLE

The method used was that of King and Armstrong in which disodium phenyl phosphate is hydrolysed with the liberation of phenol and inorganic phosphate. The liberated phenol is measured at 700 nm with Folin-Ciocalteau reagent.

REAGENTS

1. Citrate buffer: 0.1 M, pH 5.
   Solution A: Citric acid (21.01 g in 1000 ml)
   Solution B: Sodium citrate (29.41 g in 1000 ml)
   20.5 ml of A and 29.5 ml of B, diluted to a total of 100 ml

2. Disodium phenyl phosphate, 10 mmol/l: Dissolved 2.18 g in water, heated to boil, cooled and made to a litre. Added 1.0 ml of chloroform and stored in the refrigerator.

3. Buffer-substrate: Prepared by mixing equal volume of the above two solution. This has a pH of 5.0.

4. Folin Ciocalteau reagent: Mixed 1.0 ml of reagent with 2.0 ml of water.

5. Sodium carbonate solution, 15%: Dissolved 15 g of anhydrous sodium carbonate in 100 ml of water.

6. Standard phenol solution, 1 g/L: Dissolved 1 g pure crystalline in 100 mmol/L HCl and made to a litre with the acid.

7. Working standard solution: Diluted 10 ml of stock standard to 100 ml with water. This contains 100-μg phenol/ml.

PROCEDURE

Pipetted 4.0 ml of the buffer substrate into a test tube and incubated at 37°C for 5 min. Added 0.2 ml of tissue homogenate and incubated further for exactly 60
min. Removed and immediately added 1.8 ml of diluted phenol reagent. At the same time a control was set up containing 4.0 ml buffer substrate and 0.2 ml tissue homogenate to which 1.8 ml phenol reagent was added immediately. Mixed well and centrifuged. To 4.0 ml of the supernatant, added 2.0 ml of sodium carbonate. 4.0 ml of working standard solution was taken and for blank 3.2 ml water and 8.0 ml of phenol reagent was taken. Then added 2.0 ml of sodium carbonate. Incubated all the tubes at 37°C for 15 min. Read the colour developed at 700 nm. The activity in tissue homogenate was expressed as µmole of phenol liberated/min/mg protein

APPENDIX 17

ESTIMATION OF ASPARTATE TRANSAMINASE
(Reitman and Frankel, 1957)

PRINCIPLE
The enzyme catalyses the following reaction:

\[ \text{L-Aspartate} + \alpha-\text{oxoglutarate} \rightarrow \text{Oxaloacetate} + \text{L-glutamate} \]

The oxaloacetate is measured by the reaction with 2, 4 dinitrophenyl hydrazine giving a brown coloured hydrazone after the addition of sodium hydroxide. The colour developed is read at 520 nm.

REAGENTS

1. Phosphate buffer, 0.1 M, pH 7.5
   Solution A: 0.1 M solution of monobasic sodium phosphate (13.9 g/l).
   Solution B: 0.1 M solution of dibasic sodium phosphate (26.8 of Na₂PO₄·H₂O g/l) 16 ml of A and 84 ml of B, diluted to a total of 200 ml.

2. Substrate: Dissolved 146 mg of α-Ketoglutarate and 13.3 g of aspartic acid in 1N NaOH with constant stirring. Adjusted the pH to 7.4 and made up to 1000 ml with phosphate buffer.

3. Standard pyruvate, 2 mmol/l: Dissolved 22 mg of sodium pyruvate in 100 ml of phosphate buffer. 0.2 ml of standard contained 0.4 uM of sodium pyruvate.
4. Dinitrophenylhydrazine reagent, 1 mmol/l: 200 mg in 1 mol/l HCl.

5. 0.4N NaOH: Dissolved 16 g of NaOH in 1000 ml water.

PROCEDURE

0.2 ml of tissue homogenate and 1.0 ml of the buffer substrate was incubated for 60 min at 37° C. To the control tubes, enzyme was added, after arresting the reaction with 1.0 ml of DNPH and the tubes were kept at room temperature for 20 min. Then 10 ml of 0.4 N NaOH was added. A set of standard pyruvate was also treated in a similar manner. The colour developed was read at 520 nm.

The enzyme activity in liver homogenate was expressed as μ moles pyruvate liberated/minute/mg protein.

APPENDIX 18

ESTIMATION OF ALKALINE PHOSPHATASE
(King and Armstrong, 1934)

PRINCIPLE

The method used was that of King and Armstrong in which disodium phenyl phosphate is hydrolysed with the liberation of phenol and inorganic phosphate. The liberated phenol is measured at 700 nm with Folin-Ciocalteau reagent.

REAGENTS

1. Sodium carbonate – sodium bicarbonate buffer, 100mmol/l: Dissolved 6.36g anhydrous sodium carbonate and 3.36g sodium bicarbonate in water and made to a litre.

2. Disodium phenyl phosphate, 10mmol/l: Dissolved 2.18g in water, heated to boil, cooled and made to a litre. Added 1.0ml of chloroform and stored in the refrigerator.

3. Buffer substrate: Prepared by mixing equal volume of the above two solution. This has a pH of 10.

4. Folin – Ciocalteau reagent: Mixed 1.0ml of reagent with 2.0ml of water.
5. Sodium carbonate solution, 15%: Dissolved 15g of anhydrous sodium carbonate in 100ml of water.
6. Standard phenol solution, 1 g/L: Dissolved 1g pure crystalline phenol in 100mmol/L HCl and made to a litre with the acid.
7. Working standard solution: Added 100ml dilute phenol reagent to 5.0 ml of stock standard and diluted to 500ml with water. This contained 10ug phenol/ml.

PROCEDURE

Pipetted 4.0ml of the buffer substrate into a test tube and incubated at 37°C for 5 min. Added 0.2 ml of tissue homogenate and incubated further for exact 15min. Removed and immediately added 1.8ml of diluted phenol reagent. At the same time a control was set up containing 4.0 ml buffer substrate and 0.2ml tissue homogenate to which 1.8ml phenol reagent was added immediately. Mixed well and centrifuged. To 4.0ml of supernatant added 2.0ml of sodium carbonate solution. Took 4.0ml of working standard solution and for blank taken 3.2 ml water and 0.8ml of phenol regent. Then added 2.0ml of sodium carbonate. Incubated all the tubes at 37°C for 15 min. Read the colour developed at 700 nm. The activity in tissue homogenate was expressed as μmole of phenol liberated/min/mg protein.

APPENDIX 19

ESTIMATION OF ALANINE TRANSAMINASE
(Reitman and Frankel, 1957)

PRINCIPLE

The enzyme catalyses the following reaction:

L-Alanine+α-oxoglutarate → γ Pyruvate+L-glutamate

The oxaloacetate is measured by the reaction with 2,4 dinitrophenylhydrazine giving a brown coloured hydrazone after the addition of sodium hydroxide. The colour developed is read at 520 nm.
REAGENTS

1. Phosphate buffer: 0.1M, pH 7.5
2. Substrate: Dissolved 146 mg of α-ketoglutarate and 17.8 g of L-alanine in 1N NaOH with constant stirring. Adjusted the pH to 7.4 and made up to 1000 ml with phosphate buffer.
3. Standard pyruvate, 2 mM: Dissolved 22 mg of sodium pyruvate in 100ml of phosphate buffer 0.2 ml of standard contained 0.4 μM of sodium pyruvate.
4. Dinitrophenyl hydrazine reagent, 1 mmol/l: 200mg/l in 1 mol/l HCl.
5. 0.4 N NaOH: Dissolved 16 g of NaOH in 1000 ml water.

PROCEDURE

0.2 ml of enzyme and 1.0 ml of the buffer substrate were incubated for 30 min at 37°C. To the control tubes, enzyme was added after arresting the reaction with 1.0ml of DNPH and the tubes were kept at room temperature for 20 min. Then 10ml of 0.4 N NaOH was added. A set of standard pyruvate was also treated in a similar manner. The colour developed was read at 520 nm.

The enzyme activity in liver homogenate was expressed as μ moles of pyruvate liberated/minute/mg protein.

APPENDIX 20

ESTIMATION OF CATHEPSIN - D
(Barrett, 1977)

REAGENTS

1. Sodium Acetate buffer, 0.1M, pH 3.
   A: 0.1 M solution of acetic acid (5.7 ml in 1 L water)
   B: 0.1 M solution of sodium acetate (8.2 g in 1 L water) 44 ml of A and 6ml of B, diluted to a total of 100 ml.
2. Substrate: 2% hemoglobin in sodium acetate buffer.
3. 5% TCA.
4. Alkaline copper reagent.
5. Folin’s phenol reageny.
6. 5% NaOH
7. Standard: A solution of tyrosine in the concentration of 10 mg/100ml was prepared with 0.1 N Hcl.

PROCEDURE

0.75 ml of buffered substrate was mixed with 0.25 ml of homogenate and incubated for 2 hrs at 37°C. The reaction was stopped with 1.0 ml of 5% TCA and the samples were centrifuged for 10 min. To 1.0 ml of supernatant, 2.0 ml of 5% NaOH, 4.5 ml of alkaline copper reagent was added. After 20 min of incubation added 0.5 ml of Folin-Ciocalteau reagent and read immediately. 1.0 ml of standards and 1.0 ml of water as blank was taken and treated similarly as above. The colour developed was read ay 640nm.

Enzyme unit is expressed as n moles of tyrosine/min/mg protein at 37°C.

APPENDIX 21

ESTIMATION OF LACTATE DEHYDROGENASE
(King et al., 1956b)

PRINCIPLE

The lactate is acted upon by lactate dehydrogenase to form pyruvate in the presence of NAD. The pyruvate forms pyruvate phenyl hydrazone with 2,4 dinitrophenyl hydrazine. The colour developed is read in a spectrophotometer at 440 nm.

REAGENTS

1. Glycine buffer, 0.1 M, pH 10: 7.505 g of glycine and 5.85 g of sodium chloride were dissolved in 1 litre of water.

2. Buffered substrate: 125 ml of glycine buffer and 75 ml of 0.1 N NaOH were added to 4g of lithium lactate and mixed well.
3. Nicotinamide adenine dinucleotide: 10 mg of NAD was dissolved in 2.0 ml of water.
4. 2,4 Dinitrophenyl hydrazine: 20 mg of DNPH was dissolved in 100 ml of 1N HCl.
5. 0.4 N NaOH.
6. Standard, 1 µmole of pyruvate/ml: 11 mg of sodium pyruvate was dissolved in 100 ml buffered substrate (1 µmole of pyruvate/ml).
7. NADH solution, 1 µmole/ml: 8.5 mg/10 ml buffered substrate.

PROCEDURE

Placed 1.0 ml buffer substrate and 0.1 ml sample into each of two tubes. Added 0.2 ml water to the blank. Then to the test added 0.2 ml of NAD. Mixed and incubated at 37°C for 15 min. Exactly after 15 min, 1.0 ml of dinitrophenyl hydrazine was added to each (test and control) tubes. Left for further 15 min. Then added 10 ml of 0.4 N sodium hydroxide and the color developed was read immediately at 440 nm. A standard curve with sodium pyruvate solution with the concentration range 0.1 -1.0µmole was taken.

LDH activity in liver homogenate was expressed as µmoles of pyruvate liberated/minute/mg protein.

APPENDIX 22

ESTIMATION OF LIPID PEROXIDATION
(Buege and Aust, 1978)

PRINCIPLE

Malondialdehyde has been identified as the end product of lipid peroxidation that reacts with thiobarbituric acid to give a red colour, absorbing at 535 nm.

REAGENTS

1. Stock TCA-TBA-HCl reagent: 15%w/v trichloroacetic acid, 0.375%w/v Thiobarbituric acid and 0.25 N HCl. The solution was heated mildly to assist the dissolution of the TBA.
PROCEDURE

To 1.0ml of the sample, 2.0ml of TCA-TBA-HCl reagent was added and mixed thoroughly. The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1,000 rpm for 10 min. The absorbance was determined at 535 nm against a blank that contained all the reagents minus the sample. The results were expressed as nmoles of MDA formed/mg protein using an extinction coefficient of the chromophore 1.56 x 10^5 M^-1 cm^-1.

Ferrous sulphate and ascorbate induced lipid peroxidation

Ferrous sulphate and ascorbate induced lipid peroxidation was carried out as given above. The peroxidation system contained 10mM ferrous sulphate and 0.2mM ascorbate.

APPENDIX 23

ESTIMATION OF SUPEROXIDE DISMUTASE
(Das et al., 2000)

PRINCIPLE

The method involves generation of superoxide radical of riboflavin and its detection by nitrite formation from hydroxylamine hydrochloride. The nitrite reacts with sulphanilic acid to produce a diazonium compound, which subsequently reacts with naphthylamine to produce a red azo compound whose absorbance is measured at 543 nm.

REAGENTS

1. 50 mM phosphate buffer, pH 7.4
2. 20 mM L-Methionine
3. 1% (v/v) Triton X-100
4. 10 mM hydroxylamine hydrochloride
5. 50 μM EDTA
6. 50 μM Riboflavin
7. Griess reagent: 1% sulphanilamide, 2% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride.
PROCEDURE

Pipetted 1.4 ml aliquot of the reaction mixture in a test tube. 100 μl of the sample was added followed by pre incubation at 37°C for 5 min. 80 μl of riboflavin was added and the tubes were exposed for 10 min to 200 W Philips fluorescent lamps. The control tube contained equal amount of buffer instead of sample. The sample and its respective control were run together. At the end of the exposure time, 0.1 ml of Greiss reagent was added to each tube and the absorbance of the colour formed was measured at 543 nm.

One unit of enzyme activity was defined as the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition.

APPENDIX 24

ESTIMATION OF CATALASE
(Sinha, 1972)

PRINCIPLE

Catalase causes rapid decomposition of hydrogen peroxide to water.

\[
\text{Catalase} \quad \text{H}_2\text{O}_2 \xrightarrow{\text{H}_2\text{O}+\frac{1}{2}\text{O}_2} \]

The method is based on the fact that dichromate in acetic acid reduces to chromic acetate when heated in the presence of H\textsubscript{2}O\textsubscript{2} with the formation of perchloric acid as an unstable intermediate. The chromic acetate thus produced is measured colorimetrically at 610 nm. Since dichromate has no absorbancy in this region, the presence of the compound in the assay mixture does not interfere with the colorimetric determination of chromic acetate. The catalase preparation is allowed to split H\textsubscript{2}O\textsubscript{2} for different periods of time. The reaction is stopped at specific time intervals by the addition of dichromate/acidic acid mixture and the remaining H\textsubscript{2}O\textsubscript{2} is determined by measuring chromic acetate colorimetrically after heating the reaction.

REAGENTS

1. 0.01 M Phosphate buffer, pH 7.0 Solution A: 0.1 M Monobasic sodium phosphate (13.9 g/ll) Solution B: 0.1 M Dibasic sodium phosphate (26.82 g/ll)
of Na₂HPO₄ 7H₂O/ΙL) 39 ml of solution A and 61ml of solution B was
diluted to a total of 200 ml. 10ml of this solution was further diluted to
100 ml with water.

2. 0.2 M Hydrogen peroxide:

3. Stock dichromate / acetic acid solution: Mixed 5% potassium dichromate
   with glacial acetic (1:3 by volume).

4. Working dichromate / acetic acid solution: the stock was diluted to 1:5 with
   water to make the working dichromate/acetic acid solution.

PROCEDURE

The assay mixture contained 0.5 ml of H₂O₂ 11.0 ml of buffer and 0.4 ml water.
0.2 ml of the enzyme was added to initiate the reaction. 2.0 ml of the dichromate/
acetic acid reagent was added after 0, 30, 60, 90 seconds of incubation. To the control
tube the enzyme was added after the addition of the acid reagent. The tubes were then
heated for 10 min and the colour developed was read at 610 nm. The activity of
catalase was expressed as umole of H₂O₂ decomposed/min/mg protein.

APPENDIX 25

ESTIMATION OF PEROXIDASE
(Addy and Goodman, 1972)

PRINCIPLE

Pyrogallol is used as a substrate for the assay of peroxidase.

POD

Pyrogallol +H₂O₂ ➔ Oxidized pyrogallol +2 H₂O

The rate of formation of oxidized pyrogallol is a measure of peroxidase activity
and was measured at 420 nm.

REAGENTS

1. 0.1 M Phosphate buffer, pH6.5
   Solution A: 0.1 M solution of monobasic sodium phosphate
   (13.9g/1000ml water)
Solution B: 0.1 M solution of dibasic sodium phosphate (26.8g of Na₂HPO₄·7H₂O/1000 ml water) Mixed 68.5 ml of solution A and 31.5 ml of solution B and diluted to a total of 200 ml.

2. 0.05 M pyrogallol: Dissolved 630 mg of pyrogallol in 100 ml of 0.1 M phosphate buffer.

3. 1% Hydrogen peroxide.

PROCEDURE

Pipetted out 3.0 ml of 0.05M pyrogallol solution and 0.1 ml of enzyme extract in a test tube. Adjusted the spectrophotometer to read 'O' at 430 nm. Added 0.5 ml of 1% H₂O₂ in the test cuvette. Mixed and recorded the change in absorbance for every 30 seconds upto 3 min. The difference in OD change per minute with and without enzyme addition was a measure of peroxidase activity.

The activity was expressed in terms of µmoles of pyrogallol oxidized/min/mg protein.

APPENDIX 26

ESTIMATION OF TOTAL REDUCED GLUTATHIONE
(Moran et al., 1979)

PRINCIPLE

Glutathione (GSH) was measured by its reaction with DTNB to give a compound that absorbs at 412 nm.

REAGENTS

1. Metaphosphoric acid: 1.67 g of glacial metaphosphoric acid, 0.2 g EDTA and 30 g NaCl in 100ml water.

2. 0.4 M Na₂HPO₄

3. DTNB reagent: 40 mg DTNB in 100 ml of 1% trisodium citrate

4. Standard glutathione: 20mg reduced glutathione was dissolved in 100 ml water.
PROCEDURE

1.0 ml of 10% tissue homogenate was mixed with 4.0 ml of metaphosphoric acid. The precipitate was removed by centrifugation. To 2.0 ml of the supernatant, 2.0 ml disodium hydrogen phosphate and 1.0 ml of DTNB reagent were added. The absorbance was read within 2 min at 412 nm against a reagent blank. A set of standards was also treated in the above manner. The amount of glutathione was expressed as ug/mg protein.

APPENDIX 27

ESTIMATION OF ASCORBIC ACID (VITAMIN C)
(Omaye et al., 1979)

PRINCIPLE

Ascorbic acid is oxidized by copper to form dehydroascorbic acid and diketoglutaric acid. These products are treated with 2,4 dinitrophthalhydrazine to form the derivative of bis 2,4 dinitro phenyl hydrazine. This compound in strong sulphuric acid undergoes a rearrangement to from a product with an absorption band that is measured at 520 nm. The reaction is run in the presence of thiourea to provide a mildly reducing medium, which helps to prevent interference from non-ascorbic acid chromogens.

REAGENTS

1. 5%TCA
2. 65% Sulphuric acid
3. DTCS reagent: 3g 2,4 dinitrophenyl hydrazine, 0.4g thiourea and 0.05g copper sulphate were dissolved in 9 N sulphuric acid and made up to 100ml with the same.
4. Standard solution: Standard in the range of 4-20 μg/ml was prepared in 5% oxalic acid.

PROCEDURE

1.0 ml of 10% homogenate was precipitated with 5% ice-cold TCA and centrifuged for 20 min at 3,500 g. 1.0 ml of the supernatant was mixed with 0.2 ml of DTCS reagent and incubated for 3 hours at 37°C. Then 1.5 ml of ice-cold 65%
sulphuric acid was added, mixed well and the solutions were allowed to stand at room temperature for an additional 30 min. Absorbance was determined at 520 nm. This results were expressed as µg/mg protein.

APPENDIX 28

ESTIMATION OF TOTAL SUPHYDRYL GROUP
(Sedlack and Lindsay, 1968)

PRINCIPLE
The sulphydryl groups in tissues are determined using the Ellman's reagent. In this method DTNB was reduced by SH group to form 1 mole of 2 nitro 5-mercaptobenzoic acid per mole SH.

REAGENTS
1. 0.01 M DTNB in absolute methanol
2. 0.2 M Tris Hcl buffer, pH 8 containing 0.2 M EDTA
3. 0.02 M EDTA

PROCEDURE
200 mg of tissue were homogenized in 8.0 ml of 0.02M EDTA. 0.2 ml of tissue homogenate was mixed in 15 ml test tubes with 1.5 ml buffer and 0.1 ml of DTNB. The mixture was made up to 10 ml with absolute methanol. A reagent blank without the sample and sample blank without DTNB were prepared in the same manner. The test tubes were stoppered and allowed to stand for 15 min with occasional shaking. The reaction mixture was centrifuged at 3,000 rpm for 15 min at room temperature. The absorbance of the clear supernatant was read at 420 nm. Calibration curves were obtained with reduced glutathione as standard.

The values were expressed as µGSH/mg protein.
APPENDIX 29

ESTIMATION OF NON-PROTEIN AND PROTEIN SULPHYDRYL GROUPS
(Sedlack and Lindsay, 1968)

REAGENTS
1. 0.01 M DTNB in absolute methanol
2. 0.2 M Tris-HCl buffer, pH 8 containing 0.02 M EDTA
3. 0.02 M EDTA
4. 10% TCA

PROCEDURE
100 mg of the tissue was homogenized in 4.0 ml of 0.02 M EDTA. 1.0 ml of the homogenate was mixed with 1.0 ml of 10% TCA and 1 ml of water. Centrifuged to 2.0 ml of the supernatant added 4.0 ml of buffer and 0.1 ml DTNB. Mixed and read the absorbance at 420 nm. This gives the NP-SH content. The difference between T-SH and N-SH gives the P-SH content of the tissues.

The values were expressed as μg/mg protein.

APPENDIX 30

HISTOPATHOLOGICAL EXAMINATION
(John-Bancroft, 1999)

The liver samples were preserved in 20% commercial formalin immediately on removal from animal.

Tissue processing
Liver tissue was placed in 10% formal saline (10% formalin in 9% sodium chloride) for 1 hr to rectify shrinkage due to higher concentration of formalin. The tissue was dehydrated by ascending grades of Isopropyl alcohol by immersing in 80% isopropanol overnight, 100% Isopropyl alcohol for 1 hr and second change of 100% Isopropanol alcohol for 1 hr. The dehydrated tissues were cleared in two changes of xylene, 1 hr each. Then the tissues were impregnated with histology grade parafin wax (melting point 58-60°C) at 60°C for two changes of 1 hr each. The wax-impregnated
tissues were embedded in paraffin blocks using the same grade wax. The paraffin blocks were mounted and cut with rotary microtome at 3-micron thickness. The sections were floated on a tissue floatation bath at 40°C and taken on glass slides and smeared with equal parts of egg albumin and glycerol. The sections were then melted in an incubator at 60°C and after 05 min the sections were allowed to cool.

**Tissue staining**

The sections were deparaffinised by immersing in xylene for 10 min in horizontal staining jar. The deparaffinised sections were washed in 100% Isopropyl alcohol and stained in Ehrlich’s hematoxylin for 3 min in horizontal staining jar. After staining in hematoxylin, the sections were washed in tap water and dipped in acid alcohol to remove excess stain (8.3% Hcl in 70% Alcohol). The sections were then, placed in running tap water for 10 min for blueing (slow alkalization). The sections were counter stained in 1% aqueous eosin (1 g in 100 ml tap water ) for one minute and the excess stsin was washed in tap water and the sections were allowed to dry. Complete dehydration of stained sections was ensured by placing the sections in the incubator at 60°C for 5 min. When the sections were cooled, they were mounted in DPX mount having the optical index of glass (the sections were wetted in xylene and inverted on to the mountant placed on cover slip).

The architecture was observed at low power objective. The liver cell injury and other aspects were observed under high power dry objective.

**APPENDIX 31**

**SCREENING FOR SECONDARY METABOLITES**

*(Peach and Tracey, 1955)*

(a) Alkaloids *(Dragendorff's test)*

8 g of Bi (NO₃)₃ 5H₂O was dissolved in 20 ml of Nitric acid and 2.72g of Potassium iodide in 50 ml, of water. They were mixed and allowed to stand, when Potassium nitrate crystralyses out. The supernatant was decanted off and made upto
100ml with distilled water. The alkaloids were regenerated from the precipitate by treating with sodium carbonate followed by extraction of the liberated base with ether.

To 0.5 ml of alcoholic solution of *Glycyrrhiza glabra* added, 2.0 ml of HCl. To this acidic medium 1 ml of reagent was added. An orange red precipitate produced immediately indicates the presence of alkaloids.

**Wagner’s test (Iodine-Potassium-Iodide solution)**

1.0 g of iodine and 2.0 g of potassium iodide were dissolved in 5 ml sulphuric acid and solution was diluted to 100 ml. 10 ml of alcoholic extract of *Glycyrrhiza glabra* was acidified by adding 1.5% v/v HCl and a few drops of Wagner’s reagent. Formation of yellow or brown precipitate confirmed the presence of alkaloids.

**Meyer’s test (Potassium Mercuric iodide)**

1.36 g Mercuric chloride was dissolved in 60 ml of distilled water and 5 g of potassium iodide in 10 ml of water. The two solutions were mixed and diluted to 100 ml with distilled water.

To 1 ml of acidic aqueous solution of *Glycyrrhiza glabra*, few drops of reagent was added. Formation of white or pale precipitate showed the presence of alkaloids.

**(b) Flavonoids**

In a test tube containing 0.5 ml of alcoholic extract of the *Glycyrrhiza glabra*, 5-10 drops of dilute hydrochloric acid and small piece of Zn or Mg was added and the solution was boiled for few minutes. In the presence of flavonoids, reddish pink or dirty brown colour was produced.

**(c) Tannins**

**Ferric chloride test**

To 1.2 ml of an aqueous solution of *Glycyrrhiza glabra* few drops of 1% solution of lead acetate was added. A yellow or red precipitate was formed, indicating the presence of tannins.
(d) Saponins

In a tube containing about 5.0 ml of an aqueous extract of *Glycyrrhiza glabra* a drop of sodium bicarbonate was added. The mixture was shaken vigourously and kept for 3 minutes. A honey comb like froth was formed and it showed the presence of saponins.

(e) Glycosides

A small amount of alcoholic extract of *Glycyrrhiza glabra* was dissolved in 1.0 ml of water and then aqueous sodium hydroxide solution was added. Formation of a yellow colour indicates the presence of glycosides.

(f) Resins

To 2.0 ml of chloroform or ethanolic extract *Glycyrrhiza glabra* 5-10 ml of acetic anhydride was added, dissolved by gentle heating, cooling and then 0.5 ml of sulphuric acid was added. A bright purple colour was produced. It indicates the presence of resins.

(g) Phenols (Ferric Chloride test)

One ml of alcoholic solution of *Glycyrrhiza glabra* was diluted with water and followed by a few drops of 10% aqueous ferric chloride solution. Formation of blue or green colour indicates the presence of phenols.

Lead acetate test

1 ml of alcoholic solution of *Glycyrrhiza glabra* was diluted to 5 ml with distilled water and to this few drops of 1% aqueous solution of lead acetate was added. A yellow precipitate was formed which indicate the presence of phenols.

Libermann's test

A small quantity of alcoholic extract of the *Glycyrrhiza glabra* was dissolved in 0.5 ml of 20% sulphuric acid solution followed by the addition of a few drops of aqueous sodium nitrate solution. A red colour was obtained on dilution and it turned blue when made alkaline with aqueous sodium hydroxide solution.
(h) Thiols

To about 0.5 ml of plant extract enough ammonium sulphate was added to saturate the solution. 2-4 drops of 5% sodium nitroprusside was then added followed by one or more drop of concentrated nitric acid. No magenta colour was developed. This shows the absence of thiols.

(i) Steroids

Libermann-Burchard's test

To 1.0 ml of Glycyrrhiza glabra petroleum ether extract in chloroform, 1ml of concentrated sulphuric acid was added followed by the addition of 2.0 ml of acetic anhydride solution. A greenish colour developed and it turned blue. It indicated the presence of steroids.

Salkowski reaction

To 2.0 ml of chloroform extract of Glycyrrhiza glabra, 1.0 ml of concentrated sulphuric acid was added carefully along the sides of the test tube. A red colour was produced in the chloroform layer.

APPENDIX 32

QUANTITATIVE ESTIMATION FOR BIOCHEMICAL CONSTITUENTS
(Sadasivam and Manickam, 1992)

(a) Estimation of Total Carbohydrate

PRINCIPLE

Carbohydrate is first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This compound forms with anthrone a green coloured product with absorption maximum at 630 nm.

Reagents

1. **Glucose stock standard:** 100 mg of glucose was dissolved in 100 ml of water in a standard flask.
2. **Working standard:** 10 ml of the stock was diluted to 100 ml. 1.0 ml of this solution contains 100 μg of glucose.

3. **Anthrone reagent:** 0.2% anthrone was dissolved in ice cold concentrated sulphuric acid. Prepared fresh before use.

4. **2.5 N HCl.**

**PROCEDURE**

Weighed 100 mg of the sample into a boiling tube. Hydrolysed by keeping it in a boiling water bath for three hours with 5 ml of 2.5 N HCl and cooled to room temperature. Neutralised it with solid sodium carbonate until the effervescence ceases. Made up the volume to 100 ml and centrifuged. Collected the supernatant and take 0.2 to 1.0 ml for analysis. Prepared the standards by taking 0.2-1.0 ml of the working standards. 1.0 ml of water serves as a blank. Made up the volume to 1ml in all the tubes with distilled water. Then added 4 ml of anthrone reagent. Heated for eight minutes in a boiling water bath. Cooled rapidly and read the green to dark green colour at 630 nm.

**CALCULATION**

A standard graph was drawn by taking the concentration of glucose on X axis and spectrophotometer reading on Y axis. From the graph the concentration of glucose in the sample was calculated.

(b) **Estimation of Starch by anthrone reagent**

**PRINCIPLE**

The sample was treated with 80% alcohol to remove sugars and then starch was extracted with perchloric acid. In hot acidic medium starch is hydrolysed to glucose and dehydrated to hydroxymethyl furfural. This compound forms a green coloured product with anthrone.
MATERIALS
1. **Glucose stock standard:** 100 mg of glucose was dissolved in 100 ml of distilled water.

2. **Glucose working standard:** 10 ml of stock was diluted to 100 ml with distilled water.

PROCEDURE

Extracted 500 mg of sample repeatedly in hot 80% ethanol to remove sugars. Centrifuged and residue was retained. Residue was dried over a water bath. To the residue added 5.0 ml of water and 6.5 ml of 52% perchloric acid. Extracted at 0°C for 20 min. Centrifuged and supernatant was saved. Repeated the extraction using fresh perchloric acid. Centrifuged and pooled the supernatants and made up to 100 ml using distilled water. Pipetted out 75 to 375 μl of the supernatant and made up the volume to 1.0 ml with distilled water. Prepared the standards by taking 0.5 to 3.0 ml of the working standard and made up the volume to 3 ml in each tube with water. Added 4 ml of anthrone reagent to each tube. Heated for 8 min. Cooled rapidly and read the intensity of green to dark green colour at 630 nm.

CALCULATION

The glucose content in the sample was found out using a standard graph. The value was multiplied by factor 0.9 to arrive at the starch content.

(c) Estimation of Reducing Sugar (Miller, 1972)

PRINCIPLE

Several reagents have been employed which assay sugars by using their reducing properties. One such compound is 3,5, dinitrosalicylic acid which in alkaline solution is reduced to 3-amino-5-nitrosalicylic acid.

Reagents

1. **Dinitroaslicylic acid reagent (DNS reagent):**
   Dissolved by stirring 1 g of dinitrosalicylic acid, 200 mg of crystalline phenol and 50 mg of sodium sulphite in 100 ml of 1% NaOH. Stored at 4°C. Since the
reagent deteriorates due to sodium sulphite, if long storage is required, sodium
sulphite may be added at the time of use.

2. 40% Rochelle salt solution (potassium sodium tartarate).

3. **Glucose standard**: Dissolved 100 mg of glucose in 100 ml of distilled water.

4. **Working standard**: Diluted 20 ml of stock to 100 ml with distilled water
   (100 μg/ml).

**PROCEDURE**

Weighed 100 mg of the sample and extracted the sugars with hot 80% ethanol
twice (5 ml each time). Collected the supernatant and evaporated it by keeping it on a
water bath at 80°C. Added 5 ml of water and dissolved the sugars. Pipetted out 0.1 to
0.5 ml of the extract in test tubes and equalize the volume to 3 ml with water in all the
tubes. Added 3 ml of DNS reagent. Heated the contents in a boiling water bath for 5
min. When the contents of the tubes are still warm, add 1 ml of 40% Rochelle salt
solution. Cooled and read the intensity of dark red colour at 510 nm. Run a series of
standards using glucose (0 to 500 μg) and plotted a graph.

**CALCULATION**

Calculated the amount of reducing sugars present in the sample using the standard
graph.

(d) Estimation of Amino acids (Moore and Stein, 1948)

**PRINCIPLE**

Ninhydrin, a powerful oxidizing agent, decarboxylates the alpha-amino acids
and yields an intensely coloured bluish purple product which is colorimetrically
measured at 570 nm.

\[
\text{Ninhydrin} + \text{alpha-amino acid} \rightarrow \text{Hydridantin} + \text{Decarboxylated amino acid}
+ \text{Carbon dioxide} + \text{Ammonia}
\]

\[
\text{Hydridantin} + \text{Ninhydrin} + \text{Ammonia} \rightarrow \text{Purple coloured product} + \text{water}
\]
REAGENTS

1. **Ninhydrin**: Dissolved 0.8 g of stannous chloride (SnCl$_2$.2H$_2$O) in 500 ml of 0.2 M Citrate buffer (pH 5.0). Added this solution to 20 g ninhydrin in 500 ml of methyl cellosolve (2 methoxyethanol). Alternatively, acetone can also be used.

2. 0.2 M citrate buffer pH 5.0

3. **Diluent solvent**: Mixed equal volumes of water and n-propanol and used.

4. **Extraction of amino acids**: Weighed 500 mg of the plant sample and ground it in a pestle and mortar with a small quantity of acid-washed sand. To this homogenate, added 5 to 10 ml of 80% ethanol. Filtered or centrifuged. Saved the filtrate or supernatant. Repeated the extraction twice with the residue and pooled all the supernatants. Reduced the volume if needed by evaporation and use the extract for the quantitative estimation of total free amino acids. If the tissue is tough, use boiling 80% ethanol for extraction.

5. **Standard amino acids**: Dissolved 50 mg of leucine in 50 ml of distilled water in a volumetric flask. Took 10 ml of this stock standard and diluted to 100 ml in another volumetric flask for working standard solution.

PROCEDURE

A series of volumes from 0.2 to 1 ml of working standard solution giving a concentration range 20-100 µg was pipetted out. Prepared the reagent blank as above by taking 0.1 ml of 80% ethanol instead of the extract. Took 0.2 to 1.0 ml of extract. Added 1 ml of ninhydrin solution. Made up the volume to 2 ml with distilled water. Heated the tubes in a boiling water bath for 20 min. Added 5 ml of the diluent and mixed the contents. After 15 min read the intensity of the purple colour against a reagent blank in a colorimeter at 570 nm. The colour is stable for 1 hr.
APPENDIX 33

ESTIMATION OF TOTAL ALKALOIDS

Procedure

10 mg of plant material was homogenized in a mortar and pestle. Added around 20 ml of methanol : ammonia (68:2). Decanted the ammonical solution after 24 hrs and added fresh methanolic ammonia. Repeated the procedure thrice and pooled the extracts evaporated the extracts using a flash evaporator. Treated the residue with 1N HCl and kept it overnight. Extracted the acidic solution with 20 ml of CHCl₃ thrice, pooled the organic layers and evaporated to dryness, basic fraction (prserpine). Basified the acidic layer with conc. NaOH to pH-12 and extracted with CHCl₃ (20 ml) thrice, pooled the CHCl₃ layers, dry over absorbent cotton and evaporated to dryness. Weighed the fraction that contains ajmalicine and serpentine expressed as mg/100 g. (Harborne, 1973)

APPENDIX 34

EXTRACTION AND ESTIMATION OF FLAVONOIDS
(Cameron et al, 1993)

EXTRACTION

A Portion of the ground plant material was weighed out and extraction was carried out in two steps, firstly with MeOH : H₂O(1:1). At each step, sufficient solvent was added to make liquid slurry and the mixture was left for 6-12 hrs. Filtration to separate the extract from the plant material was carried out rapidly by using a glass wool or cotton wool plugged in the neck of a filter funnel. The two extracts were then combined and evaporated to about one third the original volume or until most of the MeOH has been removed. The resultant aqueous extract was cleared of low polarity contaminants such as fats, terpenes, chlorophylls and xanthophylls by extraction (in a separating funnel) with hexane or chloroform. This was repeated several times and the extracts obtained. The solvent-extracted aqueous layer containing the bulk of the flavonoids was then concentrated.

REAGENTS

1. Vanillin reagent -1% vanillin in 70% conc. H₂SO₄
2. Catechin standard -110 μg/ml
PROCEDURE

An aliquot of the extract was pipetted into a test-tube and evaporated to dryness. Then added 4 ml of vanillin reagent and heated for 15 minutes in a boiling water bath. A standard was also treated in the same manner. Then the optical density was read at 340 or 360 nm.

APPENDIX 35

ESTIMATION OF STEROIDS
(Zak’s Method)

PRINCIPLE

Steroids react with ferric chloride in the presence of concentrated sulphuric acid to give a pink color. The intensity of color developed is directly proportional to the amount of Steroids present and is read at 540 nm in a colorimeter.

REGENTS

1. Stock ferric chloride: 840 mg of pure dry ferric chloride was weighed and dissolved in 100 ml of glacial acetic acid.
2. Ferric Chloride precipitation reagent: 10 ml of stock ferric chloride reagent was taken in 10 ml of standard flask and made up to the mark with pure glacial acetic acid.
3. Ferric chloride diluting reagent: 8.5 ml of stock ferric chloride was diluted to 100 ml with pure glacial acid.
4. Standard solution: 100 mg of Steroids was dissolved in 100 ml of glacial acetic acid.
5. Working standard: 10 ml of stock was dissolved in 0.85 ml of stock ferric chloride reagent and made up to 100 ml with glacial acetic acid. The concentration of working standard is microgram/ml.

PROCEDURE

To 0.1 ml of plant extract, added 4.9 ml of ferric chloride precipitating reagent. Centrifuged and to 2.5 ml of supernatant, added 2.5 ml of ferric chloride diluting agent. Added 4.0 ml of concentrated sulphuric acid. A blank was prepared simultaneously by taking 5.0 ml of diluting reagent and 4.0 ml of concentrated sulphuric acid. A set of standards (0.5-2.5 ml) were taken and made up to 5.0 ml with ferric chloride diluting reagent. Then added 4.0 ml of concentrated sulphuric acid. After 30 minutes, the
intensity of color developed was read at 540 nm against a reagent blank. The amount of Steroids in the sample is expressed as mg/dl.

APPENDIX 36

ESTIMATION OF TANNINS
(Folin-Denis Method)

PRINCIPLE

Tannin-like compounds reduce phosphotungstomolybdic acid in alkaline solution to produce a highly colored blue solution, the intensity of which is proportional to the amount of tannins. The intensity is measured in a spectrophotometer at 700 nm.

MATERIALS

1. Folin-Denis Reagent
   Dissolved 100g sodium tungatate and 20g phosphomolybdic acid in 750 ml distilled water in a suitable flask and added 50 ml of phosphoric acid. Refluxed the mixture for 2hr and made up to one litre with water. Protect the reagent from exposure to light.

2. Sodium Carbonate Solution
   Dissolved 350g sodium carbonate in one litre of water at 70-80°C. Filtered through glasswool after allowing it to stand overnight.

3. Standard Tannic acid solution: dissolved 100mg tannic acid in 100ml of distilled water.

4. Working Standard Solution
   Dissolved 5ml of the stock solution to 100ml with distilled water. One ml contains 50g tannic acid.

PROCEDURE

   Extraction of Tannin: Weighed 0.5g of the powdered material and transferred to a 250ml conical flask. Add 75ml water, heated the flask gently and boiled for 30min. Centrifuged at 2,000 rpm for 20min and collect the supernatant in 100 ml volumetric flask and made up the volume. Transferred 1ml of the sample extract to a 100ml
volumetric flask containing 75ml water. Added 5ml of Folin-Denis reagent, 10ml of sodium carbonate solution and diluted to 100ml with water, Shook well. Read the absorbance at 700nm after 30min. If absorbance was greater than 0.7, make a 1:4 dilution of the sample. Prepared a blank with water instead of the sample. Prepared a standard graph using 0-100µg tannic acid.

CALCULATION

Calculate the tannin content of the samples as tannic acid equivalents from the standard graph.

APPENDIX 37

ESTIMATION OF TOTAL PHENOLS
(Sadasivam and Manickam, 1992)

PRINCIPLE

Phenols react with phosphomolybdic in Folin –ciocalteau reagent in an alkaline medium and produce a blue coloured complex (molybdenum blue) that can be estimated colorimetrically at 650 nm.

REAGENTS

1. 80% ethanol
2. Folin-ciocalteau reagent
3. 20% sodium carbonate
4. Stock standard: Dissolved 100 mg of Catechol in 100 ml distilled water.
5. Working standard: Diluted 12 ml of the stock to 100 ml with distilled water. 1.0 ml of this solution contains 100 µg of Catechol.
6. Sample preparation: weighed 0.5 to 1.0 g of the sample and ground it with a mortar and pestle in 10 X volume of 80% ethanol. Centrifuged the homogenate at 10,000 rpm for 20 minutes. Saved the supernatant. Re-extracted the residue with five time the volume of 80% ethanol. Centrifuged and pooled the supernatants. Evaporated the supernatant to dryness. Dissolved the residue in a known volume of distilled water.
PROCEDURE

Pipetted out 0.1 ml of sample into test tubes. Made up the volume to 3.0 ml with distilled water. Added 0.5 ml of Folin-Ciocalteau reagent. After 3 minutes added 2.0 ml of 20% sodium carbonate. Mixed thoroughly, placed the tubes in boiling water bath for exactly one minute, cooled and diluted the blue solution to 25 ml with water. Read the absorbance at 650 nm against a reagent blank. A set of standards were also treated in the above manner. The amount of phenol was expressed as mg/ g tissue.

APPENDIX 38
COLUMN CHROMATOGRAPHY

REAGENTS
1. Benzene
2. Hexane
3. Anhydrous sodium sulphate
4. Silica gel
5. Ethyl acetate
6. Samples

PROCEDURE
Silica gel was used as an adsorbent; 10 g of silica gel was weighed and made into a slurry by adding sufficient quantity of hexane. A little quantity of glass wool was already cleaned with hexane and plugged into the bottom of the column tube. This acts as a pad. In the column, the slurry of silica gel was carefully added, while pouring more hexane. It was added so that the adsorbent does not dry. The top of the column was loaded with 2 g of activated sodium sulphate. 20 ml of hexane was added to the top of the column and was allowed to pass through the column. When the solvent was 5.0 cm just above the column material, the stop cork was closed. Already dissolved plant material was transferred into the top of the column and stop cork was opened. When the solvent reached the top of the column material, 30 ml of hexane was added and 5 ml fractions were collected. The samples were eluted successively with solvents of increasing polarity in the order of hexane, benzene and ethyl acetate (Harborne, 1973).
APPENDIX 39

THIN LAYER CHROMATOGRAPHY

MATERIALS

1. Glass plate (20x20 cm or 20x10 cm)
2. Glass tank with lid
3. Spreader
4. Adsorbent silica gel G
5. Mobile phase Ethylacetate: Formic acid. H₂O (8:1:1)
6. Spraying reagent Folin’s Phenol reagent

PROCEDURE

Coated the slurry (1:2) over the glass plates at a thickness of 0.25 mm and allowed to dry at room temperature for 15 -30 minute. Heated the plates in an oven at 100-120°C for 1-2 hr to remove the moisture and to activate the adsorbent on the plate. The column eluted was applied at 2.5 cm from one end of the glass plate. Allowed the sample to dry so that spotting can be done repeatedly for a more concentrated sample spot. Poured the developing solvent into the tank to a depth of 1.5 cm. After equilibration, removed the cover plate, and placed the thin layer plate (sample applied) vertically in the tank so that it stands in the solvent with the spotted end dipping in the solvent. The separation of the compounds occurs as the solvent moves upward. Once the solvent reaches the top of the plate, removed it from the tank, dried and sprayed with spraying reagent for the identification of the separated compounds. The samples produced dark blue colour with the spraying agent (Harborne, 1973).