Chapter 2

MATERIALS AND METHODS
2. Materials and Methods

2.1. Materials

2.1.1. Plant material

*Gardenia gummifera* Linn. f. root was collected from its natural habitat (Idukki, Kerala, India). The plant was identified by Dr. V.T Antony, Department of Botany, S.B College Changanassery, Kottayam. A voucher specimen (SBSBRL.05) is maintained in the School of Biosciences, Mahatma Gandhi University, Kottayam.

2.1.2. Experimental Animals

Male albino rats of Wistar strain were used in this study. They were purchased from Small Animals Breeding Centre of Kerala Agricultural University, Mannuthy, Trichur. The animals were housed in polypropylene cages kept in the animal house of School of Biosciences, Mahatma Gandhi University, Kottayam. All the rats were maintained at a controlled condition of temperature of 26–28 °C with a 12 h light: 12 h dark cycle and were given standard rat chow (Sai Feeds, Bangalore, India) and drinking water *ad libitum*. Animal studies were followed according to Institutional Animal Ethics Committee (IAEC) regulations approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (Reg. No. B 2442009/3) and conducted humanely.

2.1.3. Chemicals and reagent kits

1-chloro-2, 4-dinitrobenzene (CDNB) : *Sisco Research Laboratories* (SRL), India.

5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB) : -do-

2,3,5-Triphenyl Tetrazolium Chloride (TTC) : -do-
Bovine Serum Albumin (BSA) : -do-
Ethylene diamine tetra acetate (EDTA) : -do-
Folin - Ciocalteau reagent : -do-
Iodine : -do-
Glucose-6-phosphate : -do-
L-malate : -do-
NADPH : -do-
Nitroblue tetrazolium (NBT) : -do-
Oxidized glutathione (GSSG) : -do-
Reduced glutathione (GSH) : -do-
Sodium azide : -do-
Trichloroacetic acid (TCA) : -do-
Tris-HCl : -do-
Cholesterol : -do-
Sodium cholate : -do-
Atorvastatin : Ranbaxy (New Delhi and Goa, India)
Chloroform : Merck, India
Citric acid : -do-
Dimethylsulphoxide (DMSO) : -do-
Di-sodium hydrogen phosphate : -do-
DPX mountant : -do-
<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acetate</td>
<td>-do-</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>-do-</td>
</tr>
<tr>
<td>Glycerol</td>
<td>-do-</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>-do-</td>
</tr>
<tr>
<td>Methanol</td>
<td>-do-</td>
</tr>
<tr>
<td>Paraffin wax</td>
<td>-do-</td>
</tr>
<tr>
<td>Petroleum ether</td>
<td>-do-</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>-do-</td>
</tr>
<tr>
<td>Potassium permanganate</td>
<td>-do-</td>
</tr>
<tr>
<td>Sodium carbonate</td>
<td>-do-</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>-do-</td>
</tr>
<tr>
<td>Sodium di-hydrogen phosphate</td>
<td>-do-</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>-do-</td>
</tr>
<tr>
<td>Sulphuric acid</td>
<td>-do-</td>
</tr>
<tr>
<td>Tri sodium citrate</td>
<td>-do-</td>
</tr>
<tr>
<td>Tween-80</td>
<td>-do-</td>
</tr>
<tr>
<td>Xylene</td>
<td>-do-</td>
</tr>
<tr>
<td>Ferric chloride</td>
<td>Central Drug House (CDH), India</td>
</tr>
<tr>
<td>Thiobarbituric acid (TBA)</td>
<td>-do-</td>
</tr>
<tr>
<td>Eosin</td>
<td>Nice chemicals, India</td>
</tr>
<tr>
<td>Formaldehyde solution</td>
<td>-do-</td>
</tr>
</tbody>
</table>
Hematoxylin : -do-
Hydrogen peroxide (H₂O₂) : -do-

2.1.4. Diagnostic reagents and kits
Aspartate aminotransferase (AST) : Agappe Diagnostic Ltd, India.
Alanine aminotransferase (ALT) : -do-
Alkaline phosphatase (ALP) : -do-
Lactate dehydrogenase (LDH) : -do-
Serum total cholesterol : -do-
Triglycerides : -do-
Haemoglobin : -do-
High density lipoprotein (HDL-C) : -do-
Creatine phosphokinase (CPK) : -do-
Creatine kinase isoenzyme (CK-MB) : -do-
Creatinine : -do-
Glucose : -do-
Uric acid : -do-
Ceruloplasmin : -do-
Iron and iron binding capacity : -do-
Urea : -do-
2.1.5. Reagents

2.1.5.1. Phosphate Buffered Saline (PBS)

NaCl 8.00 g  
KCl 0.20 g  
$\text{Na}_2\text{HPO}_4\cdot2\text{H}_2\text{O}$ 1.44 g  
$\text{KH}_2\text{PO}_4$ 0.24 g  

Dissolve in 800 mL distilled water. Adjust to pH 7.4 with 1N HCl and make up to 1000 mL.

2.1.5.2. Mayer's Hematoxylin

Ammonium alum 50 g  
Hematoxylin 1.0 g  
Sodium iodate 0.2 g  
Citric acid 1.0 g  
Distilled water 1000 mL  

Dissolve alum in distilled water. When alum is completely dissolved, add hematoxylin. When hematoxylin is completely dissolved, add sodium iodate and acetic acid. Bring to boil and cool. Filter if it is necessary.

2.1.5.3. Tris Buffered Saline (TBS)

Tris 2.42 g  
NaCl 14.6 g  

Dissolve in 800 mL distilled water. Adjust to pH 7.4 with 1N HCl and make up to 1000 mL.
2.1.5.4. Neutral buffered formalin (10%)

Formalin (40% Formaldehyde) 100 mL

Sodium phosphate – monobasic monohydrate 4.0 g

Sodium phosphate – dibasic anhydrous 6.5 g

Total volume make up to 1000 mL with distilled water.

2.1.6. Instruments

Rotary evaporator : Superfit DB3135H, India.

Spectrophotometer : Hitachi U-2800, Japan.

Weighing balance : Sartorius, Germany.

Cooling centrifuge : Remi, India.

Inverted Microscope : Motic AE 21, Germany.

Microplate reader : BioRad, USA.

Deep freezer (-20°C) : Celfrost, India.

pH meter : pH 700, Eutech Instruments, Singapore.

Microscope : Magnus, India.

Semi autoanalyzer : RMS BCA201, India.

2.1.7. Glassware and plastic wares

Glassware and plastic wares were obtained from Borosil, Mumbai and Tarsons, Kolkata, respectively.
2.2. Methods

2.2.1. Preparation of plant extracts

*G. gummifera* roots were thoroughly washed using running tap water followed by rinsing with distilled water. Roots were then chopped, shade-dried and powdered. The soxhlet extraction procedure was carried out using various solvents of increasing polarity, i.e., petroleum ether, chloroform, acetone, ethanol and methanol. About 400 ml of the solvent is poured into the round bottom extraction flask and placed on the heating mantle atop which is placed the thimble containing 50g of the dried plant powder. The condenser was placed above the thimble and the parts were fixed vertically. The extraction was carried out for 48 hours. The extract was concentrated under reduced pressure using a rotary evaporator and was kept under refrigeration. The extraction was repeated with a new set of dried powder until the required quantity was achieved.

2.2.2. Preliminary phytochemical screening

Preliminary phytochemical screening of petroleum ether (PEGG), Chloroform (CHGG), acetone (ACGG), ethanol (ETGG) and methanolic extracts (MEGG) of *G. gummifera* Linn. f. roots were carried out for the detection of phytoconstituents using standard conventional protocols (Kokate et al., 2009; Evans and Trease, 2002; Khandelwal, 1995).

2.2.2.1. Test for alkaloids

a). Dragendorff’s test: To 1 ml of the extract, 1 ml of Dragendorff’s reagent (potassium bismuth iodide solution) was added. Formation of an orange-red precipitate indicated the presence of alkaloids.
b). Mayer’s test: 1 ml of Mayer’s reagent (potassium mercuric iodide solution) was added to 1 ml of the extract and the formation of cream colored precipitate confirmed the presence of alkaloids.

c). Hager’s test: To 1 ml of the extract, 3 ml of Hager’s reagent (saturated aqueous solution of picric acid) was added. Presence of alkaloids was confirmed by the formation of yellow colored precipitate.

d). Wagner’s test: To 1 ml of the extract 2 ml of Wagner’s reagent (iodine in potassium iodide) was added. Presence of alkaloids was confirmed by the formation of reddish brown colored precipitate.

2.2.2.2. Test for Flavonoids

a). Little quantity of extract was treated with amyl alcohol, sodium acetate and ferric chloride. A yellow color solution formed, disappeared on addition of an acid indicated the presence of flavonoids.

b). Shinoda’s test: The extract was treated with magnesium foil and concentrated hydrochloric acid. An intense cherry red color formed indicated the presence of flavonones or orange red color indicated the presence of flavonols.

c). The extract was treated with sodium hydroxide; formation of yellow color indicated the presence of flavones.

d). The extract was treated with concentrated sulphuric acid; formation of yellow or orange color confirmed the presence of flavones.

e). The extract was treated with 10% sodium chloride; formation of yellow color indicated the presence of coumarins.
2.2.2.3. **Test for Phenolic compounds and Tannins**

a). 1 ml of the test solution was mixed with basic lead acetate solution and the formation of white precipitate indicated the presence of tannins and phenolic compounds.

b). To 1 ml of the extract, ferric chloride solution was added. Formation of a dark blue or greenish black color product confirmed the presence of phenolic compounds and tannins.

c). Strong potassium dichromate solution was added to the test extract, a yellow color precipitate confirmed the presence of tannins and phenolic compounds.

d). The little quantity of the extract was treated with potassium ferric cyanide and ammonia solution. Formation of a deep red color indicated the presence of tannins.

**2.2.2.4. Test for Glycosides**

a). Legal’s test: Dissolved the extract in pyridine and added sodium nitroprusside solution to make it alkaline. Pink to red color formation showed the presence of glycosides.

b). Baljet’s test: To 1 ml of the test extract, 1ml of sodium picrate solution was added and the yellow to orange color revealed the presence of glycoside.

**2.2.2.5. Test for Steroids**

Salkowski test: Dissolved the extract in chloroform and added equal volume of concentrated sulphuric acid. Formation of bluish red to cherry color in chloroform layer and green fluorescence in the acid layer indicated the steroidal components in the tested extract.
2.2.2.6. Test for Saponins

To a small quantity of the extract 20 ml of distilled water was added and shaken in a graduated cylinder for 15 minutes lengthwise. Formation of foam indicated the presence of saponins.

2.2.2.7. Test for fixed oils and fats

a). Spot test: Pressed a small quantity of extract between the filter paper. Oil stains on paper indicated the presence of fixed oils.

b). Saponification test: Few drops of 0.5N alcoholic potassium hydroxide were added to a small quantity of extract along with a drop of phenolphthalein. The mixture was heated on water bath for 1-2 hours. Formation of soap or partial neutralization of alkali indicated the presence of fixed oils and fats.

2.2.2.8. Test for carbohydrates

a). Molisch’s test: To 2 ml of the extract, added 1 ml of α-napthol solution, followed by concentrated sulphuric acid through the sides of the test tube. Purple or reddish violet color at the junction of the two liquids revealed the presence of carbohydrates.

b). Fehling’s test: To 1 ml of the extract, added equal quantities of Fehling solution A and B and heated for 2 minutes. Formation of a brick red precipitate confirmed the presence of sugars.

c). Benedict’s test: To 5 ml of Benedict’s reagent, added 1 ml of extract solution and boiled for 2 minutes and cooled. Formation of red precipitate again confirmed the presence of sugars.
2.2.2.9. Test for Protein and Amino Acids

a). Biuret test: To 2 ml of the extract added 2 ml of 10% sodium hydroxide solution followed by 2 drops of 0.1% copper sulphate solution. Formation of pinkish or purple violet color indicated the presence of proteins.

b). Ninhydrin test: Added two drops of freshly prepared 0.2% ninhydrin reagent (0.1% solution in n-butanol) to the small quantity of extract solution and heated for 2 minutes. Development of blue color confirmed the presence of proteins, peptides, or amino acids.

c). Xanthoproteic test: To 5 ml of the extract, 1 ml of concentrated nitric acid was added and boiled. Then 40% of sodium hydroxide was added. Orange color indicated the presence of aromatic amino acids.

d). Million’s test: 1 ml of test solution was acidified with sulphuric acid and then added Million’s reagent and boiled the solution. Presence of protein was confirmed by the formation of yellow precipitate.

2.2.3. In vitro antioxidant assays

The in vitro antioxidant activity of petroleum ether (PEGG), Chloroform (CHGG), acetone (ACGG), ethanol (ETGG) and methanolic extracts (MEGG) of Gardenia gummifera Linn. f. roots were measured by the following assays.

2.2.3.1. Determination of total phenolic compounds in the extracts

The amount of total phenolics was determined using the Folin-Ciocalteu method (Yu et al., 2002). In this method, to the different concentrations of standard (5 - 50 µg/ mL) and extract (100 - 1000 µg/ mL), 1 mL of diluted Folin-Ciocalteu reagent was added. To this, 2 mL of 35 % Na₂CO₃ was added and diluted to 6 mL by
the addition of 2 mL of deionized water. The absorbance was measured at 700 nm after 30 minutes. A calibration curve of gallic acid was prepared, and the results were expressed as mg GAE (gallic acid equivalents)/g dry extract.

2.2.3.2. Determination of total flavonoid content in the extracts

The total flavonoid content was determined spectrophotometrically by the method described by Quettier-Deleu et al., (2000). Briefly, 1.0 mL of 2% aluminum chloride in methanol was mixed with different concentrations of standard (5 - 50 µg/mL) and extract (100 - 1000 µg/mL). Absorption readings at 430 nm were taken after 30 min against a blank (methanol). The total flavonoid content was determined using a standard curve with quercetin and expressed as milligrams of quercetin equivalents (QE/g of dry extract).

2.2.3.3. Evaluation of total antioxidant capacity

Aliquots of 0.1 mL of extract (Conc. 10 - 100 µg/0.1 mL) and standard solution (Conc. 5 - 50 µg/0.1 mL) in DMSO were combined with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) in an Eppendroff tube. The tubes were capped, incubated in a water bath at 95°C for 90 min, cooled to room temperature, and the absorbance of each solution was measured at 695 nm against a blank solution (Jayaprakasha et al., 2004). Ascorbic acid was used as standard and the total antioxidant capacity was expressed as the equivalent of ascorbic acid per gram of the extract.

2.2.3.4. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The assay was conducted to test the scavenging activity of petroleum ether (PEGG), chloroform (CHGG), acetone (ACGG), ethanol (ETGG) and methanolic extracts (MEGG) of *G. gummifera* Linn. f. roots against DPPH radicals
(Aquino et al., 2001). Briefly, 1.0 mL of methanolic solution of 0.25 mM DPPH was added to 1.0 mL of various concentrations of extracts (10 – 100 µg/mL [for MEGG, CHGG and ACGG] and 100 – 1000 µg/mL [for PEGG and ETGG]) and standard solutions (1 - 10 µg/mL). 1.0 mL of methanol served as control. After a 20 min incubation period at room temperature in dark, the absorbance was read at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and was calculated as percentage radical scavenging activity = (control OD - sample OD/control OD) X100. Ascorbic acid standard was used for the comparison.

2.2.3.5. Assay of hydroxyl radical-scavenging activity

Hydroxyl radical-scavenging activity was determined by mixing various concentrations of extracts and standard (10 - 50 µg/ mL) in DMSO, deoxyribose (2.8 mmol/L), FeCl₃ (0.1 mmol/L), K₂HPO₄ - KOH buffer (20 mmol/L; pH 7.4), EDTA (0.1 mmol/L), H₂O₂ (1.0 mmol/L) and ascorbic acid (0.1 mmol/L) in a final volume of 1 mL (Ajith and Janardhanan, 2002). The reaction mixture was incubated at 30°C for 60 min. The TBARS formed was estimated according to the methods of Ohkawa et al (1979). The hydroxyl radical-scavenging activity was determined by comparing the absorbance of the control with that of the treatment groups. The reference standard used was quercetin.

2.2.3.6. Determination of reducing power

Adopting the method of Oyaizu (1986), different concentrations of extracts and standard (50 - 500 µg/ mL) dissolved in DMSO were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide .The mixture was then incubated at 50°C for 20 min. 2.5 mL of 10% trichloroacetic acid was added to the mixture, and centrifuged for 10 min at 3000 rpm. The upper layer of the
solution (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% FeCl₃, and the absorbance was measured at 700 nm. Ascorbic acid was used as a standard antioxidant compound.

2.2.4. In vivo experimental designs

2.2.4. 1. Toxicity studies of methanolic extract of Gardenia gummifera Lnn.f.

2.2.4. 1.1. Acute toxicity evaluation in rats

Rats were divided into three groups. Group I was treated as normal control (animals administered orally with vehicle, 5% Tween 80), Group II and III received a single dose (2.5 and 5 gm/kg) of methanolic extract of G. gummifera root and kept under observation for 14 days (Withhawaskul et al., 2003). Animals were sacrificed on 15th day. The serum levels of AST, ALT, LDH, glucose, urea, triglycerides, cholesterol and creatinine were estimated. The tissues were subjected to histopathological studies.

2.2.4. 1.2. Sub-acute toxicity studies in rats

To study the subacute toxicity (Withhawaskul et al., 2003) in rats, three groups of animals were selected, Group I was normal control (animals administered orally with vehicle, 5% Tween 80), Groups II and III were treated with daily doses of 500 mg/kg and 1000 mg/kg methanolic extract of G. gummifera respectively for 30 days. The animals were sacrificed on 31st day. Blood samples were collected from common carotid into heparinized and non-heparinized centrifuge tubes. The heparinized blood was used for haematological study that included leukocyte count, erythrocyte count and haemoglobin estimation. The serum was separated from the non-heparinized blood and was assayed for AST, ALT, LDH, creatinine, cholesterol,
triglycerides, glucose and urea. The tissues were subjected to histopathological studies.

2.6.4.2. Induction of oxidative stress

In vivo antioxidant potential of *G. gummifera* root was evaluated in male Wistar rats against thioacetamide (TAA) induced oxidative stress in preventive and curative models. In pre-treatment model, rats were treated with plant extract daily for 9 days prior to TAA administration (100 mg/kg; s.c.). In post-treatment model, rats were administered with plant extract 2, 24 and 48 h after TAA intoxication (100 mg/kg; s.c.). Antioxidant potential of the plant extract was assessed by quantifying the levels of serum marker enzymes, tissue lipid peroxidation, tissue antioxidants and histopathological evaluations.

2.6.4.3. Induction of atherosclerosis

To investigate the anti-atherogenic effect of *G. gummifera* against high fat diet induced atherosclerosis in male Wistar rats, the rats were divided into six groups, each having six animals. Group I, animals fed on normal laboratory feed and administered orally with vehicle, 5% Tween 80 instead of MEGG. Group II received atherogenic diet (AD) [10g cholesterol, 5g sodium cholate and 100g hydrogenated vegetable oil in 1000g powdered standard commercial pellet diet]. Group III served as drug control (MEGG 250 mg/kg b.w p.o) and the remaining three groups IV, V and VI received AD along with standard drug atorvastatin (5mg/kg b.w p.o) (Sai Krishna et al., 2010) and MEGG (125 and 250 mg/kg b.w p.o) respectively for 90 days. Anti-atherogenic activity of MEGG was assessed by determining the activities of serum marker enzymes, serum levels of lipoproteins, quantifying the serum and
tissue levels of total cholesterol (TC), triglycerides (TG) and phospholipids (PL), tissue lipogenic enzymes, antioxidants, lipid peroxidation and histopathological evaluations.

2.6.4.4. Induction of myocardial infarction.

Cardioprotective effect of MEGG on isoproterenol (ISO) induced myocardial infarction (MI) was studied in male Wistar rats. MI was induced by the subcutaneous injection of ISO (6mg/100g body weight) at an interval of 24h for 2 days (Vijayapadma and Shyamala Devi., 2000). MEGG (125 and 250 mg/kg b.w, p.o) was given to rats once daily for 45 days prior to the ISO challenge. Cardioprotective efficacy of MEGG was assessed by determining the serum marker enzymes, quantifying the serum levels of uric acid, ceruloplasmin, TC, TG, LDL-C, VLDL-C and PL, iron and iron binding capacity, tissue levels of TC, TG and PL, antioxidants, lipid peroxidation, Triphenyl Tetrazoleum Chloride (TTC) macroscopic enzyme mapping assay and histopathological evaluations.

2.6.4.5. Cardioprotective effect of the active fraction of methanolic extract of G. gummifera.

Cardioprotective activity of the petroleum ether fraction (PEF) of G. gummifera was examined by using ISO induced MI models. The animals were divided in to 5 groups (Six rats/group). Group I vehicle control (5% tween 80 and normal saline instead of MEGG and ISO respectively). Group II toxic control animals were given subcutaneous injection of ISO (6mg/100g) dissolved in 0.1ml normal saline at an interval of 24hrs for 2 days. Group III animals were given PEF (10 mg/kg body weight p.o.), Group IV rats pretreated with PEF (20 mg/kg body weight p.o.) and Group V animals were given PEF (30mg/kg body weight p.o.) for 15 days and on the 16th and 17th days, received ISO (6 mg /100g b.w) subcutaneously.
Cardioprotective efficacy of PEF was assessed by determining the serum marker enzymes, serum TC and TG, tissue antioxidants, lipid peroxidation and histopathological evaluations.

2.2.5. Collection of serum samples

The animals were kept starved overnight before they were sacrificed. On the next day, after recording their body weight, they were anesthetized with pentothal sodium followed by neck decapitation. The blood collected from each animal was allowed to clot for 45 min at room temperature and the serum was separated by centrifugation at 2000 rpm at 4ºC for 15 min. This was used for different biochemical estimations.

2.2.5.1. Biochemical tests

Sera from different groups were subjected to biochemical estimations of different parameters, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, lactate dehydrogenase (LDH), creatine kinase isoenzyme (CK-MB), creatine phosphokinase, cholesterol, triglycerides, lipoproteins, glucose, creatinine, uric acid, ceruloplasmin, iron and iron binding capacity.

2.2.6. Collection of tissue samples and its homogenization

Tissues were excised from sacrificed animals and washed thoroughly in ice-cold saline to remove the blood. They were then gently blotted between the folds of a filter paper and weighed in an analytical balance. Ten percent of homogenate was prepared in 0.1M Tris HCl buffer (pH-7.4) (Ilavarasan et al., 2003). The homogenate was centrifuged at 3000 rpm for 20 min at 4ºC and the supernatant was used for the estimation of reduced glutathione (GSH), glutathione-S-transferase (GST),
glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT), lipid peroxidation (Thiobarbituric Acid Reactive Substances – TBARS) and total protein. The tissue lipids were extracted (Folch et al., 1957) and used for the estimations of total cholesterol, triglycerides and phospholipids, activities of HMG Co A reductase, glucose-6-phosphate dehydrogenase (G-6-PD) and malic enzyme were also evaluated.

2.2.7. In vivo antioxidant assays

The intra cellular antioxidant system comprises of different free radical scavenging antioxidant enzymes along with some non-enzyme antioxidants. In vivo antioxidant activity of the extract was determined by the estimation of cellular enzymic antioxidants such as GST, GPx, GR and CAT, and non – enzymic antioxidant like GSH. The anti-lipid peroxidative effect of the extract was also determined in tissues by quantifying the level of malondialdehyde (MDA), an important indicator of lipid peroxidation.

(Detailed procedures are given in section 2.2.10. Procedures for in vivo antioxidant assays)

2.2.8. Histopathological studies

Small pieces of tissues were fixed in 10% buffered formalin for at least 4 hours. The tissues were then dehydrated in alcohol series, cleaned in xylene and embedded in paraffin. About 5–6 µm thick sections were taken and stained with hematoxylin and eosin and examined for histopathological changes under the microscope (Motic AE 21, Germany) (Culling, 1976). The microphotographs were taken using Moticam 1000 camera at original magnification of 100×.
2.2.9. Statistical analysis

Results are expressed as mean ± S.D and all statistical comparisons were made by means of one-way ANOVA test followed by Tukey’s post hoc analysis and p-values less than or equal to 0.05 were considered significant.

2.2.10. PROCEDURES FOR IN VIVO ANTIOXIDANT ASSAYS

2.2.10.1. ESTIMATION OF REDUCED GLUTATHIONE (GSH)

Reduced glutathione was estimated by the method of Ellman, (1959).

Principle

Reduced glutathione on reaction with DTNB (5, 5’-dithiobis nitro benzoic acid) produces a yellow coloured product with absorption maximum at 420 nm.

Reagents

1. Phosphate Buffer (0.2 M, pH - 8.0).
2. Trichloroacetic acid (TCA) (5% w/v).
3. 5, 5’- Dithio-bis-(2-nitrobenzoic acid) (DTNB).
4. Ellman’s reagent: 19.8 mg of DTNB in 100 mL of 1% sodium citrate solution.
5. Standard Glutathione (GSH) – 5mg reduced glutathione was diluted to 50ml with distilled water.

Procedure

0.5 mL of tissue homogenate was precipitated with 2.0 mL of 5% TCA and centrifuged. To 1.0 mL of the supernatant, 3.0 mL phosphate buffer and 0.5 mL Ellman’s reagent were added and incubated for 15 min at room temperature. The intensity of yellow color formed was measured at 420nm. A series of standards
(10–50 µg) were treated in a similar manner along with blank containing 1.0 mL distilled water.

The amount of GSH was expressed as nmol/mg protein.

2.2.10.2. ASSAY OF GLUTATHIONE-S-TRANSFERASE (GST) (EC 2.5.1.18)

The method of Habig et al., 1974 was adopted for the assay of GST.

Principle

The enzyme is assayed by its ability to conjugate GSH and CDNB, the extent of conjugation causing a proportionate change in the absorbance at 340 nm.

Glutathione –SH + CDNB $\xrightarrow{\text{GST}}$ Glutathione –S-CDNB

Reagents

1. Phosphate buffer (0.5 M, pH - 6.5).
2. 1- chloro-2, 4- dinitrobenzene (CDNB) in 95% ethanol (25 mM).
3. Reduced glutathione (20 mM).

Procedure

The reaction mixture containing 200 µL phosphate buffer, 20 µL of CDNB and 50 µL of tissue homogenate was made up to 1 mL with distilled water. The reaction mixture was pre-incubated at 37 °C for 10 min. 50 µL of GSH was added and the change in absorbance was measured at 340 nm for 3 min at 30 seconds intervals. To the blank, 50 µL of distilled water was added instead of tissue homogenate.

The enzyme activity is expressed as micromoles of CDNB – GSH conjugate formed/min/mg protein.
2.2.10.3. ASSAY OF GLUTATHIONE REDUCTASE (GR) (EC 1.6.4.2)

GR activity was assayed by the method of Carlberg and Mannervik, (1985).

**Principle**

The GR activity is determined by the amount of NADPH consumed in the conservation of glutathione (GSSG) to reduced glutathione (GSH). The reaction is catalysed by GR.

\[
\text{GSSG} + \text{NADPH}^+ + \text{H}^+ \xrightarrow{\text{GR}} 2\text{GSH} + \text{NADP}
\]

**Reagents**

1. Potassium phosphate buffer (0.2M, pH - 7.0), containing 2 mM EDTA.
2. NADPH (2 mM), in 10 mM Tris- HCl (pH - 7.0).
3. GSSG (20 mM).

**Procedure**

0.5mL phosphate buffer, 50 µL NADPH, 50 µL GSSG and deionized water were added in a test tube to give a total volume of 1.0 mL. The reaction was initiated by the addition of 10 µL tissue homogenate to the tube. The decrease in absorbance at 340 nm was followed at 27 °C for every 30secs for 2 min and the change in absorbance was measured.

The values are expressed as nmol of GSSG utilized/min/mg protein.

2.2.10.4. ASSAY OF GLUTATHIONE PEROXIDASE (GPx) (EC 1.11.1.9)

Glutathione peroxidase was estimated by the method of Rotruck et al. (1973).
Principle

The activity of GPx was determined by measuring the decrease in GSH content after incubating the sample in the presence of H\textsubscript{2}O\textsubscript{2} and NaN\textsubscript{3}. GSH was estimated by its reaction with dithio-bis-2-nitrobenzoic acid (DTNB) that gives a yellow colored complex with absorption maximum at 420 nm.

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

Reagents

1. Tris-HCl buffer (0.4 M, pH - 7.0).
2. Sodium azide (10 mM).
3. Trichloroacetic acid (TCA) (10% w/v).
4. EDTA (0.4 mM).
5. H\textsubscript{2}O\textsubscript{2} (0.2 mM).
6. Reduced glutathione (GSH), (2.0 mM).
7. 5, 5’- Dithio-bis-(2-nitrobenzoic acid) (DTNB).
8. Ellman’s reagent: 19.8 mg of DTNB in 100 mL of 1% sodium citrate solution.
9. Phosphate buffer (0.2 M, pH - 8.0).

Procedure

To 0.2 mL of Tris-HCl buffer, 0.2 mL EDTA, 0.1 mL of sodium azide and 0.5 mL tissue homogenate were added and mixed well. To this 0.2mL GSH followed by 0.1 mL of H\textsubscript{2}O\textsubscript{2} were added. The contents were mixed well and incubated at 37°C for 10 min. The reaction was arrested by the addition of 0.5mL of
10% TCA and then the tubes were centrifuged at 3000 rpm for 10 minutes. 1 mL of supernatant was taken and mixed with 0.5 mL Ellman’s reagent and 3 mL phosphate buffer. It was then kept at room temperature for 15 minutes and read at 420 nm. To the blank, 0.5 mL of distilled water was added instead of tissue extract.

The values are expressed as nmol of GSH oxidized/min/mg protein.

2.2.10.5. ASSAY OF CATALASE (CAT) (EC 1.11.1.6)

The catalase activity was determined by the method described by Beers and Sizer, (1952).

**Principle**

The UV absorption of hydrogen peroxide can be measured at 240nm, whose absorbance decreases when degraded by the enzyme catalase. From the decrease in absorbance, the enzyme activity can be calculated.

\[
2\text{H}_2\text{O}_2 \xrightarrow{\text{CAT}} 2\text{H}_2\text{O} + \text{O}_2
\]

**Reagents**

1. Phosphate buffer (0.1 M, pH - 7.0).
2. Hydrogen peroxide (30 mM solution in the above buffer).

**Procedure**

To 1.0 ml of phosphate buffer, 25 µL of tissue homogenate was added and the enzyme reaction was started by the addition of 250 µL of H₂O₂ solution. The decrease in absorbance was measured at 240 nm at 30 seconds intervals for 3 min. the enzyme blank was run simultaneously with 250 µL of distilled water instead of hydrogen peroxide.
The enzyme activity is expressed as U/mg protein (nmoles of \( \text{H}_2\text{O}_2 \) decomposed/min/mg protein).

### 2.2.10.6. DETERMINATION OF LIPID PEROXIDATION

Lipid peroxidation was determined by thiobarbituric acid reaction as described by Niehius and Samuelsson, (1968).

**Principle**

Malondialdehyde (MDA) formed from the breakdown of polyunsaturated fatty acids serves as a convenient index for the determination of the extent of peroxidation reaction. MDA, a product of lipid peroxidation reacts with TBA (thiobarbituric acid) to give a pink coloured product having absorption maxima at 535nm.

**Reagents**

1. Trichloroacetic acid (TCA) (15% w/v).
2. Hydrochloric acid (HCl) (0.25 N).
3. Thiobarbituric acid (TBA) (0.375% w/v).
5. Standard (1, 1’, 3, 3’ tetramethoxypropane) - 0.16 ml of 3 M solution of standard tetramethoxypropane was made up to 100 mL with double distilled water. 1 mL of this was taken and made up to 100 mL with double distilled water, which served as working standard.

**Procedure**

1.0 mL of the tissue homogenate was combined with 2.0 mL of TCA – TBA – HCl reagent and mixed thoroughly. Then the mixture was heated for 15 minutes in
a boiling water bath. The flocculent precipitate was removed by centrifugation at 2000 rpm for 10 minutes. The absorbance of the sample was read at 535 nm against a blank containing all the reagents except tissue homogenate.

Values are expressed as nmol/g tissue.

**2.2.10.7. ESTIMATION OF TOTAL PROTEIN**

The protein content was estimated by the method of Lowry et al. (1951).

**Principle**

Proteins react with Folin-Ciocalteau reagent to give a coloured complex. The colour so formed is due to the reaction of alkaline copper with protein and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein. The intensity of the colour depends on the amount of these aromatic amino acids present.

**Reagents**

1. Trichloroacetic acid (TCA) (10% w/v).
2. NaOH (0.1 N).
3. Alkaline copper reagent:
   - Solution A: 2% sodium carbonate in 0.1N NaOH.
   - Solution B: 0.5% copper sulphate in water.
   - Solution C: 1% sodium potassium tartarate in water.

50 mL of solution A was mixed with 0.5 mL of solution B and 1.0 mL of solution C just before use.

4. Folin - Ciocalteau reagent (1:1 dilution).
5. Stock standard: A stock solution was prepared by dissolving 100 mg bovine serum albumin (BSA) in 100 mL water in a standard flask. Small quantities of 0.1 N NaOH were added to complete the dissolution of BSA.

6. Working standard: 10 mL of the stock was diluted to 100 mL to obtain a working standard concentration 100 µg/mL.

**Procedure**

0.5 mL of tissue homogenate was added to equal amount of 10% TCA. The mixture was centrifuged at 2500 rpm for 10 minutes, supernatant discarded and the precipitate was dissolved in 5 mL of 0.1 N NaOH. From this, 0.5 mL was taken as test sample for the estimation. 5 mL alkaline copper reagent was added and the contents were allowed to stand at 37°C for 10 min. Then, 0.5 mL diluted Folin - Ciocalteau reagent was added, mixed well and incubated for 20 minutes at room temperature. A series of standards of concentration ranging from 20-100 µg and a blank were processed in the same way as that of the test. The blue colour developed was read at 670 nm.

The amount of total protein is expressed as mg/g tissue.

**2.2.11. PROCEDURES FOR TOXICOLOGICAL STUDY**

**2.2.11.1. DETERMINATION OF HAEMOGLOBIN (Hb) IN BLOOD**

Haemoglobin was determined according to the method of Drakin and Austin, (1932).

**Principle**

Haemoglobin was treated with a reagent containing potassium ferricyanide, potassium cyanide and potassium dihydrogenphosphate. The ferricyanide forms
methaemoglobin, which was converted to cyanmethaemoglobin by the cyanide. The intensity of the colour formed was measured at 546 nm against reagent blank. The optical density is directly proportional to the amount of haemoglobin present in the blood.

**Procedure**

The study was conducted by using Agappe diagnostic kit. 0.02 mL of fresh whole blood was mixed with 5 mL of the cyanmeth reagent. The optical density was measured at 546 nm against reagent blank after incubation for 5 min. at room temperature. The optical density of standard solution corresponding to 60 mg/dl haemoglobin at 546 nm was read against reagent blank. Haemoglobin in the blood was calculated by using the following formula.

\[
\text{Haemoglobin (g/dl)} = \frac{OD_t \times 60 \times 0.251}{OD_s}
\]

**2.2.11.2. DETERMINATION OF TOTAL RED BLOOD CELL (RBC) COUNT**

Total RBC count was determined by using Haemocytometer as described by Chaudhari, (2000a).

**Procedure**

0.02 mL of blood was added to 3.98 mL of diluting fluid. The neubauer chamber was charged with well-mixed dilute blood. The total number of red cells in the small square in the central ruled area of neubauer counting chamber was counted using 40x objective of the microscope.

Total RBC count = Number of cells counted \times 10,000 \text{ count/mm}^3
2.2.11.3. DETERMINATION OF TOTAL WHITE BLOOD CELL (WBC) COUNT

Total WBC count was determined by using Haemocytometer as described by Chaudhari, (2000b).

**Principle**

The whole blood was diluted using a diluent which haemolyses red cells, leaving all the nucleated cells intact. The number of white blood cells in a known volume and known dilution were counted using a counter chamber.

**Procedure**

0.02 mL of blood was added to 3.98 mL of diluting fluid. The neubauer chamber was charged with well-mixed dilute blood. The total number of white blood cells in the four large corner squares of chamber was counted after 3-4 min.

\[
\text{Total number of WBC} = \text{Number of cells counted} \times 50 \text{ count/mm}^3
\]

2.2.12. EXTRACTION OF SERUM AND TISSUES FOR LIPID ESTIMATION

2.2.12.1. EXTRACTION OF SERUM FOR LIPID ESTIMATION

The extraction was done on the basis of Folch et al. (1957).

**Reagents**

- Methanol
- Chloroform
- Potassium Chloride
Procedure

'n' volume of the serum sample was added drop by drop to '5n' volume of methanol in a stoppered tube. Then '5n' volume of chloroform was added and mixed. This mixture was incubated at 55° C for 15 minutes, after which another '5n' volume of chloroform was added so that the proportion of chloroform to methanol was 2:1 (v/v). After filtration and washing the residue with chloroform: methanol (2:1) atleast 3 times, the combined filtrate was washed with 0.7 % KCl solution (20 % of the total volume of the extract). The aqueous upper phase was removed with a pasteur pippette and the lower layer was washed with 5 ml of chloroform: methanol: KCl solution (3:48:47 v/v/v) for three times. The washed lower layer was evaporated to dryness and the residue redissolved in a known volume of chloroform. Aliquots were used for the lipid analysis.

2.2.12.2. EXTRACTION OF TISSUES FOR LIPID ESTIMATION

The extraction was done on the basis of Folch et al. (1957).

Reagents

1. Ethanol- Ether mixture (3:1)

2. Chloroform- Methanol mixture (CHCl₃-CH₃OH) (2:1)

Procedure

About 1g of tissue was ground in a mortar and pestle with 2ml alcohol ether mixture. The extract was then transferred to a test tube and made it up to 5ml. It was then placed at 60-70° C in water bath for 2 hrs. Contents were occasionally agitated using a glass rod. The supernatant was then transferred to a 25 ml standard flask through folded filter paper fitted in a funnel. The residue was collected and the
process was repeated three times with ethanol-ether mixture. Finally small amounts of CHCl₃-CH₃OH was added to the residue, mixed and kept at 60°C for one hour. Repeated once more with CHCl₃-CH₃OH. Pooled the filtrate and made up to 25 ml with either of the two solvents. This aliquots were used for lipid estimations.

2.2.13. ESTIMATION OF TOTAL CHOLESTEROL IN TISSUES

Cholesterol was estimated by the method of Zak, (1957).

Reagents

1. Ferric chloride - Acetic acid (FeCl₃, - CH₃COOH) Reagent (stock) dissolved 1 gm FeCl₃ 6H₂O in 100 ml acetic acid. Used analytical grade glacial acetic acid (aldehyde free).

2. FeCl₃-CH₃COOH reagent (working): Diluted 5 ml stock to 100 ml with acetic acid.

3. Cholesterol (standard) (1 mg/ml): Dissolved 100 mg cholesterol in acetic acid and made up to 100 ml in a standard flask with acetic acid and stored in the cold.

4. Cholesterol (working) (0.04 mg/ml): Diluted 4 ml to 100 ml with working FeCl₃-CH₃COOH reagent and stored in the cold.

5. Concentrated Sulphuric acid (Conc. H₂SO₄): AR

Procedure

To 1ml of the tissue extract, added 4.9 ml of working FeCl₃-CH₃COOH reagent. Mixed well and allowed to stand for 15' and then centrifuged. From this 2.5 ml was taken. 2.5 ml of working standard was taken as standard and 2.5 ml of FeCl₃-CH₃COOH was taken as blank. 1.5 ml of con. H₂SO₄ was added to all the three
tubes. The contents of each tube were mixed well and allowed to stand for 30’. The
reddish purple colour developed was measured at 560 nm.

2.2.14. ESTIMATION OF TRIGLYCERIDES IN TISSUES

Triglycerides were estimated by the method of van Handel and Zilversmith,
(1957) with the modification that florisil was used to remove phospholipids.

Reagents

1. Chloroform

2. Florisil

3. Ethanolic potassium hydroxide: 0.4 %.

4. 0.2 N H₂SO₄

5. 0.05 M sodium arsenite

6. 0.05 M sodium metaperiodate

7. Chromotropic acid: Dissolved 2g of chromotropic acid (2.4g of sodium salt)
in 200 ml distilled water. Separately added 600 ml of conc. H₂SO₄ to 300 ml
of distilled water which was already chilled in ice. This chilled and diluted
acid was then added to chromotropic acid solution.

8. Standard glycerol (9mg/ml).

Procedure

2 g of florisil was taken in a glass stoppered tube and 3ml chloroform was
added. An aliquot (0.2 ml tissue extract) was layered on the top of florisil and
mixed. Chloroform was then added to this to a total volume of 10 ml. It was then
stoppered and shaken intermittently for 10 minutes. After filtration 1 ml each was
pipetted out into three tubes. The solvent was evaporated at 60-70°C. Then 0.5 ml of ethanolic KOH was added to two of these tubes and 0.5 ml ethanol was added to the third tube. The tubes were closed and kept at 60-70°C for 15 minutes. To each tube, 0.5 ml 0.2 N H₂SO₄ was added and then placed in a gently boiling water bath for about 15 minutes to remove alcohol. They were then cooled to room temperature; 0.1 ml sodium metaperiodate was added to each tube and kept for 10 minutes. 0.1 ml sodium arsenite solution was then added. An yellow colour of iodine appeared and vanished within a few minutes. To each tube, 5 ml chromotropic acid was added and mixed. The tubes were closed and heated in a boiling water bath for 30 minutes. They were then cooled and absorbance was read at 570 nm.

2.2.15. ESTIMATION OF PHOSPHOLIPIDS IN SERUM AND TISSUES

Phospholipids were estimated by the method of Connerty et al. (1961).

Reagents

1. Trichloro acetic acid (TCA) : 5% (w/v) in water.

2. Digestion mixture : 500 ml distilled water, 25 ml con. H₂SO₄ and 25 ml 70% perchloric acid.

3. Sodium acetate : 50% of the trihydrate (w/v) in water.

4. Ammonium molybdate : 2.5% solution in water.

5. Metol (P-methyl aminophenyl sulphate) - 1 g in 100 ml of 3% sodium hydrogen sulphate.

6. Stock standard (1 mg phosphorous/ml): 4.394g anhydrous potassium dihydrogen sulphate in a litre of the solution containing 2 ml con. H₂SO₄.
7. Working standard: 1ml = 4μg of phosphorus. The stock standard was diluted 1 to 250.

Procedure

0.2 ml serum/tissue extract was pipetted into a test tube and added 5 ml 5% TCA drop by drop while shaking. Centrifuged to give tightly packed precipitate. Decanted the supernatant and kept the tube inverted on a filter paper wiped around the rim. Added 1 ml of the digestion mixture and heated gently until the liquid becomes colourless or almost so. Allowed to cool, added carefully 1 ml distilled water and boiled for 15 seconds to convert pyrophosphate to orthophosphate. Added 1 ml of 50% sodium acetate and made to 10 ml with distilled water. Then added 1 ml ammonium molybdate and 1 ml metol. Mixed well, kept for 15' and read at 700 nm. Blank was prepared by mixing 0.25 ml conc. H$_2$SO$_4$, 1 ml 50% sodium acetate, 1 ml ammonium molybdate, 1 ml metol and 8.75 ml of distilled water. As standard, took 5 ml working standard (containing 20 μg phosphorus), 0.25 ml of conc. H$_2$SO$_4$, 1 ml of acetate, 1 ml of molybdate, 1 ml metol and 3.75 ml of distilled water mixed well and read against reagent blank.

2.2.16. ESTIMATION OF TOTAL CHOLESTEROL, HDL- CHOLESTEROL, LDL-CHOLESTEROL, VLDL- CHOLESTEROL AND TRIGLYCERIDES IN SERUM

The concentration of triglycerides (TG), total cholesterol (TC) and HDL cholesterol (HDL-C) in serum were measured using a commercial diagnostic kit (AGAPPE Diagnostics, Mumbai, India). The concentration of VLDL- cholesterol (VLDL-C) and LDL cholesterol (LDL-C) were calculated by Friedwald’s formula.
(Friedwald et al., 1972). LDL-C: HDL-C ratio was taken as the Atherogenic Ratio (Bermingham et al., 1995).

2.2.17. ANALYSIS OF FAECAL STEROLS AND BILE ACIDS

24 hour stool samples, collected from the rats in metabolic cages, were homogenized with equal weight of water and lyophilized to a fine powder. Faecal sterol and bile acids were extracted by the general procedure of Grundy and Ahrens, (1969) and estimated as described by Menon and Kurup, (1976).

Reagents

1. 1N NaOH in 90 % ethanol
2. Hexane
3. Ethyl acetate

Procedure

500 mg of the sample was extracted with 10 ml of 1N NaOH in 90 % ethanol at 80°C for 2 hours. The mixture was cooled, centrifuged and the residue was re-extracted with 10 ml of 1N NaOH in 90 % ethanol. The alkaline faecal extract was diluted with an equal volume of water and the neutral sterols were extracted with hexane. The hexane layer was collected and washed with a little water. It was evaporated to dryness and redissolved in a known volume of chloroform for neutral sterol estimation. The solution left after extraction with hexane was then acidified to pH 2.0 and bile acids were extracted with ethyl acetate. The ethyl acetate layer was collected, washed with water and evaporated to dryness. The bile acids were redissolved in a known volume of ethyl acetate and aliquots were taken for the estimation of bile acids.
2.2.18. ASSAY OF $\beta$-HYDROXY $\beta$-METHYL GLUTARYL CoA REDUCTASE
[EC1.1.1.34]

The HMG CoA reductase activity was estimated by the method of Rao and Ramakrishnan, (1975).

Reagents

1. Saline arsenate: 1g of sodium arsenate in 1 litre physiological saline

2. Dilute perchloric acid: 50 ml made upto 1 litre

3. Hydroxylamine hydrochloride reagent for mevalonate: Equal volume of hydroxylamine hydrochloride reagent (138.9 g/l) and water were mixed freshly before use.

4. Hydroxylamine hydrochloride reagent for HMG CoA: Equal volume of hydroxylamine hydrochloride and sodium hydroxide (180 g/l) were mixed freshly before use.

5. Ferric chloride reagent: 5.2 g trichloroacetic acid and 10 g ferric chloride were dissolved in 50 ml of 0.65 N HCl and made up to 100 ml with water

Procedure

Equal volume of fresh 10% tissue homogenate in saline arsenate and dilute perchloric acid (50 ml made upto 1 litre) were mixed, kept for 5 minutes and centrifuged at 2000 rpm for 10 minutes. To 1 ml supernatant 0.5 ml of freshly prepared hydroxylamine reagent (alkaline hydroxylamine reagent in the case of HMG CoA) was added, mixed and after 5 minutes, 1.5 ml of ferric chloride reagent was added. After shaking well, readings were taken after 10 minutes at 540 nm against a similarly treated saline arsenate blank. The ratio of HMG CoA to
mevalonate was taken as an index of enzyme activity which catalyses the conversion of β–hydroxy-β -methyl glutaryl CoA to mevalonate. The lower the ratio the higher the activity.

2.2.19. GLUCOSE-6-PHOSPHATE DEHYDROGENASE [GLUCOSE-6 PHOSPHATE NADP+ OXIDO REDUCTASE; EC1.1.1.49]

Glucose-6-phosphate dehydrogenase was assayed by the method of Kornberg and Horecker, (1955).

Reagents

1. Glycyl glycine buffer: 0.04 M (pH 7.5)
2. Glucose-6-phosphate: 0.02 M
3. NADP+: 0.0015 M
4. MgCl₂ : 0.1 M

Procedure

The chilled tissue was homogenized with 3 volumes of 0.04 M glycyl glycine buffer (pH 7.5). The homogenate was centrifuged at 2000 g at 0°C for 10 minutes. The supernatant was used as the enzyme source.

To 1.0 ml of the substrate (0.02 M glucose-6-phosphate) in a quartz cell, 0.1 ml of NADP⁺, 0.25 ml of glycyl glycine buffer and 0.2 ml MgCl₂ were added. To this 0.05 ml of enzyme was added and the absorbance was read immediately at 340 nm at 1 minute interval. One unit of enzyme is defined as that amount which causes an initial change in OD of 1.0/ minute under the above conditions.
2.2.20. **MALIC ENZYME [L-MALATE: NADP\(^+\) OXIDO REDUCTASE; EC1.1.1.40]**

Malic enzyme was assayed by the method of Severo-Ochoa, (1955).

**Reagents**

1. Glycylglycine buffer: 0.25 M pH 7.4
2. MnCl\(_2\): 0.05M
3. NADP\(^+\): 0.135 \(\mu\)M
4. L- malate: 0.03M

**Procedure**

The chilled tissue was homogenized with 3 volumes of 0.25 M glycylglycine buffer, pH 7.4 at 0\(^\circ\)C and the supernatant obtained by centrifuging at 2000 x g for 10 minutes was used as the enzyme. The reaction mixture consisted of 0.2 M glycylglycine buffer pH 7.4, 0.06 ml of 0.05M MnCl\(_2\), 0.2 ml of NADP\(^+\), 0.05 ml of L-malate and 0.05 ml enzyme and water to a final volume of 3 ml. The assay was carried out at room temperature (23-25\(^\circ\) C). The reaction was started by the addition of either malate or enzyme and the optical density at 340 nm were taken against a blank containing all components except NADP\(^+\) at intervals of 15 seconds for 1 to 2 min. The enzyme activity is expressed in units / mg of protein.