3. MATERIALS AND METHODS

The details about the various experimental procedures followed are described in chapter 3.

3.1 CHEMICALS

Isoproterenol, diosgenin, 1,1,3’,3’-Tetraetoxy propane, 1,1-diphyenyl-2-picrylhydrazyl and 2,2’-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Trisodium citrate, glutathione, bovine serum albumin, glutathione, 2,4-dinitrophenylhydrazine, Sucrose and AAS standards were taken from MERCK.

Nitroblue tetrazolium, phenazine methosulphate, 1-chloro-2,4-dinitrobenzene, p-phenylendiamine, potassium-α-ketoglutarate, thiamine pyrophosphate, quercetin, sodium succinate, oxaloacetate and cytochrome c were brought from SRL SISCO laboratories, Mumbai.

Pyrogallol, p-nitrophenyl-N-acetyl- β-d-glucosaminide, p-nitrophenyl- β-d-glucuronide, sodium dodecyl sulphate and hemoglobin were obtained from Himedia laboratories, Mumbai.

Biochemical kits for the assay of uric acid, cholesterol, triglycerides, HDL cholesterol were procured from Randox Laboratories. CK-MB assay kit was purchased from SPINREACT. All the other reagents were of analytical grade.

The detailed methodolgy followed in this study was depicted in the Figure 3.1 given below.
FIGURE 3.1 METHODOLOGY OF THE STUDY

- **Literature Survey**
- **Department Personal Collection**
- **Libraries of various Universities and colleges**
- **Sample collection**

**Methodology**

- **Processing and powdering of tuber**
- **Identification and Authentication**

**Physico-chemical and nutritional aspect studies**

**Extraction and fractionation of crude extract**

**In vitro antioxidant activity of the different fraction**
- DPPH decolration assay
- ABTS decolration assay
- NO scavenging activity
- SOD scavenging activity
- Total reducing capacity

**Acute toxicity studies**

**In vivo cardioprotective effect of effective fraction and Diosgenin**
- Cardiac enzymes
- Oxidative status
- Hypolipidemic activity (Lipid profile)
- Cardiotonic activity (glycoprotein)
- Mitochondrial changes (Oxidative stress, energy status)
- Lysosomal enzymes
- Histopathology

**Results and Statistical Analysis**

**Interpretation**

**Conclusion and Recommendations**
3.2 COLLECTION, AUTHENTICATION AND PROCESSING OF PLANT MATERIAL

About 20 kg of fresh tubers of *Dioscorea bulbifera* Linn. was purchased from the market of Mumbai from Ayurmed Biotech Pvt.Ltd, Mumbai. The tubers were brought to the Centre for Advanced Research in Indian System of Medicine (CARISM), SASTRA University, Thanjavur and authenticated by Dr. N.Ravichandran, botanist using its morphological characters and pharmacognosy studies.

The authenticated tubers were cut into pieces of 0.5 to 1.0 cm thickness and they were shade dried for a week. The shade dried plant material was then powdered using a pulverizer and used for the further study.

### 3.2.1 EXTRACTION AND FRACTIONATION OF THE *Dioscorea bulbifera* Linn. Tuber

The extraction and partitioning of the crude extract was carried out as described by Gao *et al.*, (2002) with some modifications. The air dried tuber of *Dioscorea bulbifera* Linn. was extracted with 75 % of ethanol (v/v) using cold-extraction procedure with occasional shaking. This process was repeated thrice and all the extracts were concentrated *in vacuo* and kept in a dessicator to remove any moisture until constant weight of the crude hydroalcoholic extract (CE) was obtained. The CE was then suspended in distilled water in 1:5 ratio and they were partitioned successively with chloroform and ethyl acetate thrice. The left out extract was taken as the water soluble fraction (WSF). The individual fractions were concentrated *in vacuo* and they were stored in a desiccator to free from moisture. The chloroform soluble fraction (CSF), ethylacetate soluble fraction (ESF) and WSF were weighed in
an electronic balance and the percentage of yield was calculated on the dry weight basis.

3.2.2. **Preliminary Qualitative Phytochemical Analysis**

Phytochemical analysis was mainly to screen the presence of the various active principles in the herbal extracts in a qualitative and quantitative manner. Phytoconstituents such as phenolic compounds, reducing sugars, flavones, glycosides, saponins, alkaloids, anthraquinones, quinones, protein and tannins were qualitatively analysed using standard methods (Harbone JB, 1973).

3.2.2.1 **Sample Preparation**

Approximately 100 mg of each extract / fraction obtained was weighed and dissolved in 25 ml of respective solvents and subjected to preliminary phytochemical analysis. The standard methods are as follows.

3.2.2.2. **Test for Carbohydrates**

1. **Molisch’s Test**

   To 0.5 ml extract / fraction, 1 ml of alpha naphthol solution in alcohol was added and shaken well. Then few drops of concentrated sulphuric acid were added from the sides of the test tube. Appearance of violet ring at the junction of two liquids indicates the presence of carbohydrates.

2. **Test for reducing sugars**

   a) **Fehling test**

   1 ml of extract / fraction was mixed with equal volume of Fehling’s solution A and B and heated; the formation of red color precipitate indicates the presence of reducing sugars.
b) Benedicts test

1 ml of extract / fraction was mixed with Benedict’s reagent and heated; the formation of orange red color precipitate indicates the presence of reducing sugars.

3. Test for Glycosides

0.2 ml of extract / fraction was mixed with a pinch of anthrone on a watch glass; one drop of concentrated Sulphuric acid was added and warmed gently over water bath. Dark green coloration indicates the presence of glycosides.

3.2.2.3. Test for Steroids

1. Libermann-Burchard Test

Few mg of extract / fraction was dissolved in a few drops of chloroform. To the chloroform extract, 3 ml of acetic anhydride was added followed by drops of concentrated sulphuric acid. Bluish green appearance indicates the presence of steroids.

2. Ferric chloride –Acetic acid Test

Few mg of extract / fraction was dissolved in a few drops of chloroform. To the chloroform extract, 2 ml of ferric chloride acetic acid reagent was added followed by 1 ml of concentrated sulphuric acid. Reddish pink coloration indicates the presence of steroids.

3.2.2.4. Test for Proteins

1. Biuret Test

To 0.5 ml extract / fraction, 2.5 ml of diluted Biuret reagent was added. Formation of purple color indicates the presence of protein.
2. Million’s test

To 0.5 ml extract / fraction, 2.5 ml of Million’s reagent was added. Formation of white precipitate and then precipitate warmed turns brick red confirms the presence of protein.

3.2.2.5. Test for Total Phenols

1. Ferric Chloride test

To 0.5 ml extract / fraction, 1 ml of alcoholic ferric chloride solution was added. Bluish green or Bluish black indicates the presence of phenol.

2. Test for Tannins - Lead Acetate solution test

To 0.25 ml extract / fraction, 1 ml of basic lead acetate solution was added. Formation of orange red precipitate indicates the presence of tannins.

3. Test for Quinones - Alkali test

To 0.5 ml extract / fraction, 1 ml of 10 % sodium hydroxide was added. Blue, een or red color indicates the presence of quinones.

4. Test for Flavones

a) Shinoda Test

To 0.5 ml extract / fraction, few mg of magnesium turnings was added followed by few drops of concentrated hydrochloric acid and boiled in a boiling water bath for five minutes. Red coloration shows the presence of flavones.

b) Alkaline Reagent Test

To 0.5 ml extract / fraction, 1 ml of 10% sodium hydroxide solution or ammonia was added. Dark yellow color indicates the presence of flavones.

c) Ferric Chloride Test

To 0.5 ml extract / fraction, few drops of ferric chloride solution was added. Intense green color shows the presence of flavones.
d) Lead Acetate Solution Test

To 0.5 ml extract / fraction, few drops of lead acetate solution (10%) was added. Yellow precipitates indicates the presence of flavones.

3.2.2.6. Test for Saponins - Foam Test

0.5 ml of extract / fraction was shaken well with 5 ml water; copious lather formation indicates the presence of saponins.

3.2.2.7. Test for Alkaloids

1. Draggendroff Test

To 0.5 ml of extract / fraction, 0.2 ml of acetic acid was added followed by Draggendroff’s reagent and shaken well. Formation of orange red precipitate indicates the presence of alkaloids.

2. Meyer’s Test

To 0.5 ml of extract / fraction, little amount of dilute hydrochloric acid and 1.0 ml of Meyer’s reagent were added. Formation of white precipitate indicates the presence of alkaloids.

3. Wagnor’s Test

0.5 ml of extract / fraction was mixed with 1.0 ml of Wagnor’s reagent. Formation of yellowish orange color indicates the presence of alkaloids.

4. Hager’s test

To 0.5 ml of extract / fraction, 1 ml of Hager’s reagent (saturated aqueous solution of picric acid) was added. A yellow colored precipitate indicates the presence of alkaloids.

3.2.2.8. Test for Anthraquinones - Borntrager's Test

1 ml of extract / fraction was macerated with ether and filtered. To the filtrate, 1 ml of aqueous ammonia or caustic soda was added. After shaking, appearance of
pink or red or violet color in the aqueous layer indicates the presence of anthraquinones.

3.2.2.9. Test for Triterpenoids - Salkowski test

Few mg of extract / fraction was dissolved in a few drops of chloroform. To the chloroform solution, few drops of concentrated sulphuric acid were added. Formation of brown ring indicates the presence of phytosterols- Triterpenoids.

3.3. PHYSICO-CHEMICAL CHARACTERISTICS

The physico-chemical properties such as foreign matter, loss on drying, total ash, acid insoluble ash, water soluble ash, alcohol soluble extractive and water soluble extractive were determined as per the methods referred in Ayurvedic pharmacopoeia (2004).

3.4 ESTIMATION OF PRIMARY AND SECONDARY METABOLITES

3.4.1 DETERMINATION OF CRUDE FIBRE CONTENT

The crude fiber content was determined using the acid base method of AOAC (1999).

Two grams of the powdered sample was taken in a kjheldhal flask. Already boiled 30 mL HCl was introduced into the kjheldhal flask and allowed to digest for 30 min. After the digestion with the acid, they were filtered and the undigested material was then digested with 30 mL NaOH solution for 30 min and filtered. Then the residue was washed with hot boiling distilled water and filtered again and taken into an oven maintained at 100°C to dry before cooling in the desiccators. The undigested material was re-weighed and the, difference between the final weight and initial weight was calculated and the percentage of crude fibre content was calculated on dry weight basis.
3.4.2 ESTIMATION OF TOTAL CARBOHYDRATE

The total carbohydrate content was estimated after hydrolysis by the method of Hedge and Hofreiter (1962).

Reagents

1. 2.5 N HCl

2. Anthrone reagent: 200 mg of anthrone was dissolved in 100 ml of ice cold 95% H$_2$SO$_4$. (Fresh preparation)

3. Standard glucose stock: 100 mg of glucose was dissolved in 100 ml of distilled water.

4. Working standard: 10 ml of stock was diluted to 100 ml with distilled water.

Procedure

100 mg of the powdered tuber was weighed into a boiling tube and hydrolysed with 5.0 ml of 2.5 N HCl and cooled to room temperature. It was then neutralized using sodium carbonate salt until effervescence ceases and made upto 100 ml with distilled water and the solids were removed on centrifugation at 2000 rpm. 0.5 ml of the aliquot was made upto 1.0 ml with distilled water. 4.0 ml of anthrone reagent was added and heated for eight minutes in a boiling water bath and cooled rapidly. A series of standards at concentration 20-100 µg was also processed as that of the sample and read at 630 nm against a reagent blank. The amount of total carbohydrates in the sample was then calculated from the sample and expressed as % on dry weight basis.

3.4.3 ESTIMATION OF SOLUBLE PROTEIN

The estimation of protein was carried out by the method of Lowry et al., 1951.
Reagents

1. Alkaline copper solution:

   Reagent A: Two gm of sodium carbonate was dissolved in 100 ml of 0.1N NaOH.

   Reagent B: 5 % copper sulphate in 100 ml 1 % sodium potassium tartarate solution.

   50 ml of Reagent ‘A’ and 1.0 ml of Reagent ‘B’ was mixed prior to use.

2. Folin-Ciocalteau’s reagent: one part of commercial reagent was mixed with two part of distilled water before use.

3. Standard Bovine serum albumin (BSA): 20 mg of BSA was dissolved in 100 ml of distilled water.

Procedure

Extraction of Protein

100 mg of the powdered sample was homogenized with 20 % of TCA. Then the homogenate was centrifuged at 2000 rpm for 15 minutes. The supernatant was discarded. The pellet was re-extracted with 5.0 ml of 0.1 N NaOH and centrifuged at 2000 rpm for 15 minutes. The supernatant was saved and made upto 5.0 ml with 0.1 N NaOH.

Estimation of Protein using Folin-Ciocalteau’s reagent

To 0.5 ml of protein extract 5.0 ml of alkaline copper solution and 2.0 ml of distilled water was added and the solution was allowed to stand for 10 minutes at room temperature and then 0.5 ml of Folin-Ciocaltaeus reagent was added and mixed vigorously. The mixture was again allowed to stand for 30 minutes. The blue colour
formed was read at 620 nm. The protein content was calculated from the standard and expressed as % on dry weight basis.

### 3.4.4 Estimation of Total Tannins

Estimation of tannins is based on the measurement of a blue colour formed by the reduction of phosphotungstic acid by tannins in alkaline solution as described by Ranganna (1977).

1. Folin’s Danis reagent – 100 gm of sodium tungstate was added to 750 ml of water, 20 gm of phosphomolybdic acid and 50 ml of 85% of phosphoric acid. The whole mixture was refluxed for 2 hours, cooled and diluted to 1 liter.

2. Saturated sodium carbonate solution – 35 gm of anhydrous NaOH was dissolved in 100 ml of water at 70-80°C and cooled overnight. Clear liquid was decanted and used.

3. Working Standard solution – 10 mg of tannic acid was dissolved in 100 ml of distilled water.

#### Procedure

**Extraction of tannin**

5 gm of sample was boiled with 400 ml of water for 30 minutes, cooled and filtered through Whatmann no.1 filter paper and it was made up to 500 ml with distilled water.

About 0.5 ml of sample was made up to 10.0 ml with distilled water. To this 0.5 ml of Folin’s Danis reagent, 1.0 ml of 10% sodium carbonate was added and mixed well. The blue colour was read at 760 nm against reagent blank after 30 minutes at room temperature. A standard was also run simultaneously at concentration
20-100 µg and the amount of tannic acid equivalent was calculated. The values are expressed as % on dry weight basis.

3.4.5 Estimation of Total Phenolics

The phenolic content in the plant material was estimated by the method of Singleton et al., (1999).

Reagents

1. 1.2 M HCl in 50 % of methanol
2. Folin’s ciocalteau reagent
3. 20 % sodium carbonate
4. Working Standard pyrogallol – 5 mg of pyrogallol was dissolved in 50 ml of distilled water.

Procedure

Extraction of total phenolics

50 mg of accurately weighed dried powder was defatted with petroleum ether and then it was extracted with 5.0 ml of 1.2 M HCl in methanol and vortexed separately for 1 minute and heated at 90°C for 3 hours vortexing every 30 minutes. After 3 hours, the samples were cooled and made up to 10 ml with methanol and centrifuged for 5 minutes at 5000 rpm. The clear supernatant obtained was used for the estimation.

About 0.2 ml of supernatant was made up to 0.5 ml with methanol and it was again made up to 3.0 ml with water. 0.5 ml of Folin’s ciocalteau reagent was added, shaken well and kept at room temperature for 10 minutes. After 10 minutes, 2.0 ml of 20 % sodium carbonate was added and read against reagent blank at 765 nm after 30
minutes. Pyrogallol at concentration 3-15 µg was run as standard and the amount of phenolic content was expressed as % on dry weight basis.

### 3.4.6 Estimation of Total Flavonoid Content

The total flavonoid content in the sample was estimated by the method of Chang et al., (2002).

**Reagents**

1. 10% aluminium chloride
2. 1M NaOH
3. 5% of sodium nitrite
4. Standard quercetin – 10 mg of quercetin was dissolved in 50 ml of distilled water

**Procedure**

The extract prepared for the estimation of total phenolics was used as sample for this assay. 0.25 ml of sample was diluted to 1.25 ml with distilled water. 75 µl of 5% sodium nitrite was added and after six minutes 0.15 ml of aluminium chloride solution was added. 0.5 ml of 0.1M NaOH was added after 5 minutes and made up to 2.5 ml with distilled water. The solution was mixed well and the absorbance was read at 510 nm in comparison with standard quercetin at 5-25 µg concentration. The results are expressed as % on dry weight basis.

### 3.4.7 Estimation of Total Alkaloid Content

The total alkaloid content in the plant material was estimated gravimetrically by the method of Kokate et al., (2003)
Accurately weighed 2 gm of dried plant material was macerated for 24 hours with 50 ml of ethanol. The extract was shaken well with 25 ml of 5 % H₂SO₄ thrice. The extract was then basified using dilute ammonia solution. It was then extracted with 25 ml of chloroform thrice until complete extraction of alkaloid takes place. The chloroform extract was washed with 5.0 ml of distilled water and filtered through filter paper in a pre-weighed beaker. 2.0 ml of absolute alcohol was added to the residue and evaporated to dryness until constant weight was obtained. The percentage of total alkaloid was calculated on dry weight basis.

### 3.4.8 Estimation of Anthocyanin Content

Total anthocyanin content in the plant material was estimated using modified pH differential method (Meyers et al, 2003).

#### Reagents

1. 0.1 M Phosphate buffer, pH 4.5
2. 0.1 M Phosphate buffer, pH 1.0

#### Procedure

The plant material was subjected to continuous soxhlet extraction using acetone. The acetone extract was filtered through the Whatman filter paper and they were made to 5.0 ml with distilled water. About 1.0 ml of the extract was dissolved in 4.0 ml of the buffers at pH 1.0 and 4.5 and read at 510 and 700 nm. The absorbance was converted to total mg of cyanidin 3-glucoside per 100 gm of plant material using molar extinction coefficient of 26900 and absorbance of A= [\((A_{510\text{-}700})_{\text{pH 4.5}} - (A_{510\text{-}700})_{\text{pH 1.0}}\)] and expressed as % on dry weight basis.
3.4.9 ESTIMATION OF ASCORBIC ACID

Ascorbic acid was estimated by the method of Omaye et al., (1962). Ascorbic acid is oxidised by copper to form dehydroascorbic acid and diketoglutaric acid. These products when treated with 2,4-dinitrophenylhydrazine (DNPH) form the derivative bis-2-4-dinitrophenylhydrazone, which undergoes rearrangement to form a product with absorption maximum at 520 nm. Thiourea provides a mild reducing medium that helps to prevent interference from non-ascorbic acid chromogens.

Reagents

1. 2,4-Dinitrophenylhydrazine-thiourea-copper sulphate reagent (DTC): 0.4 gm thiourea, 0.05 gm copper sulphate and 3.0 gm DNPH were dissolved in 100 mL of 9N H₂SO₄.
2. 10 % TCA.
3. 65 % H₂SO₄.
4. Standard ascorbic acid solution: 10 mg of ascorbic acid was dissolved in 100 mL of 5 % TCA.

Procedure

1.0 gram of powdered sample was treated with 4.0 ml of 10 % TCA and centrifuged for 20 minutes at 3500 g. 0.5 ml of supernatant was then mixed with 0.1 ml DTC reagent. The tubes were incubated at 37°C for three hours. 0.75 ml of ice cold 65 % H₂SO₄ was added and the tubes were allowed to stand at room temperature for an additional 30 minutes. A set of standards containing 10-50 µg of ascorbic acid was processed similarly along with a blank containing 0.5 ml of 10 % TCA. The colour developed was read at 520 nm. Values are expressed as % on dry weight basis.
3.4.10 Estimation of α-tocopherol

Plasma α-tocopherol was estimated by the method of Baker et al., (1980). The method involves the reduction of ferric ions to ferrous ions by α-tocopherol and the formation of a red coloured 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 ethanol
4. Ferric chloride solution: 0.5 % in ethanol
5. Stock standard: 10 mg of α-tocopherol in 100 mL distilled ethanol
6. Working standard: The stock solution was diluted in distilled ethanol to a concentration of 10 µg/mL.

Procedure

1.0 gram of powdered sample was extracted with 2.0 mL petroleum ether and 1.6 mL ethanol were mixed together and centrifuged. The supernatant was mixed with 0.2 mL 2,2’-dipyridyl solution and kept in the dark for five minutes. An intense red colour was developed. To all the tubes, 4.0 mL water was added and mixed well. Standard α-tocopherol in the range of 10-100 µg were taken and treated similarly along with a blank containing only the reagent. The colour in the aqueous layer was read at 520 nm. The values are expressed as % on dry weight basis.
3.5 **ESTIMATION OF ELEMENTAL CONCENTRATIONS USING ATOMIC ABSORPTION SPECTROMETER**

**Sample preparation**

A Multiwave 3000 micro oven system containing 16 teflon vessels with capping was used for digestion process. The digestion vessels were provided with a controlled pressure, temperature and release valve. Before use, all Teflon vessels were soaked in 10 % HNO$_3$. Accurately weighed 0.4 gram of the powdered plant samples were weighed into the teflon vessels and digested using HNO$_3$ and H$_2$O$_2$ in the ratio of 3:1. The system was programmed by giving gradual rise of 20, 40 and 50 % power for 5, 15 and 20 minutes respectively. The digestion process was continued to get a clear solution. Then they were made up to 50 ml with Millipore water.

**Procedure**

The micronutrients such as Fe, Cu, Mg, Mn and Zn concentration was measured using Electrodeless Discharge Lamp (EDL) as light source and the conditions were maintained as specified by the manufacturer. The concentration of the sample was calculated from the standard graph. The wavelength used for the measurement was 248.3, 324.8, 279.5, 213.9, 285.2 nm for Fe, Cu, Mn, Zn and Mg. For Cd and Pb Hollow cathode lamp (HCL) was used as a light source to provide specific wavelength of 228.8, and 283.3 for Cd and Pb, respectively and high purity (99.999 %) acetylene was used to provide constant thermal energy for atomization process. A Solution containing only the acid mixture was used as a blank.

Estimation of Hg and As were carried out using cold vapour method using Mercury Hydride System (MHS-15). The reaction mixture consists of 10 ml of the digested sample, 5ml of sodium borohydride and 5ml of 1 % NaOH. Argon gas was used as carrier gas for purging and the cold vapour containing hydrated form of As
and Hg was passed through the quartz cell without heating and the hollow cathode lamp (HCL) was used as a light source to provide specific wavelength of 193.7 and 253.7 for As and Hg respectively. The standard was also processed as that of the sample and the results were represented as ppm or ppb or ppt.

3.6 **THIN LAYER CHROMATOGRAPHIC PROFILING**

The thin layer chromatography (TLC) technique was used for profiling the CE.

**Preparation of sample**

400 mg of CE was dissolved in 10 ml of methanol and it was filtered through Whatmann filterpaper 1 and the filtrate was used for analysis.

**Procedure:**

About 20 and 30 µl of the sample was applied on to the 10 x 10 cm TLC aluminium sheet silica gel 60 F 254 using sample applicator (LINOMAT 5) at a speed of 100 nl/s by spray on technique. The first track was applied 20 mm away from the edge of the plate and distance between the tracks was maintained at 30 mm. The plate was then air dried to remove excess methanol on the plate. Twin trough chamber was saturated with the mobile phase (Chloroform: Ethyl acetate: Methanol: Water: Formic acid (6:6.5:2.7:0.5:.3)) for 15 minutes. Filter paper immersed onto the mobile phase was also placed inside the chamber while kept for saturation. The TLC plate was developed till 8.0 cm inside the saturated chamber and dried in oven for 5 minutes at 80°C. The dried plate was then scanned using TLC scanner 3 using the slit dimension of 6.0 X 0.4 mm at 254 and 366 nm. The analog curve was recorded.
3.6.1 Quantification of Diosgenin using TLC

Diosgenin is the well known marker compound reported in *Dioscorea bulbifera* Linn. Diosgenin content in the crude alcoholic extract and fractions were estimated using a modified procedure of Suthar and Mulani (2008).

**Procedure**

Precoated TLC aluminium sheet silica gel 60F 254 (10 x 10 cm) was used as stationary phase and Chloroform: Acetone (80:20) as solvent system. Development was carried out in the twin trough chamber with lining of filter paper dipped in the mobile phase and saturating with the mobile phase for 15 minutes. Samples were spotted on to the pre-coated TLC plate using automatic applicator using spray on technique. Ascending mode was used for the development of the plate upto 8.0 cm. After development, the plate dried in oven for 5 minutes at 80°C. The dried plate was derivitized dipping in 10 % methanolic Sulphuric acid reagent (10 ml concentrated sulphuric acid added in 90 ml of methanol with cooling and the reagent must be prepared freshly), heated at 110°C for 2-3 min and brought to room temperature. Brownish spot with Rf » 0.54-0.57 was scanned at 535 nm.

Standard curve of diosgenin was also run simultaneously at concentration of 200-600 ng. The results are expressed as percentage of diosgenin present in the CE, CSF, ESF and WSF.

3.6.2 Estimation of Total Phenolics in Individual Fraction

The phenolic content in the fractions were estimated using Folin’s Ciocalteau method as described by Javanmardi (2003). An aliquot (200µl) of the each fractions were treated with 1.0 ml of Folin-Ciocalteau reagent and 0.8 ml of 7.5 % sodium carbonate and vortexed. The absorbance was measured at 765 nm after 30 min. The total phenolic content was expressed as pyrogallol equivalents (PGE).
3.7 **In-vitro free radical scavenging activity of Dioscorea bulbifera Linn.**

3.7.1 **DPPH Radical Scavenging Activity**

DPPH radical scavenging activity was carried out by the method of Molyneux (2004). To 1.0 ml of 100µM DPPH solution in methanol, equal volume of the test extracts in methanol of different concentration was added and incubated in dark for 30 minutes. The change in coloration was observed in terms of absorbance using a spectrophotometer at 514 nm. 1.0 ml of methanol instead of test extract was added to the control tube. Percentage of inhibition was calculated from the equation (Absorbance of control - Absorbance of test)* (100/ Absorbance of control).

3.7.2 **ABTS Radical Scavenging Activity**

ABTS radical scavenging activity was performed as described by Berg *et al.*, (1999) and Re *et al.*, (1999). 2.5 mM ABTS as diammonium sulphate was prepared in 100 mM phosphate buffer, pH–7.4, containing 150 mM sodium chloride. The radical formation was initiated by the addition of 1.0 mM of ammonium persulphate. The mixture was heated in a water bath at 65-70°C for 15 min. The resulting blue green ABTS radical solution was further diluted such that its absorbance is 0.650 ± 0.020 at 734 nm. Various concentrations of the sample solution (20µl) were added to 980 µl of ABTS radical solution and the mixture was incubated in darkness at 37°C for 10 min. The decrease in absorbance was read at 734 nm. A test tube containing 20 µl of methanol and processed as described above served as the control tube and the percentage of inhibition was calculated as above.

3.7.3 **Nitric Oxide Radical Scavenging Activity**

The potential of the fractions to scavenge the NO radical was carried out using the method of Asvin and Mishra (2007)
200 µl of test samples of different concentrations was taken in series of test tube. NO radical formation was initiated with the addition of 200 µl of PBS (0.1 M, pH 7.4) and 800 µl Sodium nitroprusside (10 mM), and the reaction mixture was incubated at 25°C for 150 minutes. A control tube was also processed in the same way except for the test fraction, methanol was used. After the incubation time 1.2 ml of Griess reagent was added to the tubes and they were kept at room temperature for 30 minutes and the color developed was read at 540 nm. Percentage of inhibition was calculated as said above.

3.7.4 **Superoxide Radical Scavenging Activity**

The potential of the fractions to scavenge superoxide formed on illumination of riboflavin was studied using the method of Garrat (1964). The reaction mixture consists of 100 µl riboflavin solution (20 µg), 200 µl EDTA solution (12 mM), 200 µl methanol and 100 µl NBT (Nitro-blue tetrazolium) solution (0.1 mg) and 100 µl of test substance at different concentrations were mixed in test tube and reaction mixture was diluted up to 3ml with Tris-HCl buffer (100 mM). The absorbance of solution was measured at 590 nm using phosphate buffer as blank after illumination for 5 min. A control tube without the test substance but with methanol served as control. Percentage of inhibition was calculated as above.

3.7.5 **Total Reducing Potential**

The total reducing potential of the different fractions were screened using the method of Oyaizu (1986). 0.75 ml of the sample at various concentrations was mixed with 0.75 ml of phosphate buffer (0.2 M, pH 6.6) and 0.75 ml of 1% potassium hexacyanoferrate, incubated at 50°C in a water bath for 20 min. The reaction was stopped by addition of 0.75 ml of 10 % TCA solution and then centrifuged at 800 g for 10 min. 1.5 ml of the supernatant was mixed with 1.5 ml of distilled water and 0.1
ml of 0.1 % ferric chloride solution was added and kept at room temperature for 10 min. The absorbance was read at 700 nm. Higher absorbance of the reaction mixture indicates greater reducing power.

3.8 ACUTE ORAL TOXICITY OF ESF AND DIOS

Acute oral toxicity study was carried out in adherence to OECD guidelines 423.

It is a stepwise procedure with the use of a minimum number of animals per step; so that sufficient information was obtained on the acute toxicity of the test substance to enable its classification. The substance was administered orally to a group of experimental animals at one of the defined doses. The substance is tested using a stepwise procedure, each step using three animals of a single sex (normally females). Absence or presence of compound-related mortality of the animals dosed at one step will determine the next step, i.e., no further testing is needed, dosing of three additional animals, with the same dose, dosing of three additional animals at the next higher or the next lower dose level.

3.8.1 EXPERIMENTAL ANIMALS

The acute toxicity studies were performed in female Wistar rats. The animals were housed individually in polyurethane cages in the animal house. All animals were kept in one room and with no other species being housed in the same room. The room was well ventilated with 100% fresh air. A 12-hour light /12 hour dark photoperiod was maintained. Room temperature and relative humidity was set to be maintained between 22±3°C and <55% respectively. The animals have access to pelleted feed and water ad libitum. The animals were examined at regular intervals for any behavioural abnormalities.
3.8.2 Drug dosing

Acute toxicity was performed for ESF and DIOS at a single dose level of 2000mg/kg b.w. The test materials were dissolved in 1% gum acacia.

3.8.3 Grouping

After taking the initial weights, for single test drug, the animals were distributed randomly into two cages (n=3/group) for conducting the study.

3.8.4 Experimental protocol

After 12 hours fast, the drugs were administered by oral route to all the animals. Animals were observed individually after drug dosing for the signs and symptoms such as ataxia, tremors, convulsion, hair loss, salivation, diarrhea, lethargy, lacrimation, sleep, coma, etc., at least once during first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days, except where they needed to be removed from the study and humanely killed for animal welfare reasons or are found dead. Observations also included changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. After drug administration, the mortality was noted; measured body weight once in a week and behavioural screening was recorded at 24 hours and 14 days.

3.9 Pharmacological screening of ESF and DIOS for its cardioprotective effect

3.9.1 Experimental animals

All the experiments were carried out with male albino Wistar rats weighing 140–160 g, obtained from the Central Animal House, CARISM, SAstra University, Tamil Nadu, India. They were housed in polypropylene cages (47 cm×34 cm×20 cm) lined with husk, replaced every 24 h, under a 12:12 h light dark cycle at around 22 °C and
Fig. 3.8.1 OECD PROTOCOL FOR ACUTE TOXICITY STUDY 423 WITH STARTING DOSE 2000 mg/kg
had free access to water and food. The rats were fed on a standard pellet diet (Nutri Lab- Rodent, Tetragon Chemicals Pvt. Ltd., India). The pellet diet consisted of 22.30 % crude protein, 3.44 % crude fat, 3.9 % crude fibre, 1.28 % calcium, 0.92 % phosphorous, 6.79 % total ash and 49.68 % nitrogen-free extract (carbohydrates). The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Animal Ethical Committee of SASTRA University (Approval no. 16/SASTRA/IAEC/RPP).

3.9.2 DOSE FIXATION

In our pilot study, ESF at the dose of 50, 100 and 150 mg/kg body weight and DIOS at 20, 40, 80 mg/kg body weight was used for the pretreatment of the male Wistar rats for a period of 35 days. After the last treatment, Isoproterenol (85 mg/kg) dissolved in normal saline was injected subcutaneously to all rats other than the normal group at an interval of 24 hours for two days to induce experimental MI (Rajadurai and Prince, 2006). On analyzing the serum CPK and LDH activity it was found that ESF at a dose of 150 mg/kg and DIOS at 80 mg/kg body weight dose elicited cardioprotective effect. Hence, the dose 150 mg/kg of ESF and 80 mg/kg of DIOS was used for further studies.

3.9.3 EXPERIMENTAL DESIGN

Seven groups each containing six animals was acclimatized to the laboratory conditions before a week of the experiment to be started. The animals were given free access to food and water ad libitum. The test drugs were suspended in 1 % gum acacia freshly every day and the volume of drug was kept to 10 ml/kg body weight of the animal. The various groups and their treatment was given below
Normal : Normal non-treated
ISO alone : Vehicle for 35 days and two doses of isoproterenol at 85 mg/kg b.w
Vit-E 60 : Vitamin E for 35 days and two doses of isoproterenol at 85 mg/kg b.w
ESF 150 + ISO : Ethyl acetate fraction at 150mg kg/b.w for 35 days and two doses of isoproterenol at 85 mg/kg b.w
ESF 150 alone : Ethyl acetate fraction at 150mg kg/b.w for 35 days
DIOS 80 + ISO : Diosgenin at 80mg kg/b.w for 35 days and two doses of isoproterenol at 85 mg/kg b.w
DIOS 80 alone : Diosgenin at 80mg kg/b.w for 35 days

3.9.4 Induction of Experimental MI

After the last treatment, Isoproterenol (85 mg/kg) dissolved in normal saline was injected subcutaneously to all rats other than the normal group at an interval of 24 hours for two days to induce experimental MI (Rajadurai and Prince, 2006). All the rats were sacrificed after an overnight fasting.

3.9.5 Collection of Blood

Blood was collected from the retroorbital sinus with and without anti-coagulant for plasma and serum. The blood is centrifuged and the plasma or serum was used for the biochemical assay.

3.9.6 Preparation of Heart Tissue Homogenate

The heart tissue was excised immediately and washed off from blood with ice cold physiological saline. Then the heart was blotted in between filter papers to absorb moisture and weighed in a balance. About 200 mg of the heart tissue was homogenized in 5.0 ml of 0.1 M Tris-HCl buffer (pH 7.4) solution. The homogenate
was centrifuged at 3000 rpm for 15 minutes and the supernatant was used for the various biochemical parameters.

3.10 BIOCHEMICAL ESTIMATION

3.10.1 ESTIMATION OF TISSUE AND CARDIAC MARKER ENZYMES ACTIVITY

3.10.1.1 SERUM CREATINE KINASE – MB

Serum Creatine kinase MB activity was estimated using standard kit from Spinreact Cat. No. 1001055

3.10.1.2 CREATINE PHOSPHOKINASE (CPK, EC 2.7.3.2)

The CPK activity was assayed as per the method adopted by Okinaka et al., (1961).

Reagents

1. Tris-HCl buffer - 0.1 M pH 9.0
2. ATP - 18.5 mM in Tris-HCl buffer
3. Magnesium-cysteine reagent
4. Creatine - 240 mM
5. Ammonium Molybdate
6. 10 % TCA
7. ANSA reagent
8. Standard KH$_2$PO$_4$ : 33.1 mg of KH$_2$PO$_4$ in 100 ml of double distilled water (80 µg of phosphorus/ml)

Procedure

The incubation mixture contained 0.75 ml of double distilled water, 0.05 ml serum or 0.5 ml of homogenate, 0.1 ml of ATP solution, 0.1 ml of magnesium-cysteine reagent and 0.1 ml of creatine. This was incubated at 37°C for 20 min and the reaction was stopped by adding 10 % TCA. The tubes were centrifuged and the
supernatant was used for the estimation of phosphorus by Fiske and subbarow (1925) method. 1.0 ml of the supernatant was made up to 4.3 ml with distilled water. 1.0 ml of ammonium molybdate reagent was added to the tube and kept at room temperature for 10 min. 0.4 ml of ANSA was added and the colour developed was read at 640 nm after 20 min. A series of tubes containing standard concentration 0-80 µg and a control tube was run simultaneously and the results were expressed as µM of phosphorus liberated per minute/L for serum and µM of phosphorus liberated per minute/mg of protein for tissue homogenate.

3.10.1.3 LACTATE DEHYDROGENASE (LDH, EC 1.1.1.27)

The Lactate dehydrogenase activity was assayed by the method of King (1965a).

Reagent

1. Glycine buffer: 7.505 gm of glycine and 5.85 gm NaCl was dissolved in 900 ml of double distilled water and made upto to 1000ml with double distilled water.

2. Buffered substrate: About 125 ml of glycine buffer, 75 ml of 0.1 N NaOH and 4.0 gm of Lithium lactate was mixed and the pH was adjusted to 10.0.

3. NAD⁺: 10 mg of NAD was dissolved in 2.0 ml of double distilled water (prepared freshly)

4. NADH: 0.71 mg of NADH was dissolved in 1.0 ml of buffered substrate.

5. DNPH reagent: 200 mg of DNPH was dissolved in 85 ml of conc. HCl.

The final volume was adjusted to 1000 ml with distilled water.

6. 0.4 N NaOH

7. Standard pyruvate: 22 mg of sodium pyruvate in 100 ml of distilled water.
**Procedure**

Two tubes namely “test” and “control” tube were taken and incubated with 1.0 ml of buffered substrate for 5 min. Then 0.2 ml of NAD$^+$ was added to the two tubes and 0.02 or 0.1 ml of serum or homogenate was added to the tube marked “test”. This mixture was incubated at 37°C for 15 min. Then the reaction was stopped by adding 1.0 ml of DNPH to both the tubes. 0.02 ml of serum was added to the control tube and both the tubes were kept at room temperature for 15 minutes. 10.0 ml of 0.4 N NaOH was added to both the tube and read at 445 nm after 10 min. A series of pyruvate standard at 1-5 µM concentration was run simultaneously and processed in the same way. The results were calculated and expressed as µM of pyruvate formed per minute/litre for serum and µM of pyruvate formed per min/mg of protein.

**3.10.1.4. ASPARATE TRANSAMINASE (AST, EC 2.6.1.1)**

Activity of AST in serum and homogenate was assayed by the method of Mohun and Cook (1957).

**Reagents**

1. SGOT substrate pH 7.45: 2.66 gm of aspartic acid, 30 mg of alpha-ketoglutarate and 20 ml of 1.0 N NaOH was mixed well and made up to 100 ml with phosphate buffer pH 7.45 (M/15).
2. DNPH reagent: 200 mg of DNPH was dissolved in 85 ml of concentrated HCl. The final volume was adjusted to 1 litre with distilled water.
3. 0.4 N NaOH
4. Standard pyruvate: 22 mg of sodium pyruvate in 100 ml of distilled water.

**Procedure**

Two tubes namely “test” and “control” tube were taken and incubated with 0.5 ml of buffered substrate for 5 min at 37°C. 0.1 ml of serum or homogenate was added
to the tube marked “test”. This mixture was incubated at 37°C for an hour. Then the
reaction was stopped by adding 0.5 ml of DNPH to both the tubes. 0.1 ml of serum or
homogenate was added to control tube and both the tubes were kept at room
temperature for 20 minutes. 5.0 ml of 0.4 N NaOH was added to both the tube and
read at 540 nm after 10 min. A series of pyruvate standard at 0-5 µM concentration
was run simultaneously and processed in the same way. The results are calculated and
expressed as µM of pyruvate formed per min/litre for serum and µM of pyruvate
formed per min/mg of protein.

3.10.1.5 ALANINE TRANSAMINASE (ALT, EC 2.6.1.2)

ALT activity in serum and homogenate was assayed by the method of Mohun
and Cook (1957).

Reagents

1. SGPT substrate pH 7.45: 1.79 gm of alanine, 30 mg of alpha-ketoglutarate
   and 0.5 ml of 1.0 N NaOH was mixed well and made up to 100 ml with
   phosphate buffer pH 7.45 (M/15).
2. DNPH reagent: 200 mg of DNPH was dissolved in 85 ml of conc. HCl.
   The final volume was adjusted to 1000 ml with distilled water.
3. 0.4 N NaOH
4. Standard pyruvate: 22 mg of sodium pyruvate in 100 ml of distilled water.

Procedure

Two tubes namely “test” and “control” tube were taken and incubated with 0.5
ml of buffered substrate for 5 min at 37°C. 0.1 ml of serum or homogenate was added
to the tube marked “test”. This mixture was incubated at 37°C for an hour. Then the
reaction was stopped by adding 0.5 ml of DNPH to both the tubes. 0.1 ml of serum or
homogenate was added to the control tube and both the tubes were kept at room
temperature for 20 minutes. 5.0 ml of 0.4 N NaOH was added to both the tubes and read at 540 nm after 10 min. A series of pyruvate standard at 0-5 µM concentration was run simultaneously and processed in the same way. The results were calculated and expressed as IU/L for serum and µM of pyruvate formed per min/mg of protein.

3.10.2 ASSSESSMENT OF ANTIOXIDANT STATUS

3.10.2.1 ESTIMATION OF PLASMA AND TISSUE THIOBARBITURIC ACID REACTIVE SUBSTANCES (TBARS)

The levels of lipid peroxidation in tissues were estimated by the method of Nichans and Samuelson (1968).

In this method, malondialdehyde and other thiobarbituric acid reactive substances (TBARS) were measured by their reaction with thiobarbituric acid (TBA) in acidic condition to generate a pink coloured chromophore which was read at 535 nm.

Reagents

1. TCA - 15 

2. HCl (HCl) – 0.25 N

3. Thiobarbituric acid (TBA) – 0.375 % in hot distilled water

4. TBA – TCA – HCl reagent – Solutions 1,2 and 3 reagents were mixed freshly in ratio 1:1:1.

5. Stock standard malondialdehyde (5 mM): To 50 µl of 1,1,3,3-tetraethoxy propane, 70 µl of conc. HCl was added and made up to 1.0 ml with normal saline and it was made up to 100 ml with distilled water.
6. Working standard malondialdehyde (50 nM/ml): Above prepared stock was diluted 1ml to 100 ml with distilled water.

**Procedure**

To 1.0ml of tissue homogenate or 0.3 ml of plasma was added 3.0 ml of TBA – TCA – HCl reagent and mixed thoroughly. The mixture was kept in boiling water bath for 15 minutes. After cooling, the tubes were centrifuged at 1000 g for 10 minutes and the supernatant was taken for the measurement. A series of standard solution in the range 2-10 nmole concentration were treated in a similar manner. The absorbance of chromophore was read at 535 nm against a reagent blank. Values were expressed as nM of malondialdehyde/mg of protein for heart tissue and nM/ml of plasma.

### 3.10.2.2 Estimation of Tissue and Plasma Hydroperoxides (HP)

The tissue and plasma hydroperoxides are estimated by the method of Jiang *et al.*, (1992)

**Reagent**

1. Fox reagent - 88 mg of Butylated hydroxyl toluene, 7.6 mg of xylenol orange and 0.8 mg of ammonium iron sulphate were added to 90 ml of methanol of HPLC grade and 10 ml of 250 mM of H₂SO₄ was added.

**Procedure**

0.1 ml of plasma or homogenate was treated with 1.9 ml of Fox reagent and incubated at 37°C for 30 minutes and the pink colour formed was read at 560 nm. The amount of hydroperoxide was calculated by multiplying with the molar extinction
coefficient 9.85. The values are represented as mM/dl of plasma or mM/mg of protein.

3.10.3 ESTIMATION OF ENZYMIC AND NON-ENZYMIC ANTIOXIDANTS

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

Superoxide dismutase activity was assayed by the method of Kakkar et al., (1984).

The assay of SOD was based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium complex. The reaction was initiated by the addition of NADH. After incubation for 90 seconds, the reaction was stopped by the addition of glacial acetic acid. The colour developed at the end of the reaction was extracted into butanol layer and measured at 560 nm.

**Reagents**

1. 0.025 M Sodium pyrophosphate buffer - pH 8.3
2. 186 µM Phenazine methosulphate
3. 300 mM Nitroblue tetrazolium,
4. 780 mM NADH
5. Glacial acetic acid
6. n–Butanol
7. Chloroform
8. Ethanol

**Procedure**

0.5 ml of the homogenate was diluted to 1 ml with ice cold water. 2.4 ml ethanol and 1.5 ml chloroform (in chilled condition) were added to it. This mixture
was shaken for 1 minute at 4°C and then centrifuged. The enzyme activity in the supernatant was determined.

The assay mixture contained 1.2 ml sodium pyrophosphate buffer, 0.1 ml phenazine methosulphate, 0.3 ml nitroblue tetrazolium, appropriately diluted enzyme preparation and water in a total volume of 3.0 ml. The reaction was started by the addition of 0.2 ml NADH. After incubation at 30°C for 90 seconds, the reaction was stopped by the addition of 1.0 ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4.0 ml n-butanol. The mixture was allowed to stand for 10 minutes, and then centrifuged. The colour intensity of the chromophore in the butanol layer was measured at 560 nm against butanol blank and a system devoid of enzyme served as the control. One unit of enzyme activity is defined as the enzyme reaction which gave 50% inhibition of NBT reduction in one minute under assay conditions and the activity was expressed as U/mg protein.

3.10.3.2 ASSAY OF CATALASE (CAT, EC. 1.11.1.6)

The activity of catalase was determined by the method of Sinha (1972).

Catalase was allowed to split hydrogen peroxide for different periods of time. The reaction was stopped at different time intervals by the addition of dichromate-acetic acid. Dichromate in acetic acid was converted to perchromic acid and then to chromic acetate, when heated in the presence of hydrogen peroxide. The chromic acetate formed was measured at 620 nm.

Reagents

1. 0.01 M Sodium phosphate buffer - pH 7.0
2. 0.2 M Hydrogen peroxide.
3. 5 % Potassium dichromate.

4. Dichromate acetic acid reagent: 5 % potassium dichromate was mixed with glacial acetic acid in the ratio of 1:3.

5. Standard hydrogen peroxide, 2 mM: 1 ml of 0.2 M H₂O₂ was diluted to 100 mL using distilled water.

**Procedure**

3.0 mL of phosphate buffer was mixed with 0.1 ml homogenate and 0.2 ml hydrogen peroxide. The reaction was stopped at 15, 30, 45 and 60 seconds by the addition of 1 ml dichromate-acetic acid reagent. The tubes were kept in boiling water bath for 10 minutes and the colour developed was read at 620 nm. Standards in the range of 2-10 µM were taken and treated similar to the test with a blank containing reagent alone. The activities were expressed as µM of H₂O₂ consumed/ minute/mg of protein.

**3.10.3.3 ASSAY OF GLUTATHIONE PEROXIDASE (GPₓ, EC 1.11.1.9)**

Glutathione peroxidase was estimated by the method of 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 was measured.

\[
2\text{GSH} + \text{H}_2\text{O}_2 \xrightarrow{\text{GP}_x} \text{GSSG} + 2\text{H}_2\text{O}
\]

**Reagents**

1. 0.4 M Tris-HCl buffer - pH 7.0
2. 10 mM Sodium azide solution.
3. 10 % Trichloro acetic acid.
4. 0.4 mM EDTA.
5. 20 mM H₂O₂ solution.

6. Precipitating reagent: 167 mg metaphosphoric acid, 200 mg EDTA disodium salt and 3 gm sodium chloride were dissolved in 100 ml distilled water.

7. 2 mM reduced glutathione.

Procedure

0.2 ml of tris buffer was mixed well with 0.2 ml EDTA, 0.1 ml sodium azide, 0.5 ml homogenate and 0.2 ml GSH, followed by 0.1 ml hydrogen peroxide. The contents were incubated at 37°C for 10 minutes along with a tube containing all the reagents except the homogenate. After 10 minutes, the reaction was arrested by the addition of 0.5 ml of 10 % TCA, centrifuged and the supernatant was assayed for GSH by the method of Ellman (1959).

3.10.3.4 Assay of Glutathione-S-Transferase (GST, EC. 2.5.1.18)

Glutathione-s-transferase activity was assayed spectrophotometrically at 340 nm by measuring the rate of l-chloro-2,4-dinitrobenzene conjugation with reduced glutathione as a function of time according to the established method of Habig and Jakoby (1981).

Reagents

1. 100 mM- Potassium phosphate buffer (pH 6.5).

2. 30 mM reduced glutathione: 92.1 mg of reduced glutathione in 10 ml of distilled water.

3. 30 mM l-chloro-2,4-dinitrobenzene (CDNB): mg of CDNB was dissolved in 10 ml of distilled water.
Procedure

The assay mixture contained 0.1 ml 30 mM GSH, 0.1 ml of tissue homogenate, 0.1 ml 30 mM CDNB and 2.7 ml 100 mM pH 6.5 phosphate buffer. Tube containing all reagents except the homogenate served as the control. Optical density was read at 340 nm for 5 minutes at 30 second interval. The enzyme activity was expressed as nM of CDNB conjugated /minute/mg protein.

3.10.3.5 Estimation of reduced glutathione (GSH)

Reduced glutathione was estimated by the method of Ellman (1959) in which, yellow colour developed when dithionitro-bis-benzoic acid (DTNB) was added to the compounds containing sulfhydryl groups.

Reagents

1. 0.2 M Phosphate buffer - pH 8.0.
2. 5 % TCA.
3. Ellman’s reagent: 19.8 mg of dithio-bis-2-nitro benzoic acid was dissolved in 100 ml of 1 % sodium citrate solution.
4. Precipitating reagent: 167 mg metaphosphoric acid, 200 mg EDTA disodium salt and 3 gm sodium chloride were dissolved in 100 ml distilled water.
5. Standard glutathione solution: 10 mg GSH dissolved in 100 ml distilled water (100 µg/ml).
Procedure

0.3 ml of the plasma or 0.5 ml of homogenate was mixed thoroughly with 3.0 ml of precipitating reagent and allowed to stand for 5 minutes and centrifuged. A set of standards (20-100 µg) were taken and made up to 1.0 mL with distilled water. 1.0 ml of supernatant along with 1.0 ml blank containing distilled water was taken. To all the tubes 2.0 ml of 0.3 M disodium hydrogen phosphate and 0.5 ml of DTNB reagent were added. The colour developed was read at 412 nm. Reduced glutathione levels were expressed as mg/dL plasma or mg/100 mg of wet tissue.

3.10.3.6 Estimation of ascorbic acid

Ascorbic acid content in the plasma and tissue was estimated by the method of Omaye et al. (1962) as described in section 3.3.11.

3.10.3.7 Estimation of α-tocopherol

Plasma and tissue α-tocopherol was estimated by the method of Baker and Frank (1980) as described in section 3.3.12.

3.10.3.8 Estimation of ceruloplasmin

Ceruloplasmin was estimated by the method of Ravin (1961). Ceruloplasmin an oxidase was measured on the basis of the oxidation of p-phenylene diamine to form a purple coloured compound.

Reagents

1. 0.4 M Acetate buffer - pH 5.5
2. 0.5 % Sodium azide
3. 0.5 % p-phenylene diamine hydrochloride in acetate buffer
Procedure

0.8 ml of buffer was taken in two tubes marked control and test and 0.05 ml plasma was added. 1.0 ml sodium azide was added to the control tube and mixed. 1.0 ml p- phenylene diamine was added to both the tubes, mixed and kept at 37°C for one hour. 1.0 ml sodium azide was then added to the test. All the tubes were kept at 4 to 10°C for 30 minutes. The colour developed was read at 540nm with control as blank. The values were expressed as mg/dl plasma.

3.10.4. ASSAYS OF LIPIDS AND LIPID METABOLISING ENZYMES

3.10.4.1 EXTRACTION OF LIPIDS

Lipids were extracted from heart tissues by the method of Folch et al., (1957) using chloroform-methanol mixture (2:1 v/v)

A known weight of tissue was homogenised in 7.0 ml of methanol using homogeniser. The contents were filtered into a previously weighed side arm flask, residue on the filtered paper was scraped off and homogenised in chloroform-methanol (1:1v/v and 2:1 v/v) and each time this extract was filtered. The pooled filters in the flask was adjusted to a final volume ratio using chloroform-methanol and evaporated to dryness. The dried residue of lipid was dissolved in 5 ml of chloroform-methanol mixture (2:1 v/v) and transferred into a centrifuge tube; 1.0 ml of 0.1 M potassium chloride was added, shaken well and centrifuged. The upper aqueous layer containing gangliosides was discarded. The chloroform layer was mixed with 1.0 ml of chloroform-methanol-potassium chloride mixture (1:10:10 v/v/v) and then centrifuged. This washing procedure was repeated thrice and each time the upper layer was discarded. The lower layer was made up to 5.0 ml and used for the analysis of total cholesterol, triglyceride, free fatty acid and phospholipids.
3.10.4.2 ESTIMATION OF SERUM CHOLESTEROL

Serum cholesterol was estimated using standard cholesterol estimation Randox kit (Catalogue no. CH 201) using Cholesterol oxidase – PAP assay

3.10.4.3 ESTIMATION OF TISSUE CHOLESTEROL

The total cholesterol was estimated by the method of Zlatkis et al., (1953).

Sample is treated with ferric chloride acetic acid reagent to precipitate the protein. The protein free filtrate containing cholesterol ferric chloride is treated with con. H$_2$SO$_4$. This reaction involves the dehydration of the 3-OH of cholesterol molecule to form cholesta 3-5 diene and then oxidised by H$_2$SO$_4$ to link two molecules together as bis-cholesta 3-5 diene, this material is sulphonated by H$_2$SO$_4$ to red coloured disulphonic acid in the presence of ferric ion as catalyst (Salkaowski’s reaction). The colour developed was read at 560 nm using a suitable standard and a reagent blank.

Reagents

1. Ferric chloride-acetic acid reagent – 0.05%
3. Cholesterol working standard - 40µg/ml in ferric chloride-acetic acid reagent.

Procedure

To 0.1 ml of the lipid extract, 9.9 ml of ferric chloride-acetic acid reagent was added and allowed to stand for 15 minutes and then centrifuged. To 5.0 ml of the supernatant, 3.0 ml of concentrated H$_2$SO$_4$ was added. The colour developed was read
after 20 minutes at 560 nm against a reagent blank. A set of standards was also performed in a similar manner.

Values were expressed as mg/100mg of wet tissue.

3.10.4.4 Estimation of Serum Triglycerides

Serum triglycerides level was estimated using standard Randox kit (Catalogue no. TR 1697) by Glycerol-3-phosphate oxidase-4-aminophenazone method.

3.10.4.5 Estimation of Tissue Triglycerides

Triglyceride was estimated by the method of Rice (1970).

Reagents

1. Chloroform-Methanol mixture – 2:1 (v/v)

2. Saturated sodium chloride solution.

3. Activated silicic acid – It was obtained by washing the silicic acid with HCl and then with water until it become neutral. After drying, ether was added in sufficient amounts and stirred well and left for few seconds. The supernatant was decanted and the silicic acid was dried at 60°C and then activated overnight at 100°C, prior to use.

4. H₂SO₄ – 0.2 N

5. Potassium hydroxide in alcohol – 0.4 % (prepared fresh)

6. Sodium meta periodate – 0.1 M (prepared fresh)

7. Sodium meta arsenite – 0.5 M

8. Chromotropic acid reagent – 1.41 gm of chromotropic acid was dissolved in 100 ml of water and stored as a stock solution in a brown bottle. On the
day of the experiment 10 ml of the solution was mixed with 45 ml of 
H$_2$SO$_4$ : water (2:1) and used

9. Thiourea solution – 7 %

10. Standard tripalmitin – A standard solution containing 100 mg/ml was 
prepared in chloroform. A working standard with a concentration of 0.1 
mg /ml was prepared by diluting 1 volume of stock to 10 volumes with 
chloroform.

**Procedure**

0.2 ml of tissue lipid extract was mixed with 9.8 ml of chloroform-methanol 
mixture (2:1) and shaken vigorously and allowed to stand for 30 minutes. This was 
centrifuged and 4.0 ml of lipid extract was added to tubes containing 8.0 ml of 
saturated saline solution. The tubes were stoppered, shaken vigorously and allowed to 
stand for 1 hour and centrifuged. The upper aqueous layer was discarded and the 
chloroform layer containing the lipids was filtered into a dry tube.

200 mg of activated silicic acid was added to filtered lipid extract. The mixture 
was shaken gently allowed to stand for 1 hour and then centrifuged. 0.5 ml of the 
supernatant was taken in a test tube and dried at 70°C. Standard solutions of 
tripalmitin (10-50 µg) were taken in test tubes and similarly evaporated together with 
a blank containing the solvent alone. After cooling, 0.5 ml of alcoholic potassium 
hydroxide was added to all the tubes and the mixture was saponified at 60-70°C water 
bath for 20 minutes. 0.5 ml of 0.2N H$_2$SO$_4$ was then added and placed in a boiling 
water bath for 10 minutes. Cooled and then 0.1 ml of sodium meta periodate and 
sodium meta arsenite was added. 5.0 ml of chromotropic acid was added to each tube 
mixed and kept in a boiling water bath for 30 minutes. After cooling, 0.5 ml of
thiourea solution was added. The colour developed was read at 570 nm against a reagent blank. The values were expressed as mg/100 mg of wet tissue.

3.10.4.6 Estimation of Serum HDL – Cholesterol

Serum HDL-cholesterol was estimated as that of serum cholesterol estimation after precipitating the LDL and VLDL using standard HDL-cholesterol precipitant from Randox (Catalogue no. CH 204)

3.10.4.7 Estimation of LDL-Cholesterol

The LDL cholesterol was calculated using the formula LDL = Total cholesterol – [HDL cholesterol + (triglycerides/5)]

3.10.4.8 Estimation of Serum and Tissue Phospholipids

Phospholipid was estimated by the method of Bartlette (1959) by digestion with perchloric acid and the phosphorus liberated was estimated by the method of Fiske and Subbarow (1925)

Reagents

1. Perchloric acid

2. Molybdic acid – 2.5 % of ammonium molybdate in 3 N H2SO4

3. Amino napthol sulphonlic acid (ANSA) – 500 mg of amino napthol sulphonic acid was dissolved in 195 ml 15 % sodium bisulfite and 5 ml 20 % sodium sulphite for complete solubilisation. The solution was filtered at 4°C in a brown bottle.

4. Stock standard – 35.1 mg of potassium dihydrogen phosphate was dissolved in 100 ml of water.
**Procedure**

0.1 ml of the sample (lipid extract or serum) was digested with 0.2 ml of perchloric acid over a sand bath. Digestion was continued until it turns colourless. The liberated inorganic phosphorus of the digested sample was then estimated.

4.3 ml of distilled water was added to the digested sample followed by 0.5 ml of ammonium molybdate. After 10 minutes, 0.2 ml of ANSA was added. The tubes were shaken well and kept aside for 20 min and the blue colour developed was read at 620 nm against water blank. Standards and blanks were also treated similarly. The total phospholipids were calculated by multiplying the value of phospholipids with 25 and expressed as mg/mg of wet tissue.

**3.10.4.9 Estimation of Serum and Tissue Free Fatty Acids**

Non-esterified free fatty acids were estimated by the method of Falholt *et al.*, (1973).

In the presence of phosphate buffer, the extract was shaken with a high copper reagent (pH-8.1). The free fatty acids in the copper soap were determined colorimetrically with diphenyl carbazide.

**Reagents**

1. Extraction solvent – chloroform : heptane : methanol (5:5:1)
2. 33 mM Phosphate buffer, pH-6.4
3. Stock copper solution : 500 mM
4. 1 M Triethanolamine
5. 1 M NaOH
6. Copper reagent: 10 ml of copper solution was mixed with 10 ml of triethanolamine and 6 ml of 1 M NaOH and diluted to 100 ml, then 33 gm of sodium chloride was added and the pH was adjusted to 8.1.

7. 1.5 M Diphenyl carbazide solution in ethanol

8. Standard palmitic acid – 2 mM

**Procedure**

0.1 ml of lipid extract or serum was evaporated to dryness. 1.0 ml of phosphate buffer, 6.0 ml of extraction solvent and 2.5 ml of copper reagent were added. All the tubes were shaken vigorously for 90 seconds and were kept aside for 15 minutes. The tubes were centrifuged. 3.0 ml of the copper layer was transferred to another tube containing 2.0 ml of diphenyl carbazide and mixed carefully. The absorbance was read at 550 nm immediately. 3.0ml of phosphate buffer was treated as blank.

The amount of free fatty acids estimated was expressed as mg/100 mg of tissue.

**3.10.4.10 β-HYDROXY-β-METHYLGLUTARYL COA-REDUCTASE (HMG-CoA REDUCTASE, EC 1.1.1.34)**

HMG-CoA reductase assay was carried out by the method of Rao and Ramakrishnan (1975)

**Reagent**

1. Saline arsenate – 1 gram of sodium arsenate in 1 liter of physiological saline.

2. Dilute perchloric acid – 50 ml of concentrated perchloric acid was diluted 1 liter with distilled water.
3. Hydroxylamine hydrochloride reagent – 138.98 gm of hydroxylamine hydrochloride reagent was dissolved in one litre of distilled water.

4. Hydroxylamine hydrochloride reagent for mevalonate – Equal volume of hydroxylamine hydrochloride and water was mixed freshly before use.

5. Hydroxylamine hydrochloride reagent for HMG-CoA – Equal volume of hydroxylamine hydrochloride and 4.5 N NaOH was mixed freshly before use.

6. Ferric chloride reagent – 5.2 gm of TCA and 10 gm of ferric chloride was dissolved in 50 ml of 0.65 N HCl and diluted to 100 ml with water.

**Procedure**

1.0 ml of 10 % of freshly prepared homogenate (heart and liver) and 1.0 ml of dilute perchloric acid was mixed, kept for 5 minutes and centrifuged at 2000 rpm for 10 minutes. To 0.75 ml of the supernatant 0.375 ml of freshly prepared aqueous hydroxylamine hydrochloride reagent prepared fresh for mevalonate and alkaline hydroxylamine hydrochloride reagent prepared fresh for HMG-CoA was added and mixed well. After 5 minutes, 1.0 ml of ferric chloride reagent was added and shaken well and read after 10 minutes at 540 nm against similarly treated saline arsenate blank.

The ratio between the absorbance of the HMG-CoA and mevalonate was taken as the HMG-CoA activity, lower the ratio higher the activity.

**3.10.4.11 LIPOPROTEIN LIPOASE (EC 3.1.1.34)**

LPL was assayed in plasma and tissues by the method of Korn (1955). In which albumin was added to the incubation tube to bind the unesterified fatty acids which otherwise will inhibit the reaction. Ammonium ions were also added to provide
the necessary activating cation. The amount of glycerol liberated was determined colorimetrically.

**Reagents**

1. 0.5 M Ammonium sulphate.
2. 2 % Bovine serum albumin, pH 8.5.
3. Enzyme source: plasma homogenate prepared in 0.2 M Tris–HCl buffer, pH 8.5.
4. Substrate: 13.2 mg glycerol trioleate and 18 mg BSA were homogenized in 2.0 mL of 0.2 M Tris-HCl buffer of pH 8.5.
5. 0.05 M Sodium periodate: 11.4 gm of periodic acid was dissolved in 900 mL of water, adjusted to pH 8.5 with 1N NaOH and made upto 1L with H$_2$O.
6. 0.5 M Sodium arsenate: 2.25 gm of NaOH and 50 gm arsenic oxide were dissolved in 1 L distilled water.
7. 1 N H$_2$SO$_4$
8. Chromotropic acid (1,8-dihydroxy naphthalene disulfonic acid): 1.0 gm of chromotropic acid was dissolved in 100 ml water and added to 400 ml H$_2$SO$_4$ (2:1 concentrated H$_2$SO$_4$ with water). The reagent was cooled throughout the mixing procedure and prepared fresh every day.

**Procedure**

*Preparation of sample*

A known amount of the sample (plasma or tissue) was homogenized in 20 ml of acetone. This was filtered and the residue in the filter paper was scrapped off, weighed and suspended in 1.0 mL of 0.025 M ammonia for 30 min at 0°C for complete extraction of enzyme. This extract was used as the enzyme source.
Incubation

The incubation mixture consisted of 0.4 ml albumin, 0.1 ml ammonium sulphate, 0.1 ml substrate, 0.1 ml enzyme and sufficient water to make up a final volume of 1.0 ml. The reagents were kept cold while mixing and the tubes were incubated at 37°C for 1 hour removing aliquots of 0.2 ml at 15 or 30 min intervals. The first sample was removed before placing the tube in the water bath. The samples were transferred directly into a 10 ml conical tip centrifuge tube containing 0.1 ml of 1N H$_2$SO$_4$.

Glycerol determination

0.1 ml periodate was added to the centrifuge tube, mixed well and allowed to stand at room temperature for 5 min. Then 0.1 ml sodium arsenate reagent was added, mixed well and again allowed to stand at room temperature for 10 min. This was followed by the addition of 9.0 ml chromotropic acid, mixed by inversion (covering the top of the tube by paraffin) and placed in a boiling water bath for 30 min, cooled and the volume adjusted to 10 ml with water. The optical density was read at 570 nm. The assay was standardized against a glycerol solution of known molarity.

The activity of lipoprotein lipase is expressed as $\mu$moles of glycerol liberated/hr/ml plasma or $\mu$moles of glycerol liberated per hour/mg protein.

3.10.4.12 Lecithin Cholesterol Acyl Transferase (LCAT, EC 2.3.1.43)

The activity of LCAT was assayed by the method of Hitz et al., (1983)

Reagents

1. Dextran sulphate - 0.2%
2. Isopropanol
3. Acetone
4. Digitonin - 5.0 mg/ml in 50 % of ethanol
5. Substrate – Fresh plasma was heated at 56°C for 30 minutes to inactivate LCAT. The inactivated plasma was incubated at 4°C for 15 minutes with 0.2 % of dextran sulphate. Dextran sulphate eliminates 2/3rd of the lipoprotein fraction (LDL and VLDL) which was centrifuged at 1750 x g for 15 minutes to precipitate and the supernatant containing HDL was used as substrate.

**Procedure**

To 1.0 ml of plasma or tissue (heart or liver) homogenate 0.5 ml of 0.2 % dextran sulphate was added. The mixture was incubated at 4°C for 15 minutes and centrifuged at 1750g for 15 minutes. The supernatant were separated and used as the enzyme source and its activity was assayed as given below.

0.6 ml of substrate was mixed with 0.6 ml of enzyme and was incubated at 27°C. 0.2 ml of the mixture was taken at 0, 90 and 180 minutes and mixed with 1.0 ml of isopropanol to arrest the reaction. The precipitate was removed by centrifugation and the supernatant was taken for the estimation of free cholesterol (Zlatkis et al., 1953). This represents the amount of free cholesterol present in the test sample at 0, 90 and 180 minutes. Control tubes containing only the substrate were treated similarly to check for complete inactivation of plasma during substrate preparation. LCAT activity was expressed as a function of the disappearance of free cholesterol during the incubation period given as µM of cholesterol esterified per hour per ml for plasma and mg of protein for homogenate.

### 3.10.5 MITOCHONDRIAL ENZYMES AND ANTIOXIDANT SYSTEM

#### 3.10.5.1 ISOLATION OF MITOCHONDRIA

Heart mitochondrion was isolated by the method of Takasawa et al., (1993). The heart tissue was homogenized in ice cold 50 mM Tris–HCl (pH 7.4) containing
0.25 M sucrose. The homogenates were centrifuged at 700 × g for 20 min, and then the supernatants obtained were centrifuged at 9000 × g for 15 min. The obtained pellets were washed with 10 mM Tris–HCl (pH 7.8) containing 0.25 M sucrose and finally resuspended in the same buffer.

### 3.10.5.2 Estimation of TBARS in Mitochondria

The TBARS content in mitochondrial fraction was estimated by the method of Nichans and Samuelson (1968) as described in section 3.6.4.1.

### 3.10.5.3 Mitochondrial Antioxidant System

The mitochondrial antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase and reduced glutathione were estimated by the method of Kakkar et al., (1984), Sinha (1972), Rotruck et al., (1973), Habig and Jakoby (1981) and Ellman (1959) respectively as described in the section 3.6.5.

### 3.10.5.4 Assay of Isocitrate Dehydrogenase (ICDH, EC 1.1.1.42)

The enzyme activity was assayed by the method of King (1965b).

**Reagents**

1. 0.1 M Tris-HCl, pH 7.5.
2. 0.9 M Trisodium isocitrate in 0.15 M NaCl
3. 0.015 M Manganese chloride
4. 0.001 M NADP⁺
5. 0.001 M DNPH in 1 N HCl
6. 5 % EDTA
7. 0.4 N NaOH
8. 15 mg of α-ketoglutarate in 50 ml of 0.1 M Tris-HCl, pH 7.4
Procedure

To 0.4 ml of Tris-HCl, 0.2 ml of trisodium isocitrate, 0.3 ml of manganese chloride and 0.2 ml of mitochondrial suspension and 0.2 ml of NADP+ (0.2 ml of saline for control) were added. After 60 min of incubation, 1.0 ml of DNPH was added followed by 0.5 ml of EDTA and kept at room temperature for 20 min. Then 10 ml of NaOH was added and the colour developed was read at 420 nm. A standard containing α-ketoglutarate was run simultaneously.

The isocitrate dehydrogenase activity was expressed as nmoles of α-ketoglutarate formed/hr/mg protein.

**3.10.5.5 Assay of α-Ketoglutarate Dehydrogenase (α-KGDH, EC 1.2.4.2)**

The activity of α-ketoglutarate dehydrogenase was assayed by the method of Reed and Mukherjee (1969).

**Reagents**

1. 1 M Potassium phosphate buffer, pH 6.0
2. 0.5 M α-ketoglutarate
3. 0.0002 M Thiamine pyrophosphate
4. 0.003 M Magnesium chloride
5. 0.25 M Potassium ferricyanide
6. 10 % TCA
7. 4 % Duponol
8. Ferric ammonium sulphate – duponol reagent: 1.7 g of ferric ammonium sulphate was dissolved in 10 ml of water and this mixture was filtered. Then 20 ml of a solution containing 1.5 g of duponol was added to the filtrate. To this solution, 27 ml of 85 % O-phosphoric acid was added and made up to 140 ml with water.
9. 0.01 % Potassium ferricyanide.

**Procedure**

To 0.15 ml of phosphate buffer, 0.1 ml each of thiamine pyrophosphate, magnesium sulphate, α-ketoglutarate and potassium ferric cyanide was added. The total volume was made up to 1.2 ml with water and 0.2 ml of mitochondrial suspension was added and incubated at 30 °C for 30 min. Then 1.0 ml of TCA was added and centrifuged. 0.2 ml of mitochondrial suspension was added to the control after the addition of TCA. To the supernatant, 0.1 ml of potassium ferricyanide, 1 ml of duponol and 0.5 ml of ferric ammonium sulphate-duponol reagent were added and then incubated at 25 °C for 30 min. The colour developed was measured at 540 nm. A standard containing potassium ferrocyanide was carried out simultaneously.

The activity of α-ketoglutarate dehydrogenase was expressed as nmoles of ferrocyanide formed/hr/mg protein.

**3.10.5.6 ASSAY OF SUCCINATE DEHYDROGENASE (SDH, EC 1.3.99.1)**

The activity of succinate dehydrogenase was assayed according to the method of Slater amd Bonner (1952).

**Reagents**

1. 0.3 M Phosphate buffer, pH 7.6
2. 0.03 M EDTA
3. 0.03 M Potassium cyanide
4. 0.4 M Sodium succinate
5. 3 % BSA
6. 0.075 M Potassium ferricyanide
**Procedure**

The reaction mixture containing 1 ml of phosphate buffer, 0.1 ml of EDTA, 0.1 ml of BSA, 0.3 ml of sodium succinate and 0.2 ml of potassium ferricyanide were made upto 2.8 ml with double distilled water. The reaction was started by the addition of 0.2 ml of mitochondrial suspension. The change in OD was recorded at 15 sec interval for 5 min at 420 nm.

The succinate dehydrogenase activity was expressed as nmoles of succinate oxidized/min/mg protein.

**3.10.5.7 ASSAY OF MALATE DEHYDROGENASE (MDH, EC 1.1.1.37)**

The activity of malate dehydrogenase was assayed by the method of Mehler et.al. (1948).

**Reagents**

1. 0.25 M Potassium phosphate buffer, pH 7.4
2. 0.0076 M Oxaloacetate
3. 0.005 M NADH

**Procedure**

The reaction mixture contained 0.75 ml of phosphate buffer, 0.15 ml of NADH, 0.75 ml of oxaloacetate. The reaction was carried out at 25°C and was started by the addition of 0.2 ml mitochondrial suspension. The control tubes contained all reagents except NADH. The change in OD at 340 nm was measured for 2 min at an interval of 15 seconds in UV spectrophotometer.

The activity of the enzyme was expressed as nmoles of NADH oxidized/min/mg protein.
3.10.5.8 ASSAY OF NADH-DEHYDROGENASE (EC 1.6.99.3)

The activity of NADH dehydrogenase was assayed according to the method of Minakami et al. (1962).

Reagents

1. 0.1 M Phosphate buffer, pH 7.4
2. 0.1 % NADH
3. 0.03 M Potassium ferricyanide

Procedure

The reaction mixture contained 1.0 ml of phosphate buffer, 0.1 ml of potassium ferricyanide, 0.1 ml of NADH and 0.2 ml of mitochondrial suspension. The total volume was made up to 3 ml with water. NADH was added just before the addition of the enzyme. A control was also treated similarly without NADH. The change in OD was measured at 420 nm as function of time for 3 min at an interval of 15 seconds.

The activity of NADH dehydrogenase was expressed as nmoles of NADH oxidized/min/mg protein.

3.10.5.9 CYTOCHROME–C-OXIDASE (EC 1.9.3.1)

The activity of Cytochrome-C-oxidase was assayed by the method of Pearl et al. (1963).

Reagents

1. 0.03 M Phosphate buffer
2. 0.01 % Cytochrome C
3. 0.2 % N-phenyl-p-phenylene diamine
Procedure

The reaction mixture contained 1.0 ml of phosphate buffer, 0.2 ml of 0.2 % N-phenylene diamine, 0.1 ml of 0.01 % cytochrome C and 0.5 ml of water. The sample was incubated at 25 °C for 5 min. 0.2 ml of the enzyme preparation was added and the change in OD was recorded at 550 nm for 5 min at an interval of 15 sec each. A control containing all reagents except cytochrome C was also processed in the same manner.

The enzyme activity was expressed as nmoles/min/mg protein.

3.10.6 ASSAY OF TOTAL LYSOSOMAL HYDROLASES ACTIVITIES

3.10.6.1 PREPARATION OF SAMPLE FOR TOTAL LYSOSOMAL HYDROLASES AND MEMBRANE BOUND PHOSPHATASES

About 200 mg of the heart tissue was homogenized in 5.0 ml of 0.1 M Tris-HCl buffer (pH 7.4) solution. The homogenate was centrifuged at 3000 rpm for 15 minutes and the supernatant was used for the various biochemical parameters.

3.10.6.2 ASSAY OF β-GlUCURONIDASE (EC 3.2.1.31)

The activity of β-Glucuronidase was determined by the method of Kawai and Anno (1971).

Reagents

1. 0.1 M Sodium acetate buffer, pH 5.0
2. 2 mM p-nitrophenyl β-D-glucuronide
3. 0.2 M glycine – NaOH buffer containing 2 M SDS, pH 11.7
4. 0.5 M NaOH

Procedure

Known aliquot (0.2 ml) of the enzyme source was added to 0.5 ml of incubation buffer containing 2 mM substrate (final concentration) and incubated at 37
°C for 2 hours. The substrate p-nitrophenyl β-D-glucuronide was dissolved in 0.1 M acetate buffer. At the end of the incubation period, the reaction was stopped by the addition of 4.0 ml of 0.2 M glycine-NaOH buffer (pH 11.7) containing 2 M SDS and the contents were centrifuged. To the aliquots of supernatants, 0.5 M NaOH was added and the absorbance was measured at 410 nm.

The activity was expressed as μmoles of p-nitrophenol liberated/hr/100 mg protein.

3.10.6.3 ASSAY OF β-N-ACETYL GLUCOSAMINIDASE (EC 3.2.1.30)

The activity of β-N-Acetyl glucosaminidase was determined by the procedure of Moore and Morris (1982).

Reagents

1. 0.1 M Citrate buffer, pH 4.5
2. 2 mM p-nitrophenyl β-N-acetyl glucosaminide
3. 0.2 M glycine – NaOH buffer containing 2 M SDS, pH 11.7
4. 6 mM Standard p-nitrophenol

Procedure

0.2 ml of enzyme source was added to 0.5 ml of incubation buffer containing 2 mM substrate (final concentration) and incubated at 37 °C for 2 hrs. The substrate p-nitrophenyl β-N-acetyl glucosaminide was dissolved in 0.1 M Citrate buffer. At the end of the incubation period, the reaction was stopped by the addition of 4.0 ml of 0.2 M glycine – NaOH buffer, pH 11.7 containing 2 M SDS and contents were centrifuged. To the aliquots of the supernatant 0.5 M NaOH was added and the absorbance was measured at 410 nm.

The activity was expressed as μmoles of p-nitrophenol liberated/hr/100 mg protein.
3.10.6.4 ESTIMATION OF β-D-GALACTOSIDASE (EC 3.2.1.23)

The activity of β-D-Galactosidase was assayed by the method of Conchie et al. (1967).

**Reagents**

1. 0.2 M Na$_2$HPO$_4$ – 0.1 M citric acid, pH 5.0
2. 5 mM p-nitrophenyl β-D-Galactoside
3. 0.4 M glycine – NaOH buffer, pH 10.4
4. p-nitrophenol standard

**Procedure**

The incubation mixture contained 2.0 ml of 0.2 M Na$_2$HPO$_4$ – 0.1 M citric acid buffer, 0.5 ml of 5 mM p-nitrophenyl β-D-galactoside and 0.5 ml of enzyme source. Incubation was carried out for 1 hr at 37 °C. The reaction was terminated by the addition of 4.0 ml of glycine – NaOH buffer. The reaction mixture was centrifuged and the absorbance of the released p-nitrophenol in the supernatant was measured at 410 nm using a spectrophotometer. A standard p-nitrophenol was run simultaneously. The activity was expressed as µmoles of p-nitrophenol liberated/hr/100 mg protein.

3.10.6.5 ASSAY OF CATHEPSIN D (EC 3.4.23.50)

Cathepsin D activity was determined by the method of Sapolsky et al. (1973)

**Reagents**

1. 1.5 % haemoglobin in 0.1 M acetate buffer, pH 3.0
2. 1N NaOH
3. 10 % TCA
4. Folin’s phenol reagent
Procedure

Known aliquots (0.2 ml) of the enzyme source were incubated with 1.5 % haemoglobin in 0.1 M acetate buffer, pH 3.0 for 2 hrs. The enzyme activity was arrested by the addition of 10 % TCA and the liberated TCA soluble products were filtered and neutralized with 1 N NaOH. The tyrosine content of the filtrate was determined using Folin’s phenol reagent essentially employing the procedure of Lowry et al. (1951). The blue colour developed was read at 660 nm.

The enzyme activity was expressed as µmoles of tyrosine liberated/hr/100 mg protein at 37 °C.

3.10.6.6 ACID PHOSPHATASE (ACP, EC 3.1.3.2)

Acid phosphatase was assayed by the method of King (1965c).

Reagents

1. 0.1 M Acetate buffer, pH 4.8
2. 0.01 M Disodium phenyl phosphate solution
3. Folin’s phenol reagent
4. 15 % Sodium carbonate
5. Standard Phenol: 100 mg of recrystallised phenol in 100 ml of water, 100 µg of phenol/ml was prepared by proper dilution and used as working standard.

Procedure

The incubation mixture of final volume 3.0 ml was made containing 1.5 ml of buffer, 1.0 ml of substrate and required amount of the enzyme source. The tubes were incubated at 37°C for 15 min. The reaction was arrested by the addition of 1.0 ml of Folin’s phenol reagent. To the control tubes the enzyme was added after arresting the reaction. The contents were centrifuged and 1.0 ml of 15 % sodium carbonate was
added to the supernatant. The mixture was incubated for 15 min at 37°C and the
colour was read at 640 nm using a spectrophotometer.

The enzyme activity was expressed as µmoles of phenol liberated/hr/100 mg
protein.

3.10.7 Estimation of Plasma and Tissue Glycoproteins

3.10.7.1 Preparation of Sample

About 200 mg of heart tissue was defatted as per method of Folch et al., (1957)
and the defatted tissue was suspended in 3.0 ml of 2 N Hydrochloric acid and heated at
90 °C for 4 hour. The sample was cooled and neutralized with 3.0 ml of 2 N NaOH.
Aliquots from this were used for the estimation of hexose, hexoseamine, fucose and
sialic acid.

3.10.7.2 Estimation of Protein-bound Hexoses

Protein-bound hexoses were estimated by the method of Lusting and Langer
(1931) as employed by Weimer et al., (1954).

Reagents

1. Ethanol 95 %

2. NaOH 0.1 N

3. Orcinol – H₂SO₄ reagent

   Reagent A : 60 ml of conc. H₂SO₄ and 40 ml of water

   Reagent B: 1.6 gm of orcinol (recrystallised from benzene) in 100 ml of
   water

   7.5 ml of reagent A was mixed freshly with 1 ml of reagent B.
4. Galactose – mannose standard: 0.1 mg/ml each of galactose and mannose.

**Procedure**

To 0.1 ml of plasma, 5 ml of 95 % ethanol was added, mixed and centrifuged. The process was repeated once again. The precipitate was dissolved in 1 ml of 0.1 N NaOH. 1.0 ml of distilled water and 1.0 ml of standard was set up along with the test. To all the tubes, 8.5 ml of orcinol- H$_2$SO$_4$ reagent was added and kept at 80°C water bath for exactly 15 minutes. The tubes were cooled in tap water and the colour developed was read at 540 nm against the blank.

The values were expressed as mg/dL for plasma and for tissue as mg/mg of wet tissue.

**3.10.7.3 Estimation of Fucose**

Fucose was estimated by the method of Dische and Shettles (1948) in plasma and tissue.

**Reagents**

1. 95% Ethanol
2. 0.1 N NaOH
3. Diluted H$_2$SO$_4$: 6 volumes of conc. H$_2$SO$_4$ and 1 volume of water
4. Cysteine reagent: 3 gm of cysteine hydrochloride in 100 ml of water
5. Fucose standard: 20 µg/ml

**Procedure**

To two tubes each containing 0.1 ml of plasma, 5 ml of 95 % ethanol was added, mixed and centrifuged. The process was repeated once again. The precipitate was
dissolved in 1 ml of 0.1 N NaOH. 1.0 ml of distilled water as blank and 1.0 ml of the standard were also set up along with the test. All the tubes were kept in ice and 4.5 ml of H$_2$SO$_4$ was added. The tubes were kept in boiling water bath for exactly 3 minutes and cooled.

0.1 ml of cysteine reagent was added to all the tubes (this was omitted in one of the sample) and kept for 60-90 minutes at room temperature. The colour developed was read at 396 and 430 nm against the blank. The values were expressed as mg/dl for plasma and for tissue as mg/mg of wet tissue.

### 3.10.7.4 Estimation of Sialic Acid

Sialic acid in plasma and tissue was estimated by the method of Warren (1959).

**Reagents**

1. 0.025 M Periodic acid in 0.1 N H$_2$SO$_4$
2. 4 % Sodium meta arsenate in 0.5 N HCl
3. 144 mg of Thiobarbituric acid in 10 ml hot distilled water
4. 5 % HCl in n-butanol
5. Sialic acid standard : 0.2 mg/ml

**Procedure**

To 0.5 ml of the plasma, 0.5 ml of water and 0.25 ml of periodic acid was added and incubated at 37 °C for 30 minutes. To this 0.25 ml of sodium meta arsenate and 2.0 ml of thiobarbituric acid were added and heated in a boiling water bath for exactly six minutes. It was cooled and 5.0 ml of acidified butanol was added. The
absorbance by the extract in the organic layer was read at 540 nm against a reagent blank. The values were expressed as mg/dl for plasma and for tissue as mg/mg of wet tissue.

3.10.7.5 Estimation of Hexosamines

Hexosamines was estimated in plasma and tissue by the method of Wagner (1979).

Reagents

1. Acetylacetone reagent:

   Reagent A: 1 M trisodium phosphate

   B: 0.5 potassium tetraborate

   3. 5% acetyl acetone was prepared by mixing reagent A and B in the ratio of 98:2 v/v

2. 3 N HCl

3. 6 N NaOH

4. Ehrlich’s reagent: 320 mg of p-dimethyl aminobenzaldehyde was dissolved in 21 ml of isopropanol and 3 ml of conc. HCl.

5. Standard hexosamine: Galactosamine hydrochloride solution containing 10 mg/100 ml.

Procedure:

0.1 ml of plasma is treated with 2.5 ml of 3 N HCl for 6 hours in a boiling water bath and then neutralized with 6 N NaOH. To 0.8 ml of neutralized sample, added 0.6 ml of acetyl acetone reagent was added. The tubes were heated in a boiling
water bath for 30 minutes. After cooling 2.0 ml of Ehrlichs reagent was added and mixed well. Blank contained 1.0 ml of water and standards were processed similarly. The colour developed was read at 540 nm.

3.10.8 MEMBRANE BOUND PHOSPHATASE

3.10.8.1 Na\textsuperscript{+}/K\textsuperscript{+} ATPase (EC 3.6.3.1)

Na\textsuperscript{+}, K\textsuperscript{-} ATPase was estimated by the method of Bonting (1970).

Reagents

1. 90 mM Tris-HCl buffer, pH 7.5
2. 50 mM MgSO\textsubscript{4}
3. 50 mM KCl
4. 600 mM NaCl
5. 1 mM EDTA
6. 40 mM ATP
7. 10 % TCA
8. 2.5 % Ammonium molybdate in 5N H\textsubscript{2}SO\textsubscript{4}
9. ANSA

Procedure

The incubation mixture contained 1.0 ml of Tris-HCl buffer, 0.2 ml each of magnesium sulphate and potassium chloride, sodium chloride, EDTA, ATP and the homogenate. The mixture was incubated at 37 °C for 15 min. The reaction was arrested by the addition of 1.0 ml of 10 % TCA, mixed well and centrifuged. The phosphorus content of the supernatant was estimated according to Fiske and Subborow method (1925)

The enzyme activity was expressed as µmoles of phosphorus liberated/min/mg protein under incubation conditions.
3.10.8.2 Ca$^{2+}$-ATPase (EC 3.6.1.38)

The activity of Ca$^{2+}$-ATPase was assayed according to the method of Hjerten and Pan (1983).

**Reagents**

1. 125 mM Tris-HCl buffer, pH 8.0
2. 50 mM CaCl$_2$
3. 10 mM ATP
4. 10 % TCA

**Procedure**

The incubation mixture containing 0.1 ml each of Tris-HCl buffer, calcium chloride, ATP and homogenate. After incubation at 37 °C for 15 min, the reaction was arrested by the addition of 1.0 ml TCA. The amount of phosphorus liberated was estimated according to the method of Fiske and Subbarow (1925).

The enzyme activity was expressed as µmoles of phosphorus liberated/min/mg protein under incubation conditions.

3.10.8.3 Mg$^{2+}$-ATPase (EC 3.6.3.1)

The activity of Mg$^{2+}$-ATPase was assayed according to the method of Ohnishi et al. (1982).

**Reagents**

1. 375 mM Tris-HCl buffer, pH 7.6
2. 25 mM MgCl$_2$
3. 10 mM ATP
4. 10 % TCA
Procedure

The incubation mixture contains 0.1 ml each of Tris-HCl buffer, magnesium chloride, ATP and the homogenate. The reaction mixture was incubated at 37 °C for 15 min. The reaction was arrested by the addition of 1.0 mL of TCA. The liberated phosphorus was estimated according to the method of Fiske and Subbarow (1925).

The enzyme activity was expressed as µmoles of phosphorus liberated/min/mg protein under incubation conditions.

3.10.9 ESTIMATION OF TISSUE ELECTROLYTES

3.10.9.1 ESTIMATION OF SODIUM AND POTASSIUM IN TISSUE

The tissue was digested according to the method of Ballentine and Burford (1957). About 1.0 ml of tissue homogenate was digested in 10.0 ml of diluted acid mixture (1:1 of concentrated HNO₃ and perchloric acid). The samples were digested over a sand bath until the solution became yellow in colour. If the colour of the digest was brown, more of acid mixture was added and the oxidation was repeated. The digest was then made upto 25 ml in a volumetric flask. Samples were diluted and used for the assay.

The sodium and potassium content was estimated using a flame photometer. Standard sodium and potassium solutions (0 – 10 meq/L) were first injected and readings were recorded. The sample extract was then fed and the amount of sodium and potassium in the tissue was calculated by converting the meq/L to nM per mg of protein.

3.10.9.2 ESTIMATION OF CALCIUM

Calcium in the above digested sample was estimated using O-cresolphthalein complexone method of Morin (1974).
Calcium forms a coloured product with O-cresolphthalein complexone, which is measured at 575 nm. The reagent contains ethanol that maintains a clean solution in the presence of proteins and also suppresses the ionisation of O-cresolphthalein complexone. The 8-hydroxyquinoline binds magnesium which otherwise would cause interference.

Reagents

1. Buffer – 21 gm of diethanolamine and 30 gm of urea in about 90 ml of water. pH was adjusted to 11.7 with acetic acid and diluted to 1000 ml.

2. O-cresolphthalein complexone – To 6.4 mg of O-cresolphthalein complexone, 116 mg of 8-hydroxyquinoline and 0.25 ml of acetic acid was dissolved in 25 ml of ethanol. 30 gm of urea was added and made upto 100 ml.

Working Reagent (1) and (2) was mixed in a ratio 2:1 freshly before use.

3. Stock calcium standard (25 mM): 250 mg of anhydrous calcium carbonate was dissolved in 50 ml of 0.1N HCl and made upto 250 ml with distilled water.

4. Working standard calcium (2.5 mM): About 10 ml of the stock solution was diluted to 100 ml with distilled water.

Procedure

About 500 µl of the sample was incubated with 5.0 ml of O-cresolphthalein complexone working reagent and it was read against a reagent blank at 578 nm. A series of standards of concentration 62.5 – 250 nM/L was also processed same as that of the sample and the amount of calcium was expressed as nM/mg of protein.
3.11 **Histopathological Studies**

The heart and liver tissue from all the experimental animals were washed well with physiological saline and it was fixed in neutral buffered formalin. The fixed tissue was embedded in paraffin. A thin film of 4 μm thickness was sectioned and stained with hematoxylin and eosin (H&E). The processed film was examined under the light microscope and photomicrograph was taken.

3.12 **Statistical Analysis**

Statistical analysis was performed using the SPSS software package, Version 12.0. The values were analyzed by one-way analysis of variance followed by Duncan’s multiple range test (DMRT) (Duncan, 1957). All the results were expressed as mean ± SD for six rats in each group. \( p < 0.05 \) was considered as significant. Pearson correlation coefficient was also using SPSS 12.0 for windows software.

Linear regression analysis was performed for *in vitro* free radical scavenging activities and IC\(_{50}\) was calculated and represented as mean ± standard deviation of triplicate sample using Graphpad Instat software Inc., Version 3.01.