Chapter 3

MATERIALS & METHODS
3. MATERIALS AND METHODS

3.1. Test plant (Aporosa lindleyana Baill.)

*Aporosa* (Anne M Schot, 1998) is a genus of about 80 species of small trees which inhabit the tropical rain forests of South East Asia. Six species are found in the remnant forest of South India and Sri Lanka; the others are found from the Himalaya southwards to the Solomon Islands. Borneo, with 30 species, of which 10 are endemic, and New Guinea, with probably up to 20 endemic specimen, many of which are still poorly known, are the main centres of diversity.

*Aporosa lindleyana* (Figure 4), Vettil/Kodali in Tamil, belonging to the family of *Euphorbiaceae*, Sub family of Phyllanthoideae. There are 110 taxa, including 82 species, 8 varieties, 6 forms, 7 poorly known species and a multiple outgroup. *Euphorbiaceae* comprised of 280 genera and 730 species with the largest genus *Euphorbia* having about 1600 species, it grows as small middle sized tree up to 15 m height, found throughout India and Sri Lanka (Anonymous, 1985, Kirtikar and Basu, 1993) particularly on western ghats region. The roots of *Aporosa lindleyana* was presented in Figure 5.

**Plant taxonomy**

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sub-kingdom</td>
<td>Viridaeplantae</td>
</tr>
<tr>
<td>Class</td>
<td>Magnoliopsida</td>
</tr>
<tr>
<td>Subclass</td>
<td>Dilleniidae</td>
</tr>
<tr>
<td>Order</td>
<td>Euphorbiales</td>
</tr>
<tr>
<td>Family</td>
<td>Euphorbiaceae</td>
</tr>
</tbody>
</table>
Figure 4. Habitat of *Aporosa lindleyana*
Figure 5. Root of *Aporosa lindleyana*
3.1.1. PLANT COLLECTION

*Aporosa lindleyana* (Family: *Euphorbiaceae*) root were collected from Keeriparai, Kanyakumari District, Tamil Nadu, India in the month of January. The plant was identified and authenticated by Dr. V. Chelladurai, taxonomist, Tirunelveli, Tamil Nadu. The specimen was prepared and deposited (Voucher No. SPKCDBT001) in the Department of Biotechnology, Sri Paramakalyani College, Alwarkurichi, Tirunelveli District, Tamil Nadu.

3.1.2. PREPARATION OF ETHANOLIC ROOT EXTRACT OF *Aporosa lindleyana* (EREAL)

*Aporosa lindleyana* root were shade dried and finely powdered. 100gm of the powder was extracted in soxhlet apparatus using 250 mL of ethanol for 48 hours. The extract was collected separately. The extract was concentrated using rotaevaporator under reduced pressure. A dark semisolid material (yield-14%) obtained was stored at 0-4°C until use.

**Reagents**

Isoniazid and Rifampicin were purchased from Sigma-Aldrich. Thiobarbituric acid (TBA), phenazinemethosulphate (PMS), nitroblue tetrazolium (NBT), 5,5'-dithiobis2-nitrobenzoic acid (DTNB), L-glutamyl-p-nitroanilide, α-tocopherol, 2,2’-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), (ABTS+), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid), butylated...
hydroxytoluene (BHT) and other chemicals and solvents were of analytical grade and purchased from S.D. Fine Chemicals, Mumbai and Himedia Laboratories Private Limited, Mumbai, India.

3.2. PHYTOCHEMICAL SCREENING OF Aporosa lindleyana

The preliminary phytochemical screening (Kolkate C K, 1996, Craig W J, 1997, Khandelwal K.R. 2005) was carried out for the quality of various organic compounds present in the effective crude root extracts of chloroform, methanol and ethanol.

Steroid (Liebermann- Burchard test)

With 0.5mL of EREAL in chloroform is treated with a few drops of acetic acid and acetic anhydride and two drops of concentrated H₂SO₄ along the sides of the test tube and heated gently. Development of blue or green colour indicates the presence of steroid.

Triterpenoids (Noller’s test)

With 0.5mL of EREAL in a dry test tube is treated with a bit of tin foil and 0.5ml of thionyl chloride and heated gently. Development of pink colour indicates the presence of triterpenoid.

Sugars/glycosides

With 1.0mL of EREAL is mixed with equal quantity of anthrone and treated with two drops of concentrated H₂SO₄. Heated gently on a water bath. Development of dark green colour indicates the presence of sugar/glycosides.

Aromatic acids

With 0.5mL of EREAL is treated with aqueous NaHCO₃. Effervescence showed the presence of aromatic acids.
Flavonoids (Shinoda test)

With 1.0mL of EREAL in alcohol is treated with magnesium foils and a few drops of concentrated HCl. Development of red or pink colour indicates the presence of flavonoids.

Furanoids (Ehrlich test)

With 0.5mL of EREAL in alcohol is treated with a pinch of paradimethyl amino benzaldehyde and a few drops of concentrated HCl. Development of red or pink colour indicates the presence of furanoids.

Tannin

With 0.5mL of EREAL in alcohol is treated with a few drops of aqueous basic lead acetate. A white precipitation indicates the presence of tannin.

Alkaloid (Dragendorff’s test)

With 0.5mL of EREAL in acetic acid is treated with two drops of Dragendorff reagent (potassium mercuric iodide). Development of red or orange precipitation indicates the presence of alkaloid.

Phenol

With 1.0mL of EREAL in alcohol is treated with alcoholic natural ferric chloride. Intense colouration indicates the presence of phenolic compounds.

Xanthoprotein Proteins

With 0.5mL of EREAL is treated with 1.0mL of 5% CuSO₄ and 1% NaOH. Development of deep blue colour indicates the presence of proteins.
3.3. SEPARATION AND IDENTIFICATION OF *Aporosa lindleyana*

3.3.1. PAPER CHROMATOGRAPHY PATTERN

This is the most widely used procedure (Morris & Morris, 1963) for the separation and identification of organic compounds. It is referred as Paper chromatography because filter paper is used as the supporting medium.

0.05ml of EREAL was spotted in the Whatmann No.1 filterpaper (Ascending pattern) and dried the paper and place in the different solvent system such as petroleum ether +chloroform and chloroform. After the incubation time, i.e., the solvent front reaches maximum zone, the paper was taken from the chamber and kept in the incubator for drying and the paper kept in the iodine chamber and $R_f$ was calculated.

$$R_f = \frac{\text{Distance travelled by the sample}}{\text{Distance travelled by the solvent}}$$

3.3.2. THIN LAYER CHROMATOGRAPHY PATTERN (TLC)

The TLC (Stahl, 1964) is used to identify the various phytochemicals present in the given extract. The solute which is most soluble in mobile phase has capacity to migrate through a chromoplate fastly than comparatively less soluble solutes. In other words, solutes move on the chromoplate at different rates depending on their concentration in the mobile phase. The TLC works on the difference in solubility.

The cold ethanol extract of EREAL was used for the analysis of TLC behavior. The TLC plate of commercially available 100 mesh silica gel coated in an aluminium sheet was considered and draw a line with pencil about 1cm from the bottom. 0.05ml of EREAL was spotted using microsyringe at 1-1.5cm distance
between them. Chromatographic tank is filled with the developing solvent of following.

a) Petroleum ether (100%)
b) Chloroform (100%)
c) Chloroform: Acetone (9.5:0.5)
d) Pet ether : Chloroform (1:1)
e) Benzene: Chloroform (1:1)

Thin layer plates were placed gently into the tank and allowed to stand for 15'. After developing the chromatogram, the plate were removed and the solvent front is marked with pencil immediately and allowed to air dry by placing the plate upside down.

The separated component can be detected by incubate the plate in a desicator containing a few crystals of iodine in a beaker. The Rf value at different solvent system of EREAL extract in systems were calculated by using the formula.

\[
R_f = \frac{\text{Distance travelled by the sample}}{\text{Distance travelled by the solvent}}
\]

3.3.3. TOTAL PHENOL AND FLAVONOIDS

Total Phenol

0.5 mL of freshly prepared EREAL was taken and diluted with 8 ml of disillled water. 0.5 ml of Folin Ciocalteu reagent (1N) was added and kept at 40 °C for 10min. 1.0 mL of sodium carbonate (20%) was added and kept in dark for one hour. The color was read at 650nm using shimadzu UV-1650 spectrophotometer (Malick et al, 1980). The same procedure was repeated for all standard Gallic acid
solution and standard curve obtained. The sample concentration was calculated as Gallic acid Equivalent (GE).

**Total Flavonoids**

0.5 mL of EREAL is diluted with 3.5 mL of distilled water at zero time and 0.3 mL of 5% sodium nitrate was added to the tubes. After 5 minutes, 0.3 mL of Aluminium chloride (10%) was added to all the tubes. At the 6th min. 2 ml of sodium hydroxide (1M) was added to the mixture. Immediately the contents of the reaction mixture were diluted with 2.4mL of distilled water and mixed thoroughly. Absorbance of the mixture was determined at 510 nm versus a prepared blank immediately. Gallic acid was used as the standard compound for quantification of total flavonoids as mg/100 g (Zhisen et al., 1999).

### 3.3.4. IDENTIFICATION OF COMPOUNDS BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY (GC–MS)

**GC–MS Condition**

GC–MS was performed with Hewlett–Packard (HP) 6890/5973 equipment (Japan). Mass spectrometer in the EI (Electron Impact) mode with the electron energy set at 70 eV and the mass range at m/z 100–700 (170). Compounds were separated on a 30 m × 0.25 mm capillary column coated with a 0.25 μm film of HP-5-MS (J&W Scientific, Folsom, CA, USA). Samples of BSTFA derivatized and non-derivatized were injected with a split ratio of 50:1; nitrogen was used as carrier gas at 1.0 mL min−1. The column temperature was maintained at 100 °C for 1 min after injection then increased at 10 °C min−1 to 275 °C which was sustained for 20 min. The time required for chromatography of EREAL was 40 min.
3.4. ANTIBACTERIAL ACTIVITY

Disc Diffusion Method

The paper disc diffusion method was used to determine antibacterial activity which is based on the method (Lina deodhar, 1998). Muller Hinton agar inoculated with bacteria (200 μL bacteria suspension in 20 mL of medium) was poured into petridishes to give a solid plate. Sterile paper disc (6 mm) were loaded with different concentrations (10, 20, 30, 40, 50μL/disc) of EREAL and applied to the petridishes. The plates were incubated at 37° C for 24 hours. Incubation zone diameter around each of the discs were measured and recorded at the end of the incubation time.

The antibacterial activity of EREAL was investigated against gram positive bacteria viz., Staphylococcus aureus and gram negative bacteria viz., Klebsiella pneumoniae and Escherichia coli. The stock cultures were maintained on nutrient agar medium at 4° C. In vitro antibacterial activity was determined by using Mueller Hinton Agar and Mueller Hinton Broth and they were obtained from Himedia Ltd., Mumbai.

3.5. QUANTITATIVE SCREENING OF BIOMOLECULES

Biochemical studies reveals the information on the presence of biomolecules in the EREAL. In the present study, biomolecules such as carbohydrate and protein molecules were analysed.

3.5.1. DETERMINATION OF CARBOHYDRATE (GLUCOSE) – ANTHRONE METHOD

Carbohydrates are dehydrated by Conc. H₂SO₄ to form furfural complex. This complex reacts with Anthrone reagent (Varley et al., 1980) to form a blue coloured complex which were measured colorimetrically at 620 nm.
Reagents required

1. Standard glucose – 10 mg/mL

2. Anthrone reagent

0.2-1.0 mL of standard glucose solution were pippetted out into a series of the test tubes, which are labeled as S1, S2, S3, S4, S5, which are made up to 1.0 mL using distilled water. 1.0 mL of EREAL taken in a test tube which are labeled as T (Test). 1.0 mL of water are taken in a test tube, which are labeled as B (Blank). 4.0 mL of Anthrone reagent was added to all the test tubes. The formation of blue colored complex, which were measured colorimetrically at 620 nm.

The absorbance of the EREAL was calculated by using the following formula

\[
100\text{mL of EREAL} = \frac{\text{Test OD} - \text{Standard OD}\#}{\text{Conc. of Standard} \times 100} \times \text{Standard OD}
\]

# - Optical Density.

The amount of glucose was expressed as mg/dL of EREAL.

3.5.2. DETERMINATION OF PROTEIN – MODIFIED BIURET METHOD

It is based on the fact that CO-NH groups of proteins form a purple coloured complex with copper ions in an alkaline medium.(Varley et al., 1980) The colored complex, which were measured colorimetrically at 520 nm. Previously the test samples is treated with cold TCA, because of its role in precipitation of proteins. Ethyl ether, remove excess TCA from the unknown solution.

Reagents

1. Bovine Serum Albumin – 5 mg/mL

2. Biuret reagent
3. 10% TCA

4. Ethyl ether

0.2 -1.0 mL of working standard solutions were pipetted in to the series of test tubes, which are labeled as S1, S2, S3, S4, S5. The Test (T) tube contains 1.0 mL of EREAL and made up the total volume of all to 1.0 mL by using distilled water. 1.0 mL of 10% TCA was added to all the tubes and centrifuged all for 10 min and the supernatant was decanted out and 2.0 mL of ethyl ether was added to all the tubes. Recentrifuged all the tubes and the supernatant was decanted out and 6.0 mL of biuret reagent was added to all the tubes. All the tubes were incubated at 37° C for 10 minutes and cool all the tubes. The absorbancy was read at 520 nm.

\[
100\text{mL of EREAL} = \frac{\text{Test OD} - \text{Standard OD}}{\text{Conc. of Standard}} \times 100
\]

The amount of protein was expressed as mg/dL of EREAL

3.6. In vitro FREE RADICAL SCAVENGING ASSAYS

Numerous antioxidant methods and modifications have been proposed to evaluate antioxidant activity and to explain how antioxidants function. Of these, active oxygen species such as OH', H2O2 and O2** quenching assays, DPPH*, ABTS** radical scavenging assays and reducing power assay are most commonly used for the evaluation of antioxidant activities.

Standard Antioxidants

The standard antioxidants used in the study were namely Ascorbic acid, alpha tocopherol, and Butylated Hydroxy Toluene (BHT). Ascorbic acid was dissolved in double distilled water. α-tocopherol and BHT were dissolved in methanol.
A required quantity of EREAL was suspended in 1% CMC (Carboxy Methyl Cellulose). Then the resultant suspension was taken and used for evaluating the antioxidant activity.

3.6.1. HYDROXYL RADICAL SCAVENGING ASSAY

The hydroxyl radical scavenging activity of EREAL was determined by the method (Halliwell et al., 1987). In this assay, hydroxyl radicals are produced by the reduction of \( \text{H}_2\text{O}_2 \) by the transition metal (iron) in the presence of ascorbic acid. The generation of hydroxyl radical is detected by its ability to degrade deoxyribose to form products, which on heating with TBA forms a pink colour chromogen. Addition of EREAL competes with deoxyribose for hydroxyl radicals and diminishes the colour formation.

**Reagents**

- a. Potassium phosphate buffer - 100mM, pH 6.7
- b. Ferric chloride - 500 \( \mu \)M in buffer
- c. Ascorbic acid - 1 mM in buffer
- d. EDTA - 1mM in buffer
- e. \( \text{H}_2\text{O}_2 \) - 10 mM
- f. 2-Deoxyribose - 15 mM in buffer
- g. Thiobarbituric acid (TBA) - 1% in 0.05N sodium hydroxide
- h. Trichloro acetic acid (TCA) - 28% in water

The incubation mixture in a total volume of 1 mL contained 0.1 mL of buffer, varying volumes of EREAL (10, 20, 30, 40 and 50 \( \mu \)g), 0.2 mL of ferric chloride, 0.1 mL of ascorbic acid, 0.1 mL of EDTA, 0.1 mL of \( \text{H}_2\text{O}_2 \) and 0.2 mL of 2-deoxyribose. The contents were mixed thoroughly and incubated at room
temperature for 60 min and then added 1 mL of TBA and 1 mL of TCA. All the tubes were kept in a boiling water bath for 30 min. The absorbance of the supernatant was read in a spectrophotometer at 535 nm with reagent blank containing water in place of EREAL. The efficiency of EREAL was compared with various concentrations (10, 20, 30, 40 and 50 μg) of standard ascorbic acid. Decreased absorbance of the reaction mixture indicated increased hydroxyl radical scavenging activity. The percentage of scavenging was calculated as shown below:

\[
\% \text{ Scavenging} = \frac{\text{Control OD} - \text{Test OD}^\#}{\text{Control OD}} \times 100
\]

# OD – Optical Density

3.6.2. SUPEROXIDE ANION SCAVENGING ACTIVITY

Superoxide anion scavenging activity of EREAL was determined by the method (Nishimiki et al., 1972) with modifications. The assay was based on the oxidation of NADH by phenazine methosulphate (PMS) to liberate PMS\(_{\text{red}}\). PMS\(_{\text{red}}\) converted oxidized nitroblue tetrazolium (NBT\(_{\text{oxi}}\)) to the reduced form (NBT\(_{\text{red}}\)), which formed a violet colour complex. The colour formation indicated the generation of superoxide anion, which was measured spectrophotometrically at 560 nm. Decrease in the formation of colour after addition of the antioxidant was a measure of its superoxide radical scavenging activity.

**Reagents**

- a. Methanol
- b. Phosphate buffer - 100mM, pH 7.4
- c. Nitroblue tetrazolium (NBT) - 100mM in phosphate buffer
d. Reduced nicotinamide adenine dinucleotide (NADH) - 100mM in phosphate buffer
e. Phenazine methosulphate (PMS) - 100mM in phosphate buffer

To 1 mL of NBT, 1 mL of NADH solution and varying volumes of EREAL (10, 20, 30, 40 and 50 μg) were added and mixed well. The reaction was started by the addition of 100μL of PMS. The reaction mixture was incubated at 30°C for 15 min. The absorbance was measured at 560 nm in a spectrophotometer. Incubation with water in place of EREAL was used as blank. BHT was used as standard for comparison. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage scavenging was calculated as shown below:

\[
\% \text{ Scavenging} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100
\]

3.6.3. DPPH' RADICAL SCAVENGING ASSAY

The radical scavenging activity of EREAL against DPPH* was determined spectrophotometrically by the method (Brand Williams et al., 1995).

DPPH* is a stable free radical and accepts an electron, or hydrogen radical to become a stable diamagnetic molecule. DPPH* reacts with an antioxidant compound that can donate hydrogen and gets reduced. The change in colour (from deep violet to light yellow) was measured. The intensity of the yellow colour depends on the amount and nature of radical scavenger present.

Reagent

a. 2,2-Diphenyl-1-picryl hydrazyl (DPPH*)—in 0.1mM in methanol.
The following reagents were added in the order stated below: The reaction mixture in a total volume of 3 mL contained 1 mL of DPPH*, various concentrations of \textit{EREAL} (10, 20, 30, 40 and 50 \(\mu\)g) and made up to 3 mL with water. The tubes were incubated for 10 min at 37 °C. A blue colour chromophore was formed, the absorbance of which was measured at 517 nm. Vitamin-E were used as standards for comparison.

\[
\% \text{ Scavenging} = \left(\frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}}\right) \times 100
\]

\textbf{3.6.4. EVALUATION OF TOTAL ANTIOXIDANT ACTIVITY – (ABTS** RADICAL CATION DECOLOURIZATION ASSAY)}

The generation of the ABTS** radical cation forms the basis of one of the spectrophotometric methods that has been applied for the measurement of the total antioxidant activity of solutions of pure substances (Wolfenden \textit{et al.}, 1982). The improved technique for the generation of ABTS** described here involves the direct production of the blue/green ABTS** chromophore through the reaction between ABTS** and potassium persulphate. Addition of EREAL competes with ABTS** diminishes the colour formation.

\textit{Reagents}

\begin{itemize}
  \item a. 2,2’-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) – (ABTS**) - 7 mM
  \item b. Potassium persulphate – 5 mM
  \item c. Phosphate buffer - 100 mM, pH 7.4
\end{itemize}

ABTS** was dissolved in water at a concentration of 7 mM. The stock solution was mixed with 2.45 mM potassium persulphate (final concentration). The mixture
was allowed to stand in the dark at room temperature for 12-16 h before use for incomplete oxidation of ABTS\(^{\cdot}\). The radical was stable in this form for more than two days when stored in the dark at room temperature. The following reagents were added in the following order. The incubation mixture in a total volume of 5 mL contained 0.54 mL of ABTS\(^{\cdot}\), 0.5 mL of phosphate buffer and varying concentrations of EREAL (50, 100, 150, 200 and 250 \(\mu\)g). The blank contained water in place of EREAL. The absorbance was read in spectrophotometer at 734 nm and compared with standard BHT at various concentrations (50, 100, 150, 200 and 250 \(\mu\)g).

3.6.5. REDUCING POWER

The reducing power of EREAL was determined by the method (Oyaizu M, 1986). Substances which have reduction potential react with potassium ferricyanide (Fe\(^{3+}\)) to form potassium ferrocyanide (Fe\(^{2+}\)), which then reacts with ferric chloride to form ferric-ferrous complex that has an absorption maximum at 700 nm. Increase in the reduction of ferric to ferrous ion increases the absorbance indicating the reducing ability of EREAL.

Reagents

- a. Phosphate buffer - 0.2 M, pH 6.6
- b. Potassium ferricyanide - 1% in water
- c. Trichloroacetic acid (TCA) - 10% in water
- d. Ferric chloride - 0.1 w/v in water

Varying concentrations of EREAL (50, 100, 150, 200, 250 \(\mu\)g) in double distilled water was mixed with 2.5 mL of phosphate buffer and 2.5 mL of potassium ferricyanide. The mixture was incubated at 50°C for 20 min after which, 1.5 mL of
TCA was added and centrifuged at 3000 g for 10 min. From all the tubes, 0.5 mL of supernatant was mixed with 1 mL of distilled water and 0.5 mL of ferric chloride. The absorbance was measured at 700 nm in a spectrophotometer. BHT was used as standard for comparison. Increased absorbance of the reaction mixture indicated increasing reducing power. Incubation with water in place of additives was used as the blank.

3.7. PHARMACOLOGY

3.7.1. EXPERIMENTAL ANIMALS

Healthy adult male albino Wistar rats, bred and reared in Central Animal House, Department of Pharmacology, K.M College of Pharmacy, Uthangudi, Madurai, were used for the experiment. Weight matched animals (180-200g) were selected and housed in polypropylene cages layered with husk and kept in a semi-natural light/dark condition (12 h light/12 h dark). The animals were allowed free access to water and standard pellet diet (Amrut Laboratory Animal Feed, Pranav Agro Industries Ltd., Bangalore, India). Animal handling and experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC Registration Number: 661/02/c/CPCSEA) and animals were cared in accordance with the “Guide for the care and use of laboratory animals” and “Committee for the purpose of control and supervision on experimental animals” (CPCSEA).

EXPERIMENTAL DESIGN

3.7.1.1. PRELIMINARY STUDY

Preliminary study was carried out to determine the optimum dose of EREAL by assessing serum hepatic marker enzyme activities in hepatotoxic rats. EREAL was given at different doses (i.e. 100, 200 and 300 mg/kg BW) to different groups of animals. The animals were randomly divided into seven groups of six animals each.
<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Control rats received 1% CMC only</td>
</tr>
<tr>
<td>Group II</td>
<td>Control + EREAL (300 mg/kg BW)</td>
</tr>
<tr>
<td>Group III</td>
<td>INH-RIF (50 mg/kg BW)</td>
</tr>
<tr>
<td>Group IV</td>
<td>INH-RIF + EREAL (100 mg/kg BW)</td>
</tr>
<tr>
<td>Group V</td>
<td>INH-RIF + EREAL (200 mg/kg BW)</td>
</tr>
<tr>
<td>Group VI</td>
<td>INH-RIF + EREAL (300 mg/kg BW)</td>
</tr>
<tr>
<td>Group VII</td>
<td>INH-RIF + Silymarin (70 mg/kg BW)</td>
</tr>
</tbody>
</table>
The EREAL and silymarin were dissolved in 2 mL of 1% CMC vehicle solution and fed by intubation. Among the three doses the 200 mg dose was more effective. The protective effect at the dose of 200 mg/kg BW was more pronounced than that of other two doses 100 and 300 mg dose was used for further study.

**Experimental protocol for further study**

The animals were randomly divided into five groups of six animals each. EREAL (200 mg/kg BW) and silymarin (70 mg/kg BW) was suspended in 2 mL of 1% CMC (vehicle solution) and fed by intragastric tube daily for 21 days.

After 21 days of treatment, the animals were fasted for 12 h, and sacrificed by cervical dislocation. Blood was collected in tubes with a mixture of potassium oxalate and sodium fluoride (1:3) for the estimation of various biochemical parameters. Tissue (liver and kidney) were surgically removed, washed with cold physiological saline, cleared off adherent lipids and immediately transferred to ice-cold containers. Erythrocytes were also prepared for the estimation of various biochemical preparations

### 3.7.1.2. PROCESSING OF BLOOD AND TISSUE SAMPLES

#### Serum preparation

Blood was collected in a dry test tube and allowed to coagulate at ambient temperature for 40 min. Serum was separated by centrifugation at 2000 rpm for 10 min.

#### Plasma preparation

The blood, collected in a heparinized centrifuge tube, was centrifuged at 2000 rpm for 10 min and the plasma was separated by aspiration.
Erythrocyte preparation

After the separation of plasma, the buffy coat, enriched in white cells, was removed and the remaining erythrocytes were washed three times with physiological saline. A known volume of erythrocyte was lysed with hypotonic phosphate buffer at pH 7.4. The hemolysate was separated by centrifugation at 2500 rpm for 10 min and the supernatant was used for the estimation of enzymic antioxidants.

Tissue homogenate preparation

Liver and kidney tissues (250 mg) were sliced into pieces and homogenised in appropriate buffer in cold condition (pH 7.0) to give 20% homogenate (w/v). The homogenate was centrifuged at 1000 rpm for 10 min at 0 °C in cold centrifuge. The supernatant was separated and used for various biochemical estimations.

3.7.1.3. TISSUE SAMPLING FOR HISTOLOGICAL STUDY

For histological study, three rats from each group were perfused with cold physiological saline, followed by formalin (10% formaldehyde). The liver and kidney were excised immediately and fixed in 10% formalin. Then dehydrated on treatment with a serious of different concentration of ethanol and embedded in paraffin wax. 3-5μm thick sections were cut using a microtome and stained with hemotoxyin and eosin. The specimens were evaluated with light microscope. All histopathological changes were examined by pathologist.

3.7.2. ESTIMATION OF HEPATIC MARKERS

3.7.2.1. ASSAY OF ASPARTATE AMINOTRANSFERASE (AST, EC 2.6.1.1)

Serum aspartate aminotransferase was assayed by using the diagnostic kit method (Reitman and Frankel, 1957).
# List of Parameters Analysed in This Study

| Serum       | Hepatic function markers: Aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transferase and alkaline phosphatase and bilirubin.  
|            | Renal function markers: Urea, uric acid and creatinine.  
| Plasma      | Lipid peroxidative markers: Thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides.  
|            | Non-enzymic antioxidants: Reduced glutathione, ascorbic acid and α-tocopherol.  
|            | Lipid profiles: Total cholesterol, free fatty acids, triacylglycerols and phospholipids.  
|            | Lipoprotein cholesterol: High density lipoprotein cholesterol (HDL-C), low density lipoprotein (LDL-C) and very low density lipoprotein (VLDL-C).  
| Erythrocytes| Enzymic antioxidants: Superoxide dismutase, catalase and glutathione peroxidase  
| Liver and kidney | Lipid peroxidative markers  
|              | Enzymic and non-enzymic antioxidants  
| Liver and kidney | Lipid profiles and liver glycogen.  
|              | Histopathological analysis.  

AST catalyses the transfer of amino group from L-aspartate to α-ketoglutarate with the formation of oxaloacetate and glutamate. The amount of oxaloacetate was measured by converting it into pyruvate by treating with aniline citrate and then reacting the pyruvate with 2, 4-dinitrophenylhydrazine to form 2, 4-dinitrophenyl hydrazone derivative which is brown colored in alkaline medium. The absorbance of this hydrazone derivative is correlated to AST activity.

Reagents

Buffered substrate: 2.66g of DL-aspartate and 38mg of α-ketoglutarate were dissolved in 20.5 mL of 1 N sodium hydroxide, with gentle heating. This was made up to 100 mL with phosphate buffer (0.01 M, pH 7.4).

Aniline-citrate reagent: 50 g of citric acid was dissolved in 50 mL of distilled water and mixed with equal volume of redistilled aniline.

Dinitrophenylhydrazine (DNPH) color reagent: 1.0 mM DNPH in 2N hydrochloric acid.

Sodium hydroxide: 0.4 N
Pyruvate standard: 2.0 mM

0.5 mL of buffered substrate was added to 0.1 mL of serum and placed in a water bath at 37 °C. To the blank tubes, 0.1 mL distilled water was added instead of serum. Exactly an hour later, 2 drops of aniline citrate reagent and 0.5 mL of DNPH reagent were added and kept at room temperature for 20 min. Finally, 5.0 mL 0.4 N sodium hydroxide was added. A set of standards were also treated in the same manner and read at 520 nm after 10 min. The results were expressed as IU/L of serum.
3.7.2.2. ASSAY OF ALANINE AMINOTRANSFERASE (ALT, EC 2.6.1.2)

Serum alanine aminotransferase was assayed by using the diagnostic kit based on the method (Reitman and Frankel, 1957).

ALT catalyses the transfer of amino group from L-alanine to α-ketoglutarate with the formation of pyruvate and glutamate. The pyruvate so formed, was allowed to react with 2, 4-dinitrophenylhydrazine to produce 2, 4-dinitrophenylhydrazine derivative which is brown colored in alkaline medium. The absorbance of this hydrazine derivative is correlated to ALT activity.

**Reagents**

- Buffered substrate: 1.78 g of DL-alanine and 38 mg of α-ketoglutarate were dissolved in buffer. 0.5 mL of sodium hydroxide was added and the volume was made up to 100 mL with phosphate buffer (0.01 M, pH 7.4).

- Dinitrophenylhydrazine (DNPH) color reagent: 1.0 mM DNPH in 2N hydrochloric acid.

- Sodium hydroxide: 0.4 N

- Pyruvate standard: 2.0 mM

0.5 mL of buffered substrate was added to 0.1 mL of serum and placed in a water bath at 37 °C. To the blank tubes, 0.1 mL distilled water was added instead of serum. Exactly an 30 min later, 0.5 mL of DNPH reagent were added and kept at room temperature for 20 min. Finally, 5.0 mL 0.4 N sodium hydroxide was added. A set of standards were also treated in the same manner and read at 520 nm after 10 min. The results were expressed as IU/L of serum.
3.7.2.3. ESTIMATION OF ALKALINE PHOSPHATASE (ALP, EC 3.1.2.3.1)

Plasma alkaline phosphatase was estimated by using the diagnostic kit method (Kind and King's, 1954). ALP catalyses disodium phenyl phosphate into phenol and disodium hydrogen phosphate at pH 10. Phenol so formed reacts with 4-aminoantipyrine in alkaline medium in the presence of oxidizing agent potassium ferricyanide to form a red colored complex whose absorbance is proportional to the enzyme activity.

Reagents

Buffered substrate: 0.01 M Disodium phenyl phosphate dissolved in carbonate-bicarbonate buffer (0.1 M, pH 10).

Color reagent: 4-aminoantipyrine, sodium hydroxide and potassium ferricyanide.

Phenol standard: 10 mg%

The incubation mixture, contained 1.0 mL of buffered substrate 3.1 mL of deionised water and 0.1 mL of serum, was incubated at 37 °C. Exactly after 15 min, 2.0 mL of color reagent was added to all the tubes. The control tubes received the enzyme after the addition of color reagent. 0.1 mL of standard and 0.1 mL of distilled water (blank) were also treated simultaneously and the color developed was read at 510 nm.

The enzyme activity was expressed as IU/L of serum

3.7.2.4. ESTIMATION OF γ-GLUTAMYL TRANSFERASE (GGT, EC 2.3.2.2)

The enzyme activity was assayed according to the method (Rosalki and Rau, 1972). GGT hydrolyses peptide bonds, in which a terminal glutamic acid residue is
linked by its $\gamma$-carboxyl group to an amino group. The enzyme is of low specificity for non-glutamyl moiety, so that synthetic substrates such as $\gamma$-glutamyl-p-nitroanilide are acted upon by GGT which catalyses the simultaneous transfer of glutamyl residues to an amino acid or peptide acceptor, glycyl glycine is chosen for this purpose, to form a yellow product paranitroanilide.

**Reagents**

- Tris-HCl buffer: 0.1 M, pH 8.2.
- Substrate: 30.3 mg of L-$\gamma$-glutamyl-p-nitroanilide/10 mL. The substrate was sparingly soluble and was dissolved by warming to 50-60 °C. The substrate solution was used within two hours of its preparation. 13.2 mg of glycyl glycine was dissolved in 10.0 mL of distilled water. This was used as a second substrate.
- 10% Acetic acid
- Standard p-nitroaniline: 13.8 mg of p-nitroaniline/100 mL of distilled water.

0.5 mL of serum was added to the incubation mixture containing 0.5 mL $\gamma$-glutamyl-p-nitroanilide, 2.0 mL glycyl glycine and 1.0 mL buffer. After the 30 min incubation at 37 °C the reaction was arrested by the addition of 1.0 mL of 10% acetic acid. The amount of p-nitroaniline liberated in the supernatant was measured as the difference in optical density at 410 nm, between samples, with and without substrate. The substrate incubated in the absence of serum under the same condition was used as a reference blank.

Enzyme activity was expressed as IU/L of serum.

**3.7.2.5. ESTIMATION OF SERUM BILIRUBIN**

Serum bilirubin was estimated by the method (Malloy and Evelyn, 1937).
Serum was diluted with water, and methanol added in an amount insufficient to precipitate the proteins, yet sufficient to permit all the bilirubin to react with diazo reagent.

**Reagents**

Absolute methanol

Hydrochloric acid: 1.5 %

Solution A: 1.0 g of sulphanilic acid was dissolved in 15 mL of conc. HCl and made up to one litre with water.

Solution B: 0.5 g of sodium nitrite was dissolved in water and made up to 100 mL.

Diazo-reagent: Prepared freshly before use by 0.3 mL of solution B was added to 10.0 mL of solution A.

Bilirubin standard: 10.0 mg of bilirubin was dissolved in 100 mL chloroform.

Two test tubes were taken and 0.2 mL serum and 1.8 mL of distilled water were added to each. 0.5 mL of diazo-reagent was added to the test and 0.5 mL of 1.5% HCl to the blank. Finally, to each tube 2.5 mL methanol was added and left aside for 30 min and the colour developed was read at 540 nm.

Serum bilirubin levels were expressed as mg/dL for serum.

### 3.7.3. ESTIMATION OF RENAL FUNCTION MARKERS

#### 3.7.3.1. ESTIMATION OF UREA

Serum urea was estimated by using the diagnostic kit based on the method (Fawcett and Scott, 1960).

Urea is hydrolysed in the presence of water and urease to produce ammonia and carbon dioxide. Under alkaline conditions, the ammonia so formed reacts with hypochlorite and sodium salicylate in the presence of sodium nitroprusside to form a
green colored chromophore. The intensity of the color produced is proportional to the concentration of urea in the sample.

**Reagents**

Buffered enzyme: Phosphate buffer, urease, sodium nitroprusside and ethylenediaminetetraacetic acid (EDTA).

Color developing reagent: Buffer, sodium hypochlorite, sodium salicylate and sodium hydroxide.

3. Urea standard: 40 mg/dL

To 1.0 mL of buffered enzyme, 10 μL serum was added, mixed well and kept at 37 °C for 5 min. 10 μL of standard and 10 μL of distilled water (blank) also processed simultaneously. To all the tubes, 1.0 mL of color developing reagent was added and mixed well. Exactly after 5 min of incubation at 37 °C, 1.0 mL of distilled water was added and the color developed was read at 600 nm.

The values were expressed as mG/dL of serum.

### 3.7.3.2. ESTIMATION OF URIC ACID

Serum uric acid was estimated by using the diagnostic kit enzymic method (Caraway, 1955).

Uric acid in the sample is oxidized by uricase to allantoin. In this reaction 1 mole of hydrogen peroxide is formed for every mole of uric acid oxidized. Hydrogen peroxide reacts with 3, 5-dichloro-2-hydroxybenzene sulfonic acid and 4-aminoantipyrine to give quinoneimine dye. Intensity of the color of this dye was proportional to the concentration of uric acid in the sample.

60
Reagents

Enzyme reagent: 4-Aminoantipyrine (4 mM), 3, 5-dichloro-2-hydroxybenzene sulfonate (2.0 mM), microbial uricase (150 U/L), horseradish peroxidase (10,000 U/L).

Standard uric acid: 5.0 mg/100 mL

To 1 mL of the enzyme reagent, 25 µL of serum sample was added and mixed by inversion. 25 µL of standard and 25 µL of distilled water (blank) also processed simultaneously. The tubes were incubated at 37 °C for 5 min and the color developed was read at 510 nm.

The values were expressed as mg/dL of serum.

3.7.3.3. ESTIMATION OF CREATININE

Seum creatinine was estimated using the diagnostic kit method (Tietz, 1987 using Jaffé’s 1886) color reaction.

The assay of creatinine has been based on the reaction of creatinine with alkaline picrate. Most of the contaminants reacting with the Jaffé’s reagent produce a colour at a lower rate than does creatinine. The initial rate of colour formation is proportional to the concentration of creatinine in the sample.

Reagents

1. Saturated picric acid
2. Sodium hydroxide: 0.75 N
3. Creatinine standard: 2.0 mg/dL

0.1 mL of serum was added to a reagent mixture containing 0.5 mL picric acid solution and 0.5 mL of sodium hydroxide. The tubes were mixed well and incubated
for 20s. With the spectrophotometer adjusted to zero absorbance with distilled water, reading was taken at 510 nm at 20s (A1) and exactly after 45s (A2). Change in absorbance (A2 − A1) was measured for test and standard, which was used to determine the creatinine concentration in the test sample.

The values were expressed as mg/dL of serum.

3.7.3.4. ESTIMATION OF TISSUE PROTEIN

Protein in the tissues was determined after trichloro acetic acid precipitation method (Lowry et al., 1951).

Reagents

1. Alkaline copper reagent:
   Reagent A: 2% sodium carbonate in 0.1 N NaOH
   Reagent B: 0.5 % copper sulphate in 1% sodium potassium tartarate.
   Reagent C: 50 mL of reagent A was mixed with 0.5 mL of reagent B just before use.

2. Folin’s phenol reagent: Dilute 1:2 with distilled water.

3. Stock standard: 100 mg of bovine serum albumin/ 100 mL of water.

4. Working standard: 10 mL of the stock standard was diluted to 100 mL to get a working standard containing 0.1 mg/mL.

0.5 mL of tissue homogenate was mixed with 0.5 mL of 10% TCA and centrifuged for 10 min. The precipitate was dissolved in 1.0 mL of 0.1 N NaOH. From this, an aliquot was taken, and 4.5 mL of alkaline copper reagent was added and allowed to stand at room temperature for 10 min. 0.5 mL of Folin’s phenol reagent was added and the blue color developed was read after 20 min at 640 nm. A standard
curve was obtained with standard bovine albumin and was used to assay the tissue protein level for enzyme activity.

Values were expressed as mg /g of tissue.

3.7.4. LIPID PEROXIDATION PRODUCTS

3.7.4.1. ESTIMATION OF THIOBARBITURIC ACID REACTIVE SUBSTANCES (TBARS)

The concentration of TBARS in the plasma, erythrocytes and tissues were estimated (Niehaus and Samuelson, 1968). In this method, malondialdehyde and other thiobarbituric acid reactive substances (TBARS) react with thiobarbituric acid in an acidic condition to generate a pink colour chromophore which was read at 535 nm.

Reagents

1. Trichloroacetic acid (TCA): 15%
2. Hydrochloric acid: 0.25 N
3. Thiobarbituric acid (TBA): 0.375% in hot distilled water
4. TBA-TCA-HCl reagent: Solution 1 to 3 was mixed in the ratio of 1:1:1, freshly prepared prior to use.
5. Stock standard: 4.8 molar solution of stock was prepared from 1,1,’3,’3’tetramethoxypropane purchased commercially.
6. Working standard: Stock solution was diluted to get a concentration of 48 mmol/mL.

0.5 mL of sample was diluted to 0.5 mL with double distilled water and mixed well, and then 2.0 mL of TBA-TCA-HCl reagent was added. The mixture was kept in a boiling water bath for 15 min, after cooling, the tubes were centrifuged at 1000 g for 10 min and the supernatent was estimated. A series of standard solution in the
concentration of 2-10 mmol was treated in a similar manner. The absorbance of the chromophore was read at 535 nm against reagent blank.

The values were expressed as mmol/dL of plasma or mmol/100 g of wet tissues.

3.7.4.2. ESTIMATION OF LIPID HYDROPEROXIDES

Lipid hydroperoxide in the plasma, erythrocytes and tissues were estimated (Jiang et al., 1992). Oxidation of ferrous ion (Fe²⁺) under acidic conditions in the presence of xylenol orange leads to the formation of a chromophore with an absorbance maximum at 560 nm.

**Reagents**

**Fox reagent:** 88 mg butylated hydroxytoluene (BHT), 7.6 mg xylenol orange and 9.8 mg ammonium iron (II) sulphate were added to 90 mL methanol and 10 mL H₂SO₄ (250 mM) mixture.

0.9 mL Fox reagent was mixed with 0.1 mL of the sample, incubated for 30 min at room temperature and the absorbance read in a Spectrophotometer at 560 nm.

Lipid hydroperoxides were expressed as mmol/dL of plasma or mmol/100 g of wet tissues.

3.7.5. ENZYMATIC ANTIOXIDANTS

3.7.5.1. ASSAY OF SUPEROXIDE DISMUTASE (SOD, EC 1.15.1.1)

Superoxide dismutase in the erythrocytes and tissues was assayed (Kakkar et al., 1984).
The assay is based on the inhibition of the formation of NADH-phenazinemethosulphate, nitroblue tetrazolium formazon. The reaction was initiated by the addition of NADH. After incubation for 90 sec, adding glacial acetic acid stops the reaction. The color developed at the end of the reaction was extracted into n-butanol layer and measured in a Spectrophotometer at 520 nm.

**Reagents**

1. Sodium pyrophosphate buffer: 0.025 M, pH 8.3.

2. Absolute ethanol.

3. Chloroform.

4. n-Butanol

5. Phenazine methosulphate (PMS): 186 µmol.


7. NADH: 780 µmol.

Tissue was homogenized by using sodium pyrophosphate buffer (0.025 M, pH 8.3). 0.5 mL of tissue homogenate or 0.5 mL of serum was diluted to 1.0 mL with water followed by addition of 2.5 mL of ethanol and 1.5 mL of chloroform (chilled reagents were added). This mixture was shaken for 90 sec at 4 °C and then centrifuged. The enzyme activity in the supernatant was determined as follows. The enzyme activity in the supernatant was determined as follows. The assay mixture contained 1.2 mL of sodium pyrophosphate buffer, 0.1 mL of phenazine methosulphate, and 0.3 mL of nitroblue tetrazolium and appropriately diluted enzyme preparation in a total volume of 3 mL. The reaction was started by the addition of 0.2 mL NADH. After incubation at 30 °C for 90 sec, the reaction was stopped by the addition of 1 mL glacial acetic acid. The reaction mixture was stirred vigorously and
shaken with 4mL n-butanol. The mixture was allowed to stand for 10 min; centrifuged and n-butanol layer was separated. The color density of the chromogen in n-butanol was measured at 510 nm. A system devoid of enzyme served as control. The enzyme concentration required to inhibit the chromogen produced by 50% in one minute under standard conditions was taken as one unit.

The specific activity of the enzyme was expressed as Unit/min/mg of protein for tissues or Unit/min/mg of Hb for erythrocytes.

3.7.5.2. ESTIMATION OF CATALASE (CAT, EC 1.11.1.6)

The activity of catalase in the erythrocytes and tissues was determined (Sinha, 1972). Dichromate in acetic acid was converted to perchromic acid and then to chromic acetate, when heated in the presence of H₂O₂. The chromic acetate formed was measured at 620 nm. The catalase preparation was allowed to split H₂O₂ for various periods of time. The reaction was stopped at different time intervals by the addition of dichromate-acetic acid mixture and the remaining H₂O₂ as chromic acetate was determined colorimetrically.

Reagents

1. Phosphate buffer: 0.01 M, pH 7.0
2. Hydrogen peroxide (H₂O₂): 0.2 M
3. Dichromate–acetic acid reagent:
   1:3 ratio of 5% potassium dichromate was mixed with glacial acetic acid.
   From this 1 mL was diluted again with 4 mL of acetic acid.
4. Standard hydrogen peroxide: 0.2 mM
Tissue homogenate was prepared by using phosphate buffer (0.01 M, pH 7.0). To 0.9 mL of phosphate buffer, 0.1 mL of tissue homogenate or 0.1 mL of hemolysate and 0.4 mL of hydrogen peroxide were added. The reaction was arrested after 15, 30, 45 and 60s by adding 2.0 mL of dichromate-acetic acid mixture. The tubes were kept in a boiling water bath for 10 min, cooled and the color developed was read at 620 nm. Standards in the concentration range of 20-100 µmol were taken and proceeded as for the test.

The specific activity was expressed as µmol of H₂O₂ consumed / min/ mg of protein for tissues or µmol of H₂O₂ consumed /min/mg of Hb for erythrocytes.

3.7.5.3. ESTIMATION OF GLUTATHIONE PEROXIDASE (EC 1.11.1.19)

The activity of GPx in the erythrocytes and tissues was measured (Rotruck et al., 1973). A known amount of enzyme preparation was allowed to react with H₂O₂ in the presence of GSH for a specified time period. Then the remaining GSH content was measured.

Reagents

1. Tris buffer: 0.4 M, pH 7.0
2. Sodium azide solution: 10 mM
3. TCA : 10%
4. EDTA : 0.4 mM
5. H₂O₂ solution : 0.2 mM
6. Glutathione solution: 2 mM.

The tissue was homogenized using tris buffer. To 0.2 mL of tris buffer, 0.2 mL of EDTA, 0.1 mL of sodium azide, 0.5 mL of tissue homogenate or hemolysate were
added. To the mixture, 0.2 mL of GSH followed by 0.1 mL of H$_2$O$_2$ was added. The contents were mixed well and incubated at 37 °C for 10 min, along with a control containing all reagents except homogenate. After 10 min, the reaction was arrested by the addition of 0.5 mL of 10% TCA. The tubes were centrifuged and the supernatant was assayed for GSH (Ellman, 1959).

The activity was expressed as µg of GSH consumed/min/mg of protein for tissues or µg of GSH consumed/min/mg of Hb for erythrocytes.

3.7.6. NON ENZYMATIC ANTIOXIDANTS

3.7.6.1. ESTIMATION OF REDUCED GLUTATHIONE (GSH)

Reduced glutathione in the plasma, erythrocytes and tissues was estimated (Ellman, 1959). This method was based on the formation of 2-nitro-5-thiobenzoic acid (a yellow colour compound) when 5, 5'-dithio-bis (2-nitrobenzoic acid) (DTNB) was added to compounds containing sulphydryl groups.

Reagents

1. Phosphate buffer: 0.1 M, pH 8.0
2. TCA: 5%
3. Ellman’s reagent: 34 mg of DTNB in 10 mL of 0.1% sodium citrate.
4. Disodium hydrogen phosphate: 0.3 M
5. Standard glutathione solution: 10.0 mg/100 mL.

The tissue was homogenized in phosphate buffer (0.1 M pH 7.0). 0.5 mL of homogenate or plasma was pipetted out and precipitated with 2.0 mL of 5% TCA. 2.0 mL of supernatant was taken after centrifugation and 1.0 mL of Ellman’s reagent and 4.0 mL of 0.3 M disodium hydrogen phosphate were added. The yellow color
developed was read in a Spectronic 20 at 412 nm. A series of standards (20–100 μg) was treated in a similar manner along with a blank containing 1.0 mL of buffer.

The amount of glutathione was expressed as mg/dL of plasma or mg/100g of wet tissue.

3.7.6.2. ESTIMATION OF ASCORBIC ACID (VITAMIN C)

Ascorbic acid in the plasma, erythrocytes and tissues were estimated (Roe and Kuether, 1943). The ascorbic acid was converted to dehydroascorbic acid by mixing with norit and then coupled with 2, 4 dinitrophenylhydrazine (DNPH) in the presence of thiourea, a mild reducing agent. The coupled dinitrophenylhydrazine was converted into a red colored compound when treated with sulphuric acid.

Reagents

1. TCA: 6%
2. 2, 4 DNPH reagent: 2.0 g of DNPH was dissolved in 100 mL of 9 N sulphuric acid. To this, 4.0 g of thiourea was added and mixed.
3. Acid washed norit.
4. Sulphuric acid : 85%
5. Stock ascorbic acid solution: 10 mg of L-ascorbic acid in 100 mL of 4% TCA.
6. Working ascorbic acid solution: 1 in 10 dilution of stock ascorbic acid solution with 4% TCA to obtain a concentration of 0.1 mg/mL.

To 0.5 mL of sample, 1.5 mL of 4% TCA was added and allowed to stand for 5 min and centrifuged. To the supernatant, 0.3 g of acid washed norit was added,
shaken vigorously and filtered. This converts ascorbic acid to dehydroascorbic acid. 0.5 mL of the filtrate was taken and 0.5 mL of DNPH was added, stoppered and placed in a water bath at 37 °C for exactly 3 h. Removed, placed in ice-cold water and added 2.5 mL of 85% sulphuric acid drop by drop. The contents of the tubes were mixed well and allowed to stand at room temperature for 30 min. A set of standards containing 20-100 μg of ascorbic acid were taken and processed similarly along with a blank containing 2.0 mL of 4% TCA. The color developed was read at 540 nm. The values were expressed as mg/dL of plasma or mg/100g of wet tissue.

3.7.6.3. ESTIMATION OF α–TOCOPHEROL (VITAMIN E)

α–Tocopherol in the plasma, erythrocytes and tissues were estimated (Baker et al., 1980). The method involves the reduction of ferric ions to ferrous ions by α–tocopherol and the formation of a red colored complex with 2, 2’ dipyridyl. Absorbance of the chromophore was measured at 520 nm.

Reagents

1. Petroleum ether : 60-80 °C
2. Double distilled ethanol.
3. 2, 2’ dipyridyl solution: 0.2% in double distilled ethanol.
4. Ferric chloride solution: 0.5% in double distilled ethanol.
5. Stock standard: 10 mg of α – tocopherol in 100 mL of distilled ethanol.
6. Working standard: Stock solution was diluted with ethanol to a concentration of 10 μg/mL.

To 0.5 mL of sample, 1.5 mL of ethanol was added, mixed and centrifuged. The supernatant was evaporated and to the precipitate, 3.0 mL of petroleum ether, 0.2
mL of 2, 2’ dipyridyl solution and 0.2 mL of ferric chloride solution were added. Mixed well and kept in dark for 5 min. An intense red color was developed. 4.0 mL of n-butanol was added to all the tubes and mixed well. Standard tocopherol in the range of 10-100 μg was taken and treated similarly along with a blank containing only the reagent. The color in the n-butanol layer was read at 520 nm.

The values were expressed as mg/dL for plasma or mg/100g of wet tissue.

3.7.7. ANALYSIS OF LIPID PROFILE

3.7.7.1. EXTRACTION OF LIPIDS

Lipids were extracted from plasma and tissues (Folch et al., 1957) using chloroform: methanol mixture (2:1 v/v). The tissues were rinsed in cold physiological saline thoroughly and dried by pressing between the folds of filter paper. The samples were homogenized in cold chloroform-methanol (2:1 v/v) and contents were extracted after 24 h. The extraction was repeated four times. The combined filtrate was washed with 0.7% KCl and the aqueous layer discarded. The organic layer was made upto a known volume with chloroform and used for various estimations.

3.7.7.2. ESTIMATION OF TOTAL CHOLESTEROL

Total cholesterol in the plasma and tissues was estimated enzymic method (Allain et al., 1974). Cholesterol esters were hydrolyzed by cholesterol esterase to free cholesterol and free fatty acids. The free cholesterol produced and pre-existing ones were oxidized by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. Hydrogen peroxide formed reacts with 4-aminoantipyrine and phenol in the presence of peroxidase to produce red colored quinoneimine dye. The intensity of color produced was proportional to the cholesterol concentration.
Reagents

1. Enzyme reagent: 4-aminoantipyrine, cholesterol esterase, phenol, cholesterol oxidase and horseradish peroxidase.

2. Cholesterol standard: 200 mg%

To 10 μL of plasma or 10 μL of lipid extract, 1.0 mL of enzyme reagent was added, mixed well and kept at 37 °C for 5 min. 10 μL of cholesterol standard and distilled water (blank) were also processed similarly. The absorbance was measured at 510 nm.

Cholesterol concentration was expressed as mg/dL of plasma or mg/100 g of wet tissue.

3.7.7.3. ESTIMATION OF HDL-CHOLESTEROL

HDL-cholesterol was estimated using the diagnostic kit based enzymic method (Izzo et al., 1981).

The VLDL and LDL fractions of plasma samples were precipitated using phosphotungstic acid and then HDL in the supernatant was separated by centrifugation and measured for its cholesterol content.

Reagents

1. Precipitating reagent (Phosphotungstic acid)

2. Enzyme reagent

3. HDL-cholesterol standard: 50 mg%

0.1 mL of plasma was mixed with 0.1 mL of precipitating reagent, allowed to stand at room temperature for 5 min and centrifuged at 2000-3000 rpm for 10 min. In the clear supernatant, cholesterol was estimated as described earlier.

The values were expressed as mg/dL of plasma.
3.7.7.4. ESTIMATION OF VLDL- AND LDL-CHOLESTEROL (Friedewald et al., 1972).

These were calculated using the formula

\[
\text{VLDL cholesterol} = \frac{\text{TG}}{5}
\]

\[
\text{LDL cholesterol} = \text{Total cholesterol} - (\text{HDL cholesterol} + \text{VLDL cholesterol})
\]

The values were expressed as mg/dL of plasma.

3.7.7.5. ESTIMATION OF FREE FATTY ACIDS (FFA)

Free fatty acids in the plasma and tissues were estimated (Falholt et al., 1973). Free fatty acids were extracted with chloroform-heptane-methanol mixture to eliminate interference from phospholipids and the extract was shaken with a high density copper reagent at pH 8.1. The copper soaps remained in the upper organic layer from which an aliquot was removed and copper content determined colorimetrically by treating with diphenyl carbazide.

Reagents

1. Chloroform-heptane-methanol solvent (5:5:1)
2. Stock copper solution: 500 mM
3. Triethanolamine solution: 1 M
4. Sodium hydroxide solution: 1 M
5. Copper reagent (Cu-TEA solution): 10.0 mL of stock copper solution was mixed with 10.0 mL triethanolamine and 6.0 mL sodium hydroxide. To this 33 g of sodium chloride was added, made upto 100 mL and the pH was adjusted to 8.1.
6. Diphenylcarbazide solution: 0.03 M in ethanol
7. Standard palmitic acid: 2.0 mM in chloroform-heptane-methanol solvent (5:5:1).

0.5 mL of lipid extract was evaporated to dryness and dissolved in 6.0 mL chloroform-heptane-methanol solvent and 2.5 mL of copper reagent were added. All the tubes were shaken vigorously for 90 sec and were kept aside for 15 min. The tubes were centrifuged and 3.0 mL of the copper layer was transferred to another tube containing 0.5 mL of diphenyl carbazide and mixed carefully. The color developed was read at 540 nm against a reagent blank containing 3.0 mL solvent and 0.5 mL diphenyl carbazide.

The free fatty acid content was expressed as mg/dL of plasma or mg/g wet tissue.

3.7.7.6. ESTIMATION OF TRIACYLGLYCEROL (TG)

Triacylglycerol in the plasma and tissues were estimated using the diagnostic kit enzymic method (McGowan et al., 1983). Triacylglycerol in the sample was hydrolysed by microbial lipase to glycerol and free fatty acid. Glycerol was converted by glycerol kinase into glycerol 3-phosphate (G-3-P) which was oxidized by glycerol phosphate oxidase to dihydroxyacetone phosphate and hydrogen peroxide. In this reaction hydrogen peroxide was produced in equimolar concentration to the level of triacylglycerol present in the sample. H₂O₂ reacts with 4-aminoantipyrine and 3, 5-dichloro-2-hydroxybenzene sulfonic acid in the presence of peroxidase to produce red quinoneimine colored dye. The intensity of this dye was proportional to the concentration of triacylglycerols in the sample.
Reagents

1. Triacylglycerol standard: 200 mg%

2. Enzyme reagent: Lipase, glycerol kinase, glycerol 3-phosphate oxidase, peroxidase, 4-aminoantipyrine, ATP, 3, 5-dichloro-2-hydroxybenzene sulphonate.

To 10 µL of plasma or 10 µL of lipid extract, 1.0 mL of enzyme reagent was added, mixed well and incubated at room temperature for 10 min. 10 µL of triacylglycerol standard and distilled water (blank) were also processed similarly. The absorbance was measured at 510 nm.

The triacylglycerol content was expressed as mg/dL of plasma or mg/g wet tissue.

3.7.7.7. ESTIMATION OF PHOSPHOLIPIDS (PL)

Phospholipids in the plasma and tissues were estimated (Zilversmit and Davis, 1950).

Phospholipids were digested with concentrated sulphuric acid to liberate the lipid bound inorganic phosphorus. Then it reacted with ammonium molybdate to form phosphomolybdic acid which was treated with 1-amino-2-naphthol-4-sulphonic acid (ANSA) to form a stable blue color.

The intensity of the color was proportional to the amount of phospholipids in the sample.
Reagents

1. Sulphuric acid: 5.0 N

2. Ammonium molybdate: 2.5% in 5.0 N sulphuric acid. 25 g of ammonium molybdate was dissolved, in about 200 mL of water. It was transferred to a one litre volumetric flask containing 500 mL of 10 N sulphuric acid and made up to the mark with water.

3. ANSA reagent: 0.2 g of ANSA was mixed with 1.2 g of sodium bisulphite and 1.2 g of sodium sulphite. 0.25 g was taken from this mixture and dissolved in 10 mL water.

4. Stock standard phosphorus: 35.1 mg of potassium dihydrogen phosphate was dissolved in water. To this, 1 mL of 10 N sulphuric acid was added and made up to 100 mL with water (80 μg/mL phosphorus).

5. Working standard phosphorus: Stock solution was diluted to get a concentration of 8 μg/mL of phosphorus.

0.5 mL of lipid extract was evaporated to dryness. 1.0 mL of 5.0 N sulphuric acid was added and digested till light brown. Then 2 to 3 drops of concentrated nitric acid was added and the digestion was continued till it became colorless. After cooling, 1 mL of water was added and heated in a boiling water bath for about 5 min. Then, 1.0 mL of ammonium molybdate and 0.1 mL of ANSA were added. The volume was then made up to 10.0 mL with distilled water and the absorbance was measured at 680 nm within 10 min. Standards in the concentration range of 2-8 mg were treated in the similar manner. The values obtained were multiplied with a factor 25 to convert inorganic phosphorus to its phospholipids equivalents.

The amount of phospholipids was expressed as mg/dL of plasma or mg/g wet tissue.
3.7.7.8. ESTIMATION OF LIVER GLYCOGEN

Liver glycogen was extracted and estimated by the method (Morales et al., 1975).

Reagents

1. Potassium hydroxide: 30%
2. Absolute alcohol.
3. Anthrone reagent: 0.2% of anthrone in concentrated sulphuric acid was prepared just before use.
4. Ammonium acetate: 1 M.
5. Stock standard: 1 mg/mL-100 mg of anhydrous D-glucose was dissolved in 100 mL of distilled water containing 0.01% benzoic acid.
6. Working standard: 100 μg/mL-10 mL of stock standard was diluted to 100 mL with distilled water.

The alkali extract of the tissue was prepared by digesting 50 mg of fresh tissue with 3 mL of 30% potassium hydroxide solution in boiling water bath for 15 min. The tubes were cooled and a drop of 1 M ammonium acetate was added to precipitate glycogen and left it in a freezer overnight for complete precipitation. Glycogen was collected by centrifuging at 3000 rpm for 20 min. The precipitate was dissolved with the aid of heating and again the glycogen was reprecipitated with alcohol and 1 M ammonium acetate and centrifuged. The final precipitate was dissolved in saturated ammonium chloride solution by heating in a boiling water bath for 5 min. Aliquots of glycogen solution were taken up for suitable dilution and 4 mL of anthrone reagent was added by cooling the tubes in an ice bath. The tubes were shaken well, covered with marble caps and heated in a boiling water bath for 20 min. After cooling, the
absorbance was read at 640 nm against water blank treated in a similar manner. Standard glucose solution was also treated similarly.

The glycogen content was calculated from the amount of glucose present in the sample, by multiplying with the factor 0.91 and expressed as mg/100 g of tissue.

3.8. STATISTICAL ANALYSIS

All quantitative measurements were expressed as means ± SD for control and experimental animals. The data were analyzed using one way analysis of variance (ANOVA) on SPSS/PC (statistical package for social sciences, personal computer) and the group means were compared by Duncan’s Multiple Range Test (DMRT). The results were considered statistically significant if the $P$ value is less than 0.05.