CHAPTER 4

MATERIAL AND METHODS
EXPERIMENTAL ANIMALS: Albino Wistar rats weighing 180 to 250gms were obtained from animal house of Shri B M Patil Medical college Hospital & Research Centre, BLDE University, Bijapur. All the five group animals were acclimatized for 7 days to the laboratory conditions at 22-24°C and maintained 12 HR. Light/dark cycle. All the experimental procedures were performed in accordance with the approval of the Institutional Animal Ethics Committee (IAEC) of Shri B M Patil Medical college Hospital & Research Centre, Bijapur.

All the care has been taken on animals during experimental as well as at the time scarification as per the guidelines of ICMR (Indian Council of Medical Research) on animal research 2006. An experiment was performed according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), INDIA. After completion of the experimental protocol, animals were sacrificed by cervical dislocation and incinerated electrically\(^1\).

AUTHENTICATION OF DRUGS:

Fresh fruits of *Emblica Officinalis (Amla)* and Bark of *Terminalia Arjuna* were collected from the local market in Bijapur and obtained the botanical authentication of these drugs in the Dept of Botany, KCP Science College, Bijapur.

PERMISSION EXTRACTION OF DRUGS:

Obtained permission for ethanolic extraction of *Emblica Officinalis (Amla)* and *Terminalia (Arjuna)* drugs from the Department of Pharmacology BLDEA College of Pharmacy Bijapur

EXTRACTION OF DRUG:

Ethanolic extract preparation: Powder of dry bark of *Terminalia Arjuna* and powder of dry fruits of *Emblica Officinalis (Amla)* was extracted with 99% ethanol using a soxhlet
apparatus at a temperature below 60°C for 22 hours. The solvent was evaporated under vacuum which gave semisolid mass with respect to the dried powder.

Percent of Yield was calculated as follows:

\[
\text{Extract yield \%} = \frac{W_1}{W_2} \times 100
\]

Where, \(W_1\) = Net weight of powder in grams after extraction and \(W_2\) = Total weight of wood powder in grams taken for extraction\(^2\).

Extraction of drugs by Soxhlet apparatus

**Phytochemical Screening**

Different extracts were screened for the presence of phenols, flavonoids, tannin, saponin, alkaloids, glycosides, phytosterols and carbohydrate by using standard protocols\(^3\).

**Phytochemical Investigations:**

**Chemical and reagents:** Laboratory grade chemicals were used for routine work. Analytical grade reagents (A.R.) were used for analytical work

**Absorbents:** Silica gel GF254 (RFCL Ltd Renkem production, New Delhi) was used for TLC

**Preliminary Phytochemical studies:** The crude extract obtained by extraction from ethanolic extraction was subjected phytochemical studies
A. Detection of Carbohydrates

Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

1. Molisch’s test: To 1 ml of test solution added a few drops of 1 % alpha-napthol and 2-3 ml concentrated sulfuric acid along the side of test tube. The reddish violet or purple ring formed at the junction of two liquids confirmed the test.

2. Benedict’s test: Filtrates were treated with Benedict’s reagent and heated on a water bath. Formation of an orange red precipitate indicates the presence of reducing sugars.

3. Fehlings test: Dissolved 2 mg dry extract in 1 ml of distilled water and added 1ml of Fehling’s (A+B) solution, shooked and heated on a water bath for 10 minutes. The brick red precipitate formed confirmed the test.

B. Detection of fixed oils & fats

1. Stain Test: Small quantities of extracts were pressed between two filter papers. An oily stain on filter paper indicates the presence of fixed oil.

C. Detection of phytosterols

1. Salkowski’s Test: Extracts were treated with chloroform and filtered. The filtrates were treated with a few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.

2. Libermann Burchard’s test: Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Conc. Sulphuric acid was added carefully along the sides of the test tube. Formation of brown ring at the junction indicates the presence of phytosterols.

D. Detection of glycosides

Extracts were hydrolysed with dil. HCl, and then subjected to test for glycosides.
1. Modified Borntrager’s Test: Extracts were treated with a ferric chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and shaken with an equal volume of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammoniacal layer indicates the presence of anthranol glycosides.

E. Detection of flavonoids

1. Alkaline Reagent Test: Extracts were treated with a few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

2. Lead acetate Test: Extracts were treated with a few drops of lead acetate solution. Formation of a yellow colour precipitate indicates the presence of flavonoids.

3. Shinoda Test: To the alcoholic solution of extracts, a few fragments of magnesium ribbon and Conc. HCl was added. Appearance of magenta colour after few minutes indicates presence of flavonoids. Alcoholic solution of extracts, a pinch of zinc dust and conc. HCl was added. Appearance of magenta colour after few minutes indicates presence of flavonoids.

F. Detection of alkaloids

Approximately 50 mg of extract was dissolved in 5 ml of distilled water. Further 2M hydrochloric acid was added until an acid reaction occurred and filtered. The filtrate was tested for the presence of alkaloids as detailed below. Extracts were dissolved individually in dilute hydrochloric acid and filtered. The filtrates were used to test for the presence of alkaloids.

1. Mayer’s Test: Filtrates were treated with mayer’s reagent (Potassium Mercuric iodide). Formation of a yellow, cream precipitate indicates the presence of alkaloids.
2. Wagner’s Test: Filtrates were treated with wagner’s reagent (Iodine in potassium iodide).
   Formation of brown/reddish brown precipitate indicates the presence of alkaloids.

3. Dragendorff’s Test: To 2 ml of the filtrate was added 1 ml of dragendorff’s reagent along the side of the test tube. Formation of orange or orange reddish brown precipitate indicated the test as positive.

4. Hager’s Test: Filtrates were treated with Hager’s reagent (saturated picric acid solution).
   Formation of a yellow coloured precipitate indicates the presence of alkaloids.

G. Detection of saponins

1. Froth Test: Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

2. Foam test: Small amount of extract was shaken with little quantity of water. If foam produced persists for ten minutes it indicates the presence of saponins.

3. Olive oil test: - Added a few drops of olive oil to 2ml of the test solution and shaken well.
   The formation of a soluble emulsion confirmed the test.

H. Detection of resins

1. Acetone-water Test: Extracts were treated with acetone. Small amount of water was added and shaken. Appearance of turbidity indicates the presence of resins.

I. Detection of phenols

1. Ferric Chloride Test: Extracts were treated with few drops of ferric chloride solution.
   Formation of bluish black colour indicates the presence of phenols.

J. Detection of tannins

1. Ferric chloride Test: Added a few drops of 5% ferric chloride solution to 2 ml of the test solution. Formation of blue colour indicated the presence of hydrolysable tannins.
2. Gelatin Test: Added five drops of 1% gelatin containing 10% sodium chloride to 1 ml of the test solution. Formation of white precipitates confirmed the test.

STUDY DESIGN

**Experimental protocol:** All the rats were divided into following five groups with 6 rats in each group. Group-I, fed Iso-caloric diet and serve as a control, the Group II Fed with high fat diet, Group-III fed with high fat diet with ethanolic extract of *Terminalia Arjuna (Arjuna)*, Group IV fed with high fat diet with ethanolic extract of *Emblica Officinalis (Amla)* and Group V fed with high fat diet with ethanolic extract of *Terminalia Arjuna and Emblica Officinalis (Amla)*. It was given daily *Terminalia Arjuna (Arjuna)* 500mg/kg B². wt and *Emblica Officinalis(Amla)* 100mg/kg B.Wt, I.P. for three weeks

<table>
<thead>
<tr>
<th>Rats Group</th>
<th>Normal &amp; Hyperlipidemic</th>
<th>Food Dosages</th>
<th>Dosages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>Terminalia Arjuna (Arjuna)</em></td>
<td><em>Emblica Officinalis (Amla)</em></td>
</tr>
<tr>
<td>Group1. Normal diet</td>
<td>a. Carbohydrate 62%.</td>
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<tr>
<td></td>
<td>b. Protein 18%.</td>
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<td>c. Fat 20%.</td>
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<td></td>
<td>d. Salt 4%.</td>
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<td>e. Multi vitamins 1%</td>
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<tr>
<td>Group 2. Hyperlipidemic diet</td>
<td>a. Carbohydrate 52%.</td>
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<tr>
<td></td>
<td>b. Protein 18%.</td>
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<td></td>
<td>c. Fat 30%.</td>
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<td></td>
<td>d. Salt 4%.</td>
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<td></td>
<td>e. Multi vitamins 1%</td>
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</tr>
<tr>
<td>Group 3. Hyperlipidemic diet</td>
<td>a. Carbohydrate 52%.</td>
<td>100mg/200gm</td>
<td>--</td>
</tr>
<tr>
<td>Treated with <em>Terminalia Arjuna</em></td>
<td></td>
<td>of Rats. For 3 weeks</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b. Protein 18%.</td>
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<tr>
<td></td>
<td>c. Fat 30%.</td>
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<td></td>
<td>d. Salt 4%.</td>
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<td></td>
<td>e. Multi vitamins 1%</td>
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<td>Group 4. Hyperlipidemic diet</td>
<td>a. Carbohydrate 52%.</td>
<td>--</td>
<td>100mg/Kg</td>
</tr>
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<td>For 3 weeks</td>
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<tr>
<td></td>
<td>b. Protein 18%.</td>
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<td></td>
<td>c. Fat 30%.</td>
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<td>d. Salt 4%.</td>
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<td></td>
<td>e. Multi vitamins 1%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 5. Hyperlipidemic diet</td>
<td>a. Carbohydrate 52%.</td>
<td>100mg/200gm</td>
<td>100mg/Kg</td>
</tr>
<tr>
<td>Treated with <em>Terminalia Arjuna</em> and <em>Emblica Officinalis (Amla)</em></td>
<td></td>
<td>of Rats. For 3 weeks</td>
<td>For 3 weeks</td>
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</table>
PREPARATION OF ISO-CALORIC AND HYPERLIPIDEMIC DIET

PREPARATION OF ISO-CALORIC DIET:

Normal diet (Group 1) 1kg of Iso-caloric diet contains 620gms of carbohydrate, 180gms of protein, 200gms of fat, 2gms of Na+ and 1% of multivitamins.

In this group each rat fed with Iso-caloric diet per day on and average 50gms. We observed that all rats consumed not more than 40gms. Each rat consumed 35gms instead of 40gms, the left over 5gms of food discarded and next day fresh 40gms of food was served5.

PREPARATION OF HYPERLIPIDEMIC DIET:

Hyperlipidemic diet (Group 2 to 5) 1kg of hyperlipidemic diet contains 520gms of carbohydrate, 180gms of protein, 300gms of fat, 2gms of Na+ and 1% of multivitamins.

In these groups, each rat fed with hyperlipidemic diet per day on and average 50gms. We observed that few of rats consumed more than 45gms of diet and a few of them consumed less than 40gms of diet6.
Sample collection

Every alternate week (six rats) one group of animals was sacrificed by cervical dislocation at the end of the last dose with an overnight fast. Blood was collected in normal tubes for the separation of serum, by doing retro-orbital puncture, before sacrificing the animals.

Cervical dislocation method

1. Restrain the rodent in a normal standing position on a firm, flat surface and grasp the base of the tail firmly with one hand. Performing the procedure on a surface that the animal can grip (such as the wire bar grid of the cage top) may make it easier to gain access to the base of the skull because rodents often stretch themselves forward when held by the tail.

2. Place a sturdy stick-type pen, a rod-shaped piece of metal, closed scissors/hemostats or the thumb and first finger of the other hand against the back of the neck at the base of the skull.

3. To produce the dislocation, quickly push forward and down with the hand or object restraining the head while pulling backward with the hand holding the tail base.

4. The effectiveness of dislocation can be verified by feeling for a separation of cervical tissues. When the spinal cord is severed, a 2-4 mm space will be palpable between the occipital condyles and the first cervical vertebra. Occasionally, however, the dislocation occurs between thoracic vertebrae.

5. Check closely to confirm respiratory arrest, and when possible verify, by palpation, that there is no heart beat.
HAEMATOLOGY

After the experimental period blood was collected from the rats by retro orbital venous puncture and blood was collected in heparinized tubes and non heparinized tubes. Haematological parameters were evaluated by XS-1000i closed tube sampling sysmax automated analyzer (Calibrated by Sysmex division of MYCO Instrumentation Ltd).

Measurement principle:

RBC/PLT- sheath flow direct current on XE and XT series. WBC-semiconductor laser flow cytometry, HGB-clorometric method (SLS).

Testing parameters CBC and S-Part differential 21 parameters like Hb% RBC WBC Platelet PCV & MCHC were analyzed.

LIPID PROFILE

Serum triglycerides (TG), Serum total cholesterol (TC), High-density lipoprotein (HDL), Low-density lipoprotein (LDL) and Very Low-density lipoprotein (VLDL) were analysed.

i) Total cholesterol

**Principle:** Enzymatic determination of total cholesterol was performed according to the following equation

\[
\text{Cholesterol ester} + \text{H}_2\text{O} \xrightarrow{\text{Esterase}} \text{Cholesterol} + \text{Fatty acids},
\]

\[
\text{Cholesterol} + \text{O}_2 \xrightarrow{\text{Esterase}} 4\text{-Cholesten-3-one} + \text{H}_2\text{O}_2
\]

2 \text{H}_2\text{O}_2 + \text{Phenol} + 4\text{-aminoantipyrene} \text{Red quinine} + 4\text{H}_2\text{O}

**Cholesterol reagent**

Pipes buffer, pH 6.7- 50 mmol/l

Phenol- 24 mmol/l

Sodium cholate- 0.5 mmol/l

4- aminoantipyrene- 0.5 mmol/l
Material and Methods

Cholesterol esterase > 180U/l

Cholesterol oxidase > 200U/l

Peroxidase > 1000U/l

Cholesterol standard solution 200 mg/dl

The reagent is stable for 18 months when stored at 2-8° C. Animal serum was used as the sample. 10 μl of serum was mixed with 1000 μl of reagent, incubated for 5 min at 37 °C and estimated at 630 nm using a Biochemical Analyzer.

Calculation

\[
\text{Cholesterol (mg/dl)} = \frac{\text{Absorbance of sample} \times 200}{\text{Absorbance of standard}}
\]

ii) Triglycerides

Principle: Enzymatic determination of triglycerides was performed according to the following equation:\(^9\).

\[
\begin{align*}
\text{Triglyceride} + \text{H}_2\text{O} & \longrightarrow \text{Glycerol} + \text{Fatty acids} \\
\text{Glycerol} + \text{ATP} & \longrightarrow \text{glycerol-3-phosphate} + \text{ADP} \\
\text{Glycerol-3-PO}_4 + \text{O}_2 & \longrightarrow \text{dihydroxy acetone phosphate} + \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + 4\text{- aminoantipyrine} + \text{p-chlorophenol} & \longrightarrow \text{Red quinoneimine}
\end{align*}
\]

Reagent Composition

Pipes buffer, pH 7.0-50 mmol/l

p-chlorophenol - 5.3 mmol/l

Potassium ferrocyanate - 10 mmol/l

Magnesium salt - 17 mmol/l

4- aminoantipyrine - 0.9 mmol/l

ATP - 3.15 mmol/l

Lipoprotein lipase > 1800 U/l

Glycerol kinase > 450 U/l
Glycerol-3- phosphate oxidase > 3500 U/l

Peroxidase > 450 U/l

Triglyceride standard solution- 200 mg/dl

The reagent was stable for 18 months when stored at 2-8° C. Animal serum was used as the sample. 10 μl of serum was mixed with 1000 μl of reagent, incubated for 5 min at 37 °C and estimated at 630 nm using a biochemical analyzer.

**Calculation:**

\[
\text{Triglyceride (mg/dl)} = \frac{\text{Absorbance of sample} \times 200}{\text{Absorbance of standard}}
\]

**iii) HDL cholesterol**

**Principle:** The chylomicrons, VLDL and LDL of serum were precipitated by phosphotungstic acid and magnesium ions. After centrifugation, HDL in the supernatant solution was measured by enzymatic method\(^\text{10}\).

**HDL cholesterol reagent**

Phosphotungstate- 14 mmol/l

Magnesium chloride- 1 mmol/l

**Preservative**

HDL cholesterol standard- 50 mg/dl.

Animal serum was used as the sample. 300 μl of serum was mixed with 300μl of HDL reagent, allowed to stand for 10 min at room temperature, mixed again and centrifuged for 10 min at 4000 rpm. After centrifugation the clear supernatant was separated from the precipitate within 1hr and HDL was determined using cholesterol reagent. 50 μl of supernatant was mixed with 1000 μl of cholesterol reagent, incubated for 5 min at 37 ° C and estimated at 630nm using a biochemical analyzer.
Calculation

\[
\text{HDL cholesterol conc. (mg/dl)} = \frac{\text{Absorbance of sample} \times N \times 2}{\text{Absorbance of standard}}
\]

N= Standard concentration (50 mg/dl)

iv) Estimation of LDL

**Formula:** \( \text{LDL} = \frac{\text{TC}}{1.19} + \frac{\text{TG}}{1.9} - \text{HDL} \times 1.1 - 38 \) (mg/dl)\(^\text{11}\).

v) Estimation of VLDL

**Formula**

\[
\text{VLDL} = \frac{\text{triglycerides}}{5} \text{ (mg/dl)}\(^\text{11}\).
\]

1. LIVER FUNCTION TEST:

i) Assessment of liver function test

Serum was separated by centrifuging blood at 2500 rpm for 10 minutes and the levels of SGOT, SGPT, bilirubin, ALP, albumin, A/G ratio and total protein were analyzed by using a commercially available enzymatic kit (AGAPPE, India) and an autoanalyser (Chemistry Analyser (CA 2005), B4B Diagnostic Division, China).

i) Estimation of Serum Glutamate Pyruvate Transaminases (SGPT/ ALT)

Principle: Alanine aminotransferase catalyses the transfer of amino group from alanine to 2-oxoglutarate, resulting in the formation of pyruvate and glutamate. The catalytic concentration is determined from the rate of decrease of NADH, measured at 340 nm, by means of lactate dehydrogenase coupled reaction\(^\text{12}\).

The enzymatic reaction employed in the assay of SGPT is as follows.

\[
\text{L- Alanine} + 2\text{-oxoglutarate } \xrightarrow{\text{ALT}} \text{ Pyruvate} + \text{L- Glutamate}
\]

\[
\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{D- Lactate} + \text{NAD}^+
\]

**Reagent Preparation: Reagent A:** Tris 150 mmol/l, L- Alanine 750 mmol/l, lactate dehydrogenase >1350U/l, pH 7.3. **Reagent B:** NADH 1.3 mmol/l, 2-oxoglutarate 75 mmol/l, sodium hydroxide 148 mmol/l, sodium azide 9.5 g/l. Auxillary Reagent **Reagent C:**
Pyridoxal phosphate 10 mmol/l. Working Reagent: Reagent A (4 parts) is mixed with 1 part of Reagent B. The combined reagent was stable for 2 months at 2-8° C. The mixed reagent was stored in a dark place and protected from light. Working Reagent with Pyridoxal phosphate: 10ml of working reagent was mixed with 0.1 ml of reagent C. The solution was stable for 6 days at 2-8° C.

Procedure: Animal serum was used as the sample. 50 µl of serum was mixed with 1000 µl of mixed reagent and estimated in kinetic mode using a Biochemical Analyzer.

Calculations

\[
\text{SGPT/ ALT concentration (U/l )} = \frac{\text{delta } A/\text{min } \times Vt \times 10^6}{E \times L \times Vs}
\]

Molar absorbance (E) of NADH at 340 nm is 6300

L - Light path 1cm

Vt - Total reaction volume is 1.05 at 37° C

Vs - Sample volume is 0.05 at 37° C

ii) Estimation of Serum Glutamate Oxaloacetate Transaminases (SGOT/ AST)

Principle: Aspartate aminotransferase catalyzes the transfer of the amino group from aspartate to 2-oxoglutarate, forming oxaloacetate and glutamate. The catalytic concentration is determined from the rate of decrease of NADH, measured at 340nm, by means of malate dehydrogenase (MDH) coupled reaction12.

The enzymatic reaction employed in the assay of SGOT is as follows.

\[
\begin{align*}
\text{2-oxoglutarate } & \text{ Glutamate} + \text{ Oxaloacetate} \\
\text{Oxaloacetate} + \text{ NADH+ H+ D-Malate} & \text{ + NAD+}
\end{align*}
\]

Reagent Preparation: Reagent A: Tris 121 mmol/l, L- aspartate 362 mmol/l, malate dehydrogenase >460 U/l, lactate dehydrogenase > 660 U/l, sodium hydroxide 255 mmol/l, pH 7.8. Reagent B: NADH 1.3 mmol/l, 2-oxoglutarate 75 mmol/l, sodium hydroxide 148 mmol/l, sodium azide 9.5 g/l.
Auxillary reagent – Reagent C: Pyridoxal phosphate 10 mmol/l.

Working reagent: Reagent A (4 parts) is mixed with 1 part of Reagent B. The combined reagent is stable for 2 months at 2-8° C. The mixed reagent was stored protected from light.

Working reagent with pyridoxal phosphate: 10ml of working reagent was mixed with 0.1 ml of Reagent C. Stable for 6 days at 2-8° C. Animal serum was used as the sample.

Procedure: 50 µl of serum was mixed with 1000 µl of mixed reagent and estimated in kinetic mode using a biochemical analyzer.

Calculations:

\[ \text{SGOT/ AST concentration (U/l)} = \frac{\text{delta A/min} \times V_t \times 10^6}{E \times L \times V_s} \]

Molar absorbance (E) of NADH at 340nm is 6300

L - Light path 1cm

Vt - Total reaction volume is 1.05 at 37° C

Vs - Sample volume is 0.05 at 37° C

iii) Estimation of Alkaline Phosphatase

Principle: Alkaline Phosphatase catalyses in alkaline medium the transfer of phosphate group from 4-nitrophenyl phosphate to 2-amino-2-methyl-1-propanol, liberating 4-nitrophenol. The catalytic concentration is determined from the rate of 4-nitrophenol formation, measured at 405nm\(^3\).

The enzymatic reaction employed in the assay of alkaline phosphatase is as follows.

4- Nitrophenyl phosphate + H\(_2\)O \[\xrightarrow{\text{ALP}}\] Phosphate + 4- Nitrophenol

Reagents: Reagent A: 2- Amino-2- methyl- 1- propanol 0.4 mol/l

Zinc sulphate 1.2 mmol/l

N hydroxy ethylene diamine tri aceticacid 2.5 mmol/l

Magnesium acetate 2.5 mmol/l, pH 10.4.

Reagent B: 4- Nitrophenyl phosphate 60 mmol/l.
Working reagent: 4 parts of reagent A is mixed with 1 part of reagent B. The combined reagent is stable for 2 months at 2-8° C. Animal serum was used as the sample 20 µl of serum was mixed with 1000 µl of mixed reagent and estimated in kinetic mode using a biochemical analyzer.

Calculations

\[
\text{ALP concentration (U/l)} = \frac{\text{delta A/min x Vt X 10}^6}{E X L X Vs}
\]

Molar absorbance (E) of NADH at 405nm is 18450

L- Light path 1cm

Vt - Total reaction volume is 1.02 at 37° C

Vs - Sample volume is 0.02 at 37° C

iv. Total bilirubin

**Principle:** Direct bilirubin in the sample reacts with diazotised sulfanilic acid forming a coloured complex that can be measured by spectrophotometry. Both direct and indirect bilirubin couple diazo in the presence of cetrimide\(^{14}\).

The terms direct and total refer to the reaction characteristics of serum bilirubin in the absence or presence of solubilizing reagents. The direct and indirect bilirubin is approximately equivalent to the conjugated and unconjugated fractions.

**Composition** (Bilirubin)

**Reagent A:** Sulfanilic acid 29 mmol/l

Hydrochloric acid 0.2 mol/l

Cetrimide 50 mmol/l

**Reagent B:** Sodium nitrite- 11.6 mmol/l. Stored at 15-30° C. Reagents were stable until the expiry date shown on the label when stored tightly closed and if contaminations was avoided during use. Presence of particulate matter, turbidity, absorbance over 0.05 at 540nm, indicate deterioration.
Working Reagent preparation: Mixture of 1 ml of Reagent B and 4 ml of Reagent A. This was Stable for 20 days at 2-8° C.

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Reagent Blank</th>
<th>Sample Blank</th>
<th>Sample</th>
<th>Standard</th>
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</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>100 µl</td>
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</tr>
<tr>
<td>Sample</td>
<td>--</td>
<td>100 µl</td>
<td>100 µl</td>
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<td>Reagent A</td>
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<td>1000 µl</td>
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<tr>
<td>Working Reagent</td>
<td>1000 µl</td>
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</tbody>
</table>

Mixed thoroughly and was allowed to stand for 2 min at room temperature. Absorbance of Sample Blank was read at 540nm against distilled water and absorbance of Sample was read at 540nm against reagent blank.

Calculations

Bilirubin content in the sample = \( \frac{A \text{ (sample)} - A \text{ (sample blank)}}{A \text{ (standard)}} \times C \text{ (standard)} \)

Mass concentration (mg/dl) x 17.1 = Substance concentration in µmol/l.

v) Total protein estimation

**Principle:** The enzymatic reaction sequence employed in the assay of total protein was as follows:

Protein + Cu2+ Cu – Protein complex

Total proteins were estimated using Total protein reagent from AGAPPE Diagnostics, Kerala, India.¹⁵

**Composition of Total protein reagent**

Potassium iodide- 6 mmol/l. Potassium sodium tartrate- 21 mmol/l. Copper sulphate- 6 mmol/l. Sodium hydroxide- 58 mmol/l. Total Protein Standard- 6 g/dl.

The reagent was stable for 18 months when stored at 2-8° C. To 20 µl of serum, 1ml of total protein reagent was added and mixed. The mixture was incubated at 37°C for 15 minutes and the absorbance was measured at 546 nm using a biochemical analyzer.

The protein content was calculated by using the following formula and expressed as total protein in g/dl.
Total protein in g/dl = \frac{\text{Absorbance of sample} \times C}{\text{Absorbance of standard}}

Where C refers to the protein concentration in standard protein solution in g/dl.

vi) **Albumin estimation**

**Principle:** The reaction between albumin in serum or plasma and the dye bromocresol green produces a change in colour, which is proportional to albumin concentration\(^{16}\).

**Reagent Composition**

- **Albumin reagent:** Succinate buffer (pH 4.2) 75 mmol/l. Bromocresol green 0.14 g/l.
- **Albumin Standard:** Albumin Standard concentration 3 g/dl. The reagent is stable for 18 months when stored at 2-8°C. Animal serum was used as sample. 10 μL of serum was mixed with 1000 μL of reagent, mixed and incubated for 1 minute. The absorbance was measured against blank at 630 nm.

**Calculation**

\[
\text{Albumin (g/dl)} = \frac{\text{Absorbance of sample} \times C}{\text{Absorbance of standard}}
\]

Where C refers to the albumin concentration in standard albumin solution in g/dl

2. **SERUM ELECTROLYTE**

Serum Na\(^+\) (Sodium), K\(^+\) (Potassium) and Ca\(^{++}\) (Calcium) were analysed by Meril Diagnostic Kit Method: Merilyzer Cliniquant easylyte analyzer

3. **GLUCOSE ESTIMATION:** Random blood sugar glucose oxidase method

**Principle:** The substrate β-D-glucose is oxidized by glucose oxidase to from gluconic acid and hydrogen peroxide. The hydrogen peroxide so generated oxidizes the chromogen system consisting of 4-aminoantipyrine and phenolic compound to a red quinoeimine dye. The intensity of the colour produced is proportional to the glucose concentration and is measured at 505 nm (490-530 nm) or with green filter\(^{17}\).
Glucose + O2 $\rightarrow$ Gluconic acid + H2O2

\[
\text{Oxydase} \quad \text{H2O2+Phenolic compound+4-Aminoantipyrine} \rightarrow \text{Red Quinoemine+H2O}
\]

**Kit contents** Reagent1: Glucose reagent. Reagent 2: Glucose standard (For calibration)

**Procedure for estimation of glucose**

<table>
<thead>
<tr>
<th>Pipetted into microcentrifuge tubes</th>
<th>Blank (μl)</th>
<th>Standard (μl)</th>
<th>Test (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose Reagent</td>
<td>500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Calibrator (Standard)</td>
<td>--</td>
<td>5</td>
<td>--</td>
</tr>
<tr>
<td>Sample (Serum)</td>
<td>--</td>
<td>--</td>
<td>5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>5</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Mixed and incubated at 37°C for 10 minutes. Absorbance of the Test (AT), Standard (AS) and Reagent Blank (AB) at 505 nm was read against distilled water using biochemical analyzer.

**Calculations:** Glucose (mg/dl) = (AT-AB/AS-AB) x100

Where 100 = Standard concentration of Glucose (mg/dl)\(^{18}\).

4. **ESTIMATION OF NITRIC OXIDE:** Method: Griess method (Kinetic cadmium reduction)

Nitric oxide was determined as nitrite in serum by a kinetic cadmium reduction method by Najwa cortas and Nabil wakid\(^{19}\).

**Principle:** Nitrate the stable product of nitric oxide is reduced to nitrite by cadmium reduction method after deproteinization of sample by somogyi reagent. The nitrite produced is determined by diazotization with sulphanilamide and coupling to Nnaphthylethlenediamine. The intensity of coloured complex is measured at 540 nm.

**Reagents:**

1. Cadmium granules (2.5 – 3 gram granules in assay, stored in 0.1M/L H2SO4)
2. Glycine–NaOH buffer (pH 9.7): 7.5 gm of glycine was dissolved in 200ml deionised water, then the pH was adjusted to 9.7 by 2M NaOH and was diluted to 500 ml by deionised water.
3. Sulfanilamide: 2.5 gm of sulfanilamide was dissolved in 250 ml of warm 3M/L HCl and allowed to cool.

4. N-naphthylethylenediamine: 50 mg of N-naphthylethylenediamine was dissolved in deionised water and the volume was adjusted to 250 ml.

5. Stock standard sodium nitrite solution (0.1 mol/L): 690 mg sodium nitrite was dissolved in 100 ml of 10 mmol/L of sodium borate solution.

6. Working standard solution (10 µmol/L): 10µl of stock was diluted to 100 ml with 10mmol/L solution of sodium borate.

7. ZnSO4 solution (75 mmol/L)

8. NaOH solution (55 mmol/L)

9. H2SO4 solution (0.1 mol/L)

10. CuSO4 solution (5 mmol/L): 125 mg of CuSO4 was dissolved in 100 ml of glycine - NaOH buffer.

**Procedure: Part I:** - Deproteinization: A centrifuge tube was taken and additions were made as follows:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum.</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>75 mM ZnSO4</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>55 mM NaOH</td>
<td>2.5 ml</td>
</tr>
</tbody>
</table>

Tube was centrifuged at the speed of 1500 rpm for 10 min. and supernatant was collected.

**Part II:** Activation of Cadmium granules

1. Cadmium granules were stored in 0.1 mol/L H2SO4 solution.

2. At the time of assay the acid from granules was rinsed three times with deionized water.

3. Then the granules were swirled in 5 mmol/L CuSO4 solution for 1-2 minutes.

4. These copper coated granules were drained and washed by glycine - NaOH buffer.

5. These activated granules were used within 10 minutes after activation.
6. The granules after use were washed by deionized water and stored in 0.1 mol/L H2SO4 solution. Same procedure for activation was followed each time.

**Part III**: Nitrite assay a set of three test tubes was arranged as follows and respective additions were made as follows.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Test</th>
<th>Standard</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine-NaOH Buffer</td>
<td>500 µl.</td>
<td>500 µl.</td>
<td>500 µl.</td>
</tr>
<tr>
<td>Supernatant</td>
<td>500 µl.</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Standard 10 µmol/L</td>
<td>--</td>
<td>500 µl.</td>
<td>--</td>
</tr>
<tr>
<td>D/W</td>
<td>--</td>
<td>--</td>
<td>500 µl.</td>
</tr>
<tr>
<td>Cadmium granules</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Cadmium granules were swirled and tubes were kept at RT for 90 min.

D/W                        | 1.0 ml. | 1.0 ml. | 1.0 ml. |

Content from all this tube was mixed well and diluted solutions were taken in following tubes

| Above diluted solutions   | 1.0 ml. | 1.0 ml. | 1.0 ml. |
| Sulfanilamide            | 0.5 ml. | 0.5 ml. | 0.5 ml. |
| N-naphtylethylendiamine  | 0.5 ml. | 0.5 ml. | 0.5 ml. |

After 20 minutes waiting all tubes were read at 540 nm.

**Calculation**: Serum Nitrite (µm/L) = (T-B) ÷ (S-B) X 100
HISTOLOGY

Fixation of tissues:

The fixation of tissues in 10% of formalin

Dissection and Fixation of Tissues:

Rats were carefully dissected. Taking midline incision opened the anterior of the chest wall and neck. The thoracic cage opened and muscles were separated and the collected the blood directly from the heart and heart was removed. Then it was weighed immediately and fixed in 10% formalin. The heart was cut into pieces and fixed in bouins medium for 24 hrs. After fixation, it was placed in 70% alcohol for 6-8 hours during the day, then in 90% alcohol for overnight. Next day three changes of absolute alcohol were given for one hour each.

The tissue was blotted with blotting paper and placed in xylene for about 30 min for clearing. Then tissue was subjected to 3 changes of paraffin wax at 56°C temp 60°C for one hour each. Then tissue was embedded. ‘L’ shaped moulds were smeared with glycerine and fresh filtered wax poured into it to fill it almost. Any air bubbles formed were removed by hot spatula. Then the tissue was fixed on one side of the mould and label was placed on the opposite side of the mould. After a skin of wax has formed completely over the surface of the block, its solidification was hastened by careful immersion in cold water, for 15 min. Then the block was removed from the mould.

The blocks were prepared for cutting. Finally tissue was cut in the sizes of 5 to 7 μ in the form of a ribbon.

The individual sections were gently lowered onto the surface of water at 5°C to 10°C to remove the folds. The sections were taken on egg albumin coated slides. Slides were kept for drying on a hot plate at 45°C - 50°C for 2 hours or more as per requirement.
Tissue collection:

Tissue collection for histopathology: after proper dissection of the animal heart (Atrium & Ventricle), aorta and muscular artery was isolated immediately and fixed in 10% neutral buffered formalin solution for 24 hours. The fixed tissues were processed routinely and then embedded in paraffin, sectioned to 3–5 µm thickness, de-paraffinized, and rehydrated using standard techniques. The extent of hyperlipidemic (high fat diet) induced variations was evaluated by assessing morphological changes in the heart (Atrium & Ventricle), aorta and muscular artery sections stained with hematoxylin and eosin (H and E), using standard techniques.
**Staining preparation:**
1. Removal of paraffin wax was done by dipping into xylene one or two min in each of two changes of xylene.
2. Removal of xylene was done by dipping into two changes of absolute alcohol for one half to one min each.
3. Then it was followed by treatment for a minute or two with 90% alcohol and then 70% alcohol.
4. After this, slides were kept under running tap water for about 5 minutes.
5. Then stained in haematoxylin for about 10-15 min and again kept under tap water for 5 min.
6. Excess stain was removed by dipping into acid alcohol for a few seconds. Here the blue colour was changed to red because of acid.
7. The blue colour (bluing) was regained by washing in alkaline, running tap water for 5-10 min. The stain was checked.
8. The slides were stained by eosin for 3-5 min, surplus stain was washed off in water.
9. Dipping into ascending grades of alcohol did the dehydration of slides and clearing was done by two changes of xylene for about 1 min each.
10. The slides were mounted with DPX and cover slips were applied. The slides were kept at room temperature for some hours to allow, firm adhesion of the cover slip to the section.
11. The slides were observed under light microscope for microscopic differentiation and the photographs were taken.
12. Few slides, were stained by verhoeffs stain technique.

**VERHOEFF’S SPECIAL STAIN**

This stain is useful in demonstrating atrophy of elastic tissue in cases of emphysema, and the thinning and loss of elastic fibres in arteriosclerosis, and other vascular diseases.

**PRINCIPLE:** The tissue is stained with a regressive hematoxylin, consisting of ferric chloride and iodine. The differentiating is accomplished by using excess mordant (ferric chloride) to break the tissue-mordant dye complex. The dye will be attracted to the larger amount of mordant in the differentiating solution and will be removed from the tissue. The
elastic tissue has the strongest affinity of the ironhematoxylin complex and will retain the dye longer than the other tissue elements.

**CONTROL:** skin.

**FIXATIVE:** Any well fixed tissue.

**TECHNIQUE:** Cut paraffin sections 4µ or 5µ.

**EQUIPMENT:** Rinse glassware in DI water: coplin jars, graduated cylinders

**REAGENTS:** Alcohol Hematoxylin:

- Hematoxylin 5.0 gm
- Absolute alcohol 100.0 ml
  
Dissolve hematoxylin into alcohol with the aid of gentle heat, do not boil. Label with date and initials, solution are stable for 1 year.

**CAUTION:** Flammable, avoid contact and inhalation.

**10% Ferric Chloride:**

- Ferric chloride 10.0 gm
- Distilled water 100.0 ml mix well. Label with date and initials, solution are stable for 1 year.

**CAUTION:** Avoid contact and inhalation

- Verhoeff's Hematoxylin:
- Alcoholic hematoxylin 20.0 ml
- 10% ferric chloride 8.0 ml
- Lugol's iodine 8.0 ml
  
Add in the order given, mixing between additions. Make fresh, discard.

Caution: Avoid contact and inhalation.

**Differentiating Solution:**

- (2% Ferric Chloride) 10% ferric chloride 10.0 ml
Distilled water 40.0 ml make fresh, discard.

CAUTION: Avoid contact and inhalation 5% Hypo: See stock solutions

PROCEDURE:

1. Deparaffinize and hydrate to distilled water.
2. Verhoeff’s hematoxylin for 30 minutes (save solution until stain is completed)
3. Wash in tap water.
4. Differentiate in 2% ferric chloride solution, check microscopically for black fibers on a gray background.
5. Rinse in water.
6. Hypo for 1 minute to remove iodine.
7. Wash in water.
8. Counterstain in van gieson's for 5 minutes.
9. Dehydrate, clear in xylene, and coverslip.

RESULTS: Elastic fibers and nuclei black collagen red other tissue elements yellow\textsuperscript{22,23&24}. 

![Image of laboratory equipment]
VASCULAR INTEGRITY PARAMETERS

Morphometric study

Elastic arterial wall thickness
  ➢ Tunica intima and media

Muscular arterial wall thickness
  ➢ Tunica intima and media

Coronary arterial wall thickness

Lumen diameter
  ➢ Anteroposterior
  ➢ Transverse
  ➢ Arterial lumen

Normalized wall index

**Morphometry of Arterial Wall Thickness, Lumen Diameter and Calibration:**

**Procedure of Arterial Wall Thickness measurement in Histological:**

Morphometry of arterial wall thickness & lumen diameter

➢ Arterial wall Thickness and its lumen diameter was measured by using Digimizer image analyzer version 4.3.0 copyright 2005-2014 Medclac software

➢ 8 megapixel Picture in 10X and 40X of microscopic (MIPS) and calibrated with Digimizer image and measured the thickness of arterial wall

➢ Whereas arterial lumen diameter was measured in 4X

Digimizer version 4.3.0 copyright C 2005-2014 Medclac software and following things were used

1. Strengthen the image.

2. Calibrate measurement

3. Performed the measured manually like: Length, Area, Centre, Unit, Area etc
4. Analyse the object Average Intensity defined using the circle tool, area tool, rectangle tool digimizer can calculate the average intensity

Normalized wall index of coronary artery

- **Normalized Wall Index**
  - The outer and inner vessel wall counters were manually traced for the coronary artery using the digimizer image analyzer software.
  - Wall area, lumen area, and total vessel area were automatically calculated based on the counters drawn by the software program.
  - The normalized wall index was calculated by dividing the wall area by the total vessel area.

\[
\frac{\text{Wall area}}{\text{Total Vessel Area}} = \text{Normalized wall Index}^{25}.
\]

Before manual tracing the counter on arterial wall the 40X of microscopic image of the artery was calibarated with digimizer image analyzer.

**DESCRIPTION OF CAMERA:** ZEN 2 core package hardware license key with Axiocam 105 color (D) consisting of the camera Axiocam 105 color and the ZEN 2 core basic software plus the ZEN module image analysis camera specification: microscopy camera with driver software 64bit, USB 3.0 PCIe x1 interface, USB 3.0 connection cable 3.0 m, SATA power cable and molex power cable Sensor: Aptina CMOS color sensor basic resolution: 2560 (H) x 1920 (V) = 5.0 mega pixels color pixel size: 2.2 μm x 2.2 μm chip size: 5.70 mm x 4.28 mm, equivalent to 1/2.5” (diagonal 7.1 mm) Live frame rates (depending on hardware and software configuration): H x V binning factor frame rate@1ms 2560 x 1920 1 15 1280 x 960 2 37 640 x 480 4 47 frame rates for time series recording (depending on hardware and software configuration): H x V binning factor frame rate@1ms 2560 x 1920 1 8 1280 x 960 2 16 640 x 480 4 19 readout of sensor sub- regions ("ROI"): 
Adjustable digitization: 3 x 8 Bit / Pixel integration Time: 100 µs up to 2 s interface (camera): USB 3.0 Micro-B Interface (PC / board): USB 3.0 standard a spectral range: Approx. 400 nm - 650 nm, IR filter read-out mode: Progressive optical interface: C-Mount (adapter 0.5x recommended) Size / Weight: approx. 2.9 cm x 2.9 cm x 4.76 cm / 50 g Housing: Aluminum, magnesium, stainless steel registration: CE, FCC class B, RoHS power supply: via USB 3.0 interface, max. 1.7 W (5 V, 0.34 A)\textsuperscript{26}.

MIPS (Magnified Image Processing System)

Environmental conditions: 0° ... +50° celsius, max. 80% relative air humidity, no condensation, free air circulation required Supported operating systems: Windows 7 x64 Prof./Ultimate SP1
REFERENCE:


STATISTICAL ANALYSIS
STATISTICAL ANALYSIS:

- Values are expressed as Mean ± SD.
- To determine the significance between 5 groups, One Way ANOVA was applied.
- If one way ANOVA shows significant difference, then Post Hoc test was used to find the difference between two groups.
- Statistical significance was established at P≤0.05.
- Data were analysed by using SPSS software version 16.