CHAPTER 4
MATERIALS AND METHODS

4.1 MATERIALS

4.1.1. Drugs

(±)-Isoproterenol hydrochloride (ISO) and Vitamin E (DL-α-Tocopherol acetate) were purchase from Sigma Aldrich Co. St. Louis. MO. USA. Alcoholic extract of green tea (GT) (*Camellia sinensis*) containing 60% polyphenols was gifted by K. Patel phytoextract Ltd. Mumbai. Lycopene powder (LYP) was obtained as gift sample from Genesis Laboratory Ltd, Mumbai. Alcoholic extract of Pomegranate fruit (PGFE) was gifted by Cherain Chemicals, Baroda, India. Fresh fruits of *Lagenaria siceraria* (LS) were collected from nearby farm at Baroda, Gujarat.

4.1.2. Chemicals

Superoxide Dismutase, Catalase, triphenyl tetrazolium chloride (TTC), 1, 1-diphenyl-2-picryl hydrazyl (DPPH), 1, 1, 3, 3-tetraethoxypropane from Sigma Chemicals, St. Louis, M.O., U.S.A.

Epinephrine bicarbonate, Thiobarbituric acid, sucrose, Adenosine triphosphate (ATP), reduced glutathione, 5, 5'-dithiobis (2-nitro benzoic acid) (DTNB), Sodium azide, bovine serum albumin, riboflavin, naphthylenediamine di hydrochloride, nitro blue tetrazolium (NBT), 1-chloro, 2, 4-dinitrobenzene (CDNB), 1-Amino 2-naphthol 4-sulphonic acid (ANSA), Dextran sulphate, Copper triethanolamine, Diethyl dithiocarbamate, HTAB (hexadecyltrimethylammonium bromide), o-dianisidine, N-(1-naphthyl ethylenediamine dihydrochloride) and phenazine methosulphate from Hi-Media Laboratories Pvt. Ltd., Mumbai, India.

Hydrogen peroxide, disodium hydrogen orthophosphate (Na_2HPO_4), potassium dihydrogen orthophosphate (KH_2PO_4), , Magnesium sulphate, Potassium chloride, Sodium chloride, Calcium chloride, Magnesium chloride, Ammonium molybdate reagent, sodium metabisulphite, sodium sulphite, potassium dihydrogen orthophosphate, sodium hydroxide, Copper sulphate, sodium potassium tartarate, Sodium carbonate, Activated silicic acid, palmitic acid, olive oil, steric acid,

Sulphosalicyclic acid, sulfanilamide, Agarose, lithium lactate, Riboflavin, Sodium nitroprusside, Adenosine diphosphate, Ferric chloride, Chloroform, methanol, sulphuric acid, glacial acetic acid, hydrochloric acid, Perchloric acid, Heptane, Isopropanol, Acetone, Digitonin, diethyl ether, ethanol, Sodium deoxy cholate and Folin's phenol reagent from S.D. Fine Chemicals, Mumbai, India.All the reagents and chemicals used in the entire study were of analytical grade.

4.1.3. Sources of diagnostic kits

Kits for the estimation of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), uric acid, total protein, lactate dehydrogenase (LDH), creatine phospokinase-MB (CK-MB), total cholesterol (TC), triglycerides (TG), high density lipoproteins (HDL), Na^+, K^+ and Ca^{++} were purchase from Span Diagnostic Pvt Ltd., India, Reckon Diagnostic Ltd., India and Monozyme India Ltd, Secunderabad. The level of inflammatory marker i.e. C-reactive protein (CRP) was estimated using standard diagnostic kit from Spinreact, S. A. Ctra. Santa Coloma, Spain. Caspase-3 protease activity was estimated using kit from BioVision (Caspase-3/CPP32 Colorimetric Assay Kit, USA). Tissue DNA extraction was carried out as per the instruction provided by GeNei™, Bangalore-India DNA extraction kit.

4.1.4. Collection and authentication of *Lagenaria siceraria* (LS) fruits

Fresh fruits of LS were collected from nearby farm at vadodara. The fruits collected were semiriped, bottle shape, weighted between 500-700g and of the same plant. The fruit of LS was authenticated by the authority from Botany Department, The M.S.University of Baroda, Vadodara.

4.1.4.1. Preparation of Fruit Juice of *Lagenaria siceraria*

The fresh juice of LS was prepared with the help of juicer without addition of water. 250 g of fresh fruit was chopped into small pieces and 135 ml juice was

collected. The juice was filtered with sterile cloth and the resultant filterate was used for oral dosing to animals.

4.1.4.2. Test dose Preparation

10ml of fresh juice was subjected for drying in previously dried and weighed petridish, then the juice was evaporated to complete dryness in a hot air oven (45ºC) and then weight of petridish containing dry residue of juice was taken and milligram equivalent dose of 10ml juice was calculated by subtracting initial weight of dried petridish. The same procedure was repeated for six times at different days. It was clear from the mean that, 10ml of juice gives 602.00mg of total solid residue in dried juice, which is equivalent to 250gm of fresh fruit of LS. The dose of fresh juice of *Lagenaria siceraria* in (ml) equivalent to 100, 200 and 400mg was administered orally to rats /kg/day for 30 days.

ISO was dissolved in normal saline. Green tea and pomegranate fruit extract were dissolving in distilled water. However, Vitamin E and Lycopene is dissolving in olive oil as vehicle (Kucukatay *et al*, 2005; Pour *et al*, 2008; Bansal *et al*, 2006). Same amount of olive oil was administered to control and ISO injected rats.

4.1.5. Experimental animals

All experiments were carried out on male albino Wistar rats weighing 200-250gm, obtained from in house animal breeding. They were housed in polypropylene cages (47×34×20cm) lined with husk, renewed every 24h under a 12:12h light dark cycle at around 22ºC and had free access to water and food. The animals were fed on a standard pellet diet (Pranav Agro Industries Ltd., Maharashtra, India). The pellet diet consisted of crude protein (22.12%), crude oil (4.05%), crude fibre (4.11%), ash (9.13%) and sand silica (0.75%) which provides the energy of 3630 Kcl/Kg. All the protocols of animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) in accordance to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals

(CPCSEA), Ministry of Social Justice and Empowerment, Government of India, New Delhi.

4.1.6. Fixation of optimum dosage of drugs

A pilot study was carried out to establish the optimum dose of the drugs which exhibits maximum cardioprotective effect during 30days. Rats were divided into different groups with six animals in each group. Rats were treated with Vit.E (25, 50 and 100 mg/kg/day, p.o), GT (10, 30 and 100 mg/kg/day, p.o), LYP (5, 10 and 15 mg/kg/day, p.o), PGFE (25, 50 and 100 mg/kg/day, p.o) and LSFJ (100, 200 and 400 mg/kg/day, p.o) for 30 days and ISO on 29th and 30th day. At the end of treatment period serum lactate dehydrogenase, creatine phospokinase-MB, tissue Lipid peroxidation and reduced glutathione were evaluated. The dose which was found to be most effective in functional recovery of above biochemical alterations were selected for further evaluation as single as well as in combination with Vitamin E in the present study (Jayachandran *et al*, 2009: Priscilla and Prince, 2009). Dose of ISO (200mg/kg, s.c) was selected based on the previous study carried out in our laboratory (Trivedi *et al*, 2006).

4.1.7. EXPERIMENTAL DESIGN

Set I: Effect of vitamin E alone and its combination with green tea (GT)

Group 1: Control rats received distilled water for 30 days and normal saline (1ml/kg, s.c) on 29th and 30th day.

Group 2: Rats received distilled water for 30 days and ISO (200mg/kg, s.c) in normal saline on 29th and 30th day at an interval of 24hr.

Group 3: Rats received Vit.E (100 mg/kg/day, p.o) + GT (100 mg/kg/day, p.o) for 30 days.

Group 4: Rats received Vit.E (100 mg/kg/day, p.o) for 30 days and ISO on 29th and 30th day.

Group 5: Rats received GT (100 mg/kg/day, p.o) for 30 days and ISO on 29th and 30th day.

Group 6: Rats received Vit. E (100 mg/kg/day, p.o) + GT (100 mg/kg/day, p.o) for 30 days and ISO on 29th and 30th day.

Set II: Effect of vitamin E alone and its combination with Lycopene (LYP)

Group 1: Control rats received distilled water for 30 days and normal saline (1ml/kg, s.c) on 29th and 30th day.

Group 2: Rats received distilled water for 30 days and ISO (200mg/kg, s.c) in normal saline on 29th and 30th day at an interval of 24hr.

Group 3: Rats received Vit.E (100 mg/kg/day, p.o) + LYP (10 mg/kg/day, p.o) for 30 days.

Group 4: Rats received Vit. E (100 mg/kg/day, p.o) for 30 days and ISO on 29th and 30th day.

Group 5: Rats received LYP (10 mg/kg/day, p.o) for 30 days and ISO on 29th and 30th day.

Group 6: Rats received Vit. E (100 mg/kg/day, p.o) + LYP (10 mg/kg/day, p.o) for 30 days and ISO on 29th and 30th day.

Set III: Effect of vitamin E alone and its combination with Pomegranate fruit extract (PGFE)

Group 1: Control rats received distilled water for 30 days and normal saline (1ml/kg, s.c) on 29th and 30th day.

Group 2: Rats received distilled water for 30 days and ISO (200mg/kg, s.c) in normal saline on 29th and 30th day at an interval of 24hr.

Group 3: Rats received Vit.E (100 mg/kg/day, p.o) + PGFE (100 mg/kg/day, p.o) for 30 days.

Group 4: Rats received Vit. E (100 mg/kg/day, p.o) for 30 days and ISO on 29th and 30th day.

Group 5: Rats received PGFE (100 mg/kg/day, p.o) for 30 days and ISO on 29th and 30th day.

Group 6: Rats received Vit. E (100 mg/kg/day, p.o) + PGFE (100 mg/kg/day, p.o) for 30 days and ISO on 29th and 30th day.

Set IV: Effect of vitamin E alone and its combination with *Lagenaria siceraria* fruit juice (LSFJ)

Group 1: Control rats received distilled water for 30 days and normal saline (1ml/kg, s.c) on 29th and 30th day.

Group 2: Rats received distilled water for 30 days and ISO (200mg/kg, s.c) in normal saline on 29th and 30th day at an interval of 24hr.

Group 3: Rats received LSFJ (400 mg/kg/day, p.o) for 30 days.

Group 4: Rats received Vit.E (100 mg/kg/day, p.o) + LSFJ (400 mg/kg/day, p.o) for 30 days

Group 5: Rats received Vit. E (100 mg/kg/day, p.o) for 30 days and ISO on 29th and 30th day.

Group 6: Rats received LSFJ (400 mg/kg/day, p.o) for 30 days and ISO on 29th and 30th day.

Group 7: Rats received Vit. E (100 mg/kg/day, p.o) + LSFJ (400 mg/kg/day, p.o) for 30 days and ISO on 29th and 30th day.

The change in body weight was recorded at the end of experimental period. After 24 hours of second injection (i.e on 48th hour) of Isoproterenol, electrocardiographic changes were recorded. Blood was collected and serum was separated. The animals were sacrificed and the heart was dissected out and weighed. Serum and heart tissue homogenate was used for the estimation of following biochemical parameters.

4.2. Assessment of Biochemical parameters

4.2.1. Removal and Processing of Serum and Tissues for Various Estimations

At the end of the treatment period, rats were anaesthetised using mild ether anaesthesia. Blood was collected from the retro-orbital plexus without any anti-coagulant and allowed to clot for 10 minutes at room temperature. It was then centrifuged at 2500 rpm for 20 minutes. The serum obtained was stored at 4°C till further use.

4.2.2. Tissues homogenization

The animals were euthanasiously sacrificed, after blood collection; heart was quickly transferred to ice-cold phosphate buffered saline (pH 7.4). It was blotted free of blood and tissue fluids, weighed on a Single Pan Electronic Balance (Precisa 205 ASCS). The hearts were cross-chopped with surgical scalpel into fine slices, suspended in chilled 0.25M sucrose solution and quickly blotted on a filter paper. The tissues were then minced and homogenised in chilled tris hydrochloride buffer (10mM, pH 7.4) to a concentration of 10% w/v. Prolonged homogenisation under hypotonic condition was designed to disrupt, as far as possible, the structure of the cells so as to release soluble proteins. The homogenate was centrifuged at 7,000 rpm at 0°C for 25 minutes using Remi C-24 high speed cooling centrifuge. The clear supernatant was used for the determination of lipid peroxidation and endogenous antioxidant activities. The sediment was re-suspended in ice-cold tris hydrochloride buffer (10mM, pH 7.4) to get a final concentration of 10% w/v and was used for the estimation proteins and membrane bound enzymes.

4.2.3. Assessment of general parameters

The final body weight along with heart weight was recorder. From this values heart weight to body weight ratio was calculated.

4.2.4. Hemodynamic measurements

4.2.4.1. Electrocardiographic measurements

After 48 hours of the first injection of either isoproterenol or vehicle, ECG was recorded under light ether anesthesia through needle electrodes (Lead II) using Biopac MP30 data acquisition system (Biopac Systems, Santa Barbara, CA). The changes in ST interval, QT interval, RR interval, QRS complex, P wave amplitude and Heart rate were determined from ECG.

4.2.4.2. Measurement of Blood pressure by Noninvasive method (indirect method)

For arterial blood pressure measurements using tail cuff method, rats were trained for at least one week until the blood pressure was recorded with minimal stress and restraint. Systolic BP, Diastolic BP and Mean BP were measured at the end of treatment period (Tail cuff) using LE 5002 storage pressure meter.

4.2.5. Assessment of serum cardiac marker enzymes

4.2.5.1. Assay of Aspartate transaminase (AST/GOT)

Quantitative estimation of serum Glutamate Oxaloacetate Transaminase (GOT) or Aspartate aminotransferase (AST) was done as per method of Reitman and Frankel 1957 and performed according to instructions provided for diagnostic reagent kit (Span Diagnostic) using SHIMADZU 1601 UV spectrophotometer at 505nm.

4.2.5.2. Assay of Alanine transaminase (ALT)

Quantitative estimation of serum Glutamate Pyruvate Transaminase (GOT) or Alanine aminotransferase was done as per method of Reitman and Frankel 1957

and performed according to instructions provided for diagnostic reagent kit (Span Diagnostic) using SHIMADZU 1601 UV spectrophotometer at 505nm.

4.2.5.3. Assay of Alkaline phosphatase (ALP)

Quantitative estimation of alkaline phosphatase was done according to instructions provided for diagnostic reagent kit (Span Diagnostic) using SHIMADZU 1601 UV spectrophotometer at 505nm.

4.2.5.4. Assay of Lactate dehydrogenase (LDH)

Quantitative estimation of serum Lactate dehydrogenase was done as per the instructions provided for diagnostic reagent kit (Reckon Diagnostic) using SHIMADZU 1601 UV spectrophotometer at 340nm.

4.2.5.5. Assay of Creatine kinase-MB (CK-MB)

Quantitative estimation of Serum Creatine kinase-MB was done as per the instructions provided for diagnostic reagent kit (Reckon Diagnostic) using SHIMADZU 1601 UV spectrophotometer at 340nm.

4.2.5.6. Assay of serum total protein (TP)

Quantitative estimation of Serum total protein was done as per the instructions provided for diagnostic reagent kit (Reckon Diagnostic) using SHIMADZU 1601 UV spectrophotometer.

4.2.5.7. Assay of Uric acid (UA)

Quantitative estimation of Serum Uric acid was done as per the method of Uricase/PAP method according to instructions provided for diagnostic reagent kit (Crest Biosystems) using SHIMADZU 1601 UV spectrophotometer at 540nm.

4.2.6. Assessment of lipid peroxidation and endogenous antioxidants

4.2.6.1. Assay of Lipid Peroxidation (MDA content)

It was estimated using the method described by Slater and Sawyer (1971).

Reagents:

1. Thiobarbituric acid (0.67% w/v): 0.67gm of thiobarbituric acid was dissolved in 50 ml of hot distilled water and the final volume was made up to 100 ml with hot distilled water.
2. Trichloroacetic acid (10% w/v):10gms of trichloroacetic acid was dissolved in 60 ml of distilled water and the final volume was made up to 100 ml with distilled water.
3. Standard Malondialdehyde stock solution (50mM): A standard malondialdehyde stock solution was prepared by mixing 25µl of 1, 1, 3, 3-tetraethoxypropane up to 100 ml with distilled water. 1.0 ml of this stock solution was diluted up to 10 ml to get solution containing 23µg of malondialdehyde/ml. One ml of this stock solution was diluted up to 100 ml to get a working standard solution containing 23ng of malondialdehyde/ml.

Procedure:

2.0 ml of the tissue homogenate (supernatant) was added to 2.0 ml of freshly prepared 10% w/v trichloroacetic acid (TCA) and the mixture was allowed to stand in an ice bath for 15 minutes. After 15 minutes, the precipitate was separated by centrifugation and 2.0 ml of clear supernatant solution was mixed with 2.0 ml of freshly prepared thiobarbituric acid (TBA).

The resulting solution was heated in a boiling water bath for 10 minutes. It was then immediately cooled in an ice bath for 5 minutes. The colour developed was measured at 532nm against reagent blank. Different concentrations (0-23nM) of standard malondialdehyde were taken and processed as above for standard graph. The values were expressed as nM of MDA/mg protein.

4.2.6.2. Assay of Reduced Glutathione (GSH)

Reduced glutathione was determined by the method described by Moron *et al.* (1979).

Reagents:

1. Trichloroacetic acid (20% w/v): 20gms of trichloroacetic acid was dissolved in sufficient quantity of distilled water and the final volume was made up to 100 ml with distilled water.
2. Phosphate Buffer (0.2M, pH 8.0): 0.2M sodium phosphate was prepared by dissolving 30.2gms sodium phosphate in 600 ml of distilled water, the pH was adjusted to 8.0 with 0.2M sodium hydroxide solution and the final volume was adjusted up to 1000 ml with distilled water.
3. DTNB reagent (0.6mM): 60mg of 5, 5'-dithiobis (2-nitro benzoic acid) was dissolved in 50 ml of buffer and the final volume was adjusted to 100 ml with buffer.
4. Standard Glutathione: 10mg of reduced glutathione was dissolved in 60 ml of distilled water and the final volume was made up to 100 ml with distilled water.

Procedure:

Equal volumes of tissue homogenate (supernatant) and 20% TCA were mixed. The precipitated fraction was centrifuged and to 0.25ml of supernatant, 2ml of DTNB reagent was added. The final volume was made up to 3ml with phosphate buffer. The colour developed was read at 412nm against reagent blank. Different concentrations (10-50μg) of standard glutathione were taken and processed as above for standard graph. The amount of reduced glutathione was expressed as μg of GSH/mg protein.

4.2.6.3. Assay of Glutathione peroxidase (GPx)

The activity of glutathione peroxidase was assayed by the method of Rotruck *et al* (1973)

Reagents:

1. Phosphate buffer (0.32M, pH7.0)

2. EDTA (0.8mM)
3. Sodium azide(10 mM)
4. Standard Glutathione (3mM)
5. Hydrogen peroxide (2.5mM)
6. Disodium hydrogen phosphate (0.3 M)
7. DTNB (40mg DTNB in 100ml of 1% sodium citrate)

Procedure:

0.2ml each of EDTA, sodium azide, reduced glutathione, hydrogen peroxide, 0.4ml of phosphate buffer and 0.1ml of homogenate were mix and incubated at 37ºC for 10 min. The reaction was arrested by the addition of 0.5ml of 10% TCA and the tubes were centrifuged at 10,000rpm. To 0.5 of supernatant, 4ml of disodium hydrogen phosphate and 1ml of DTNB were added and the colour developed was read at 420nm immediately using Shimadzu UV spectrophotometer. Graded concentration of the standards was also treated similarly. GPx is expressed as µmoles of glutathione oxidised/min/mg protein.

4.2.6.4. Assay of Glutathione S-transferase (GST)

The activity of glutathione peroxidase was assayed by the method of (Habig *et al* 1974).

Reagents:

1. Phosphate buffer (0.1M, pH6.5)
2. 1-chloro, 2,4-dinitrobenzene (CDNB) 30mM in 95% ethanol
3. Standard Glutathione (3mM)

Procedure:

To 1ml of phosphate buffer, 0.1 ml of homogenate, 1.7ml of water and 0.1ml of 1-chloro, 2, 4-dinitrobenzene in 95% ethanol were added and incubated at 37ºC for 15min. After incubation, 0.1ml of reduced glutathione was added. The increase in

optical density was measured against that of the blank at 340nm. GST activity was expressed as μmoles of CDNB conjugated/min/mg protein.

4.2.6.5. Assay of Superoxide dismutase (SOD)

Superoxide dismutase was estimated using the method developed by Misra and Fridovich (1972).

Reagents:

1. Carbonate Buffer (0.05M, pH 10.2):16.8gms of sodium bicarbonate and 22gms of sodium carbonate was dissolved in 500 ml of distilled water and the volume was made up to 1000 ml with distilled water.
2. Ethylenediaminetetra acetic acid (EDTA) solution (0.49M):1.82gm of EDTA was dissolved in 200 ml of distilled water and the volume was made up to 1000 ml with distilled water.
3. Hydrochloric acid (0.1N):8.5ml of conc. hydrochloric acid was mixed with 500 ml of distilled water and the volume was made up to 1000 ml with distilled water.
4. Epinephrine solution (3mM): 0.99gm epinephrine bitartarate was dissolved in 100 ml of 0.1N hydrochloric acid and the volume was adjusted to 1000 ml with 0.1N hydrochloric acid.
5. Superoxide Dismutase (SOD) standard (100 U/L):1mg (1000 U/mg) of SOD from bovine liver was dissolved in 100 ml of carbonate buffer.

Procedure:

0.5ml of tissue homogenate was diluted with 0.5ml of distilled water, to which 0.25ml of ice-cold ethanol and 0.15ml of ice-cold chloroform was added. The mixture was mixed well using cyclo mixer for 5 minutes and centrifuged at 2500 rpm. To 0.5ml of supernatant, 1.5ml of carbonate buffer and 0.5ml of EDTA solution were added. The reaction was initiated by the addition of 0.4ml of epinephrine and the change in optical density/minute was measured at 480nm

against reagent blank. SOD activity was expressed as units/mg protein. Change in optical density per minute at 50% inhibition of epinephrine to adrenochrome transition by the enzyme is taken as the enzyme unit. Calibration curve was prepared by using 10-125 units of SOD.

4.2.6.6. Assay of Catalase (CAT)

It was estimated by the method of Hugo Aebi as given by Colowick *et al.* (1984).

Reagents:

1. Phosphate Buffer (50mmol/l, pH 7.0): (a) 6.81gms of potassium dihydrogen orthophosphate (KH_2PO_4) was dissolved in distilled water and made up to 1000 ml. (b) 8.90gms of disodium hydrogen orthophosphate (Na_2HPO_4) was dissolved in distilled water and made up to 1000 ml. The solutions (a) and (b) were mixed in the proportion of 1: 1.5 (v/v).
2. Hydrogen Peroxide (30 mmol/l): 0.34ml of 30% hydrogen peroxide was diluted with phosphate buffer to 100 ml. This solution was prepared fresh each day.
3. Catalase standard (65,000 U/mg protein; 1mg protein/ml): Crystalline beef-liver catalase suspension was centrifuged to isolate the crystals of the enzyme that was dissolved in 0.01M phosphate buffer (pH 7.0) to give a final concentration of 1.0mg protein/ml. Before assay, it was diluted with distilled water to obtain 1000 U/ml.

Procedure:

To 2ml of diluted sample 1ml of hydrogen peroxide (30 mmol/l) was added to initiate the reaction. The blank was prepared by mixing 2ml of diluted sample (similar dilution) with 1ml of phosphate buffer (50mmol/l; pH 7.0). The dilution should be such that the initial absorbance should be approximately 0.500. The decrease in absorbance was measured at 240nm. Catalase activity was expressed as μmoles of H_2O_2 consumed/min/mg protein.

4.2.6.7. Estimation of Vitamin E

The level of vitamin E was estimated as suggested by Desai (1984).

Reagents:

1. Ethanol
2. Petroleum ether
3. Bathophenanthroline
4. O-Phosphoric acid

Procedure:

To, 1ml of homogenate, 1ml of ethanol was added and thoroughly mixed. 3ml of petroleum ether was added, shaken rapidly and centrifuged. 2ml of supernatant was taken and evaporated to dryness. To this 0.2ml of bathophenanthroline (0.2% 4, 6-diphenyl-1,10-phenanthroline in ethanol) was added. The assay mixture was protected from light and 0.2ml of ferric chloride (0.001M) was added followed by 0.2ml of o-phosphoric acid (0.001M). The total volume was made upto 3ml with ethanol. The colour developed was read at 530nm. The level of vitamin E is expressed as μg/mg protein.

4.2.7. Assessment of membrane bound phosphatases (ATPases)

4.2.7.1. Sodium-Potassium dependent adenosine triphosphatase (Na^+K^+ ATPase)

Na^+K^+ATPase were assayed according to the method described by Bonting (1970).

Reagents:

1. Tris hydrochloride buffer (92mM, pH 7.5):11.13gms of tris was dissolved in 900ml of distilled water and the pH was adjusted to 7.5 with 1M hydrochloric acid. The resulting solution was diluted to 1000ml with distilled water.
2. Magnesium sulphate solution (5mM):1.232gm of magnesium sulphate dissolved and made upto 1000ml with distilled water.

3. Potassium chloride solution (5mM):0.372gm of potassium chloride was dissolved in 400ml of distilled water and the final volume was made upto 1000ml with distilled water.
4. Sodium chloride solution (60mM):3.231gms of sodium chloride was dissolved in 400ml of distilled water and the final volume was made upto 1000ml with distilled water.
5. EDTA solution (0.1mM): 0.372gm of EDTA was dissolved in 400ml of distilled water and the final volume was made upto 1000ml with distilled water.
6. Adenosine triphosphate (ATP) solution (40mM):0.220gm of ATP was dissolved in 4ml of distilled water and the final volume was made upto 10ml with distilled water.
7. Trichloroacetic acid (10% w/v);10gms of trichloroacetic acid was dissolved in sufficient quantity of distilled water and the final volume was made upto 100ml with distilled water.

Procedure:

1.0ml of tris-hydrochloride buffer and 0.2ml each of magnesium sulphate, sodium chloride, potassium chloride, EDTA, ATP were added to test tube containing 0.2ml of homogenate. The mixture was incubated at 36°C for 15 minutes. The reaction was arrested by addition of 1.0ml of 10% TCA, mixed well and centrifuged. The phosphorus content of the supernatant was estimated as described in section 3.2.6.4.

The enzyme activity was expressed as μM of inorganic phosphorus liberated/mg protein/min.

4.2.7.2. Calcium dependent adenosine triphosphatase (Ca^{2+}ATPase)

Ca^{2+}ATPase was assayed according to the method described by Hjerken and Pan (1983).

Reagents:

1. Tris hydrochloride buffer (125mM, pH 7.5): 15.12gms of tris was dissolved in 900ml of distilled water and the pH was adjusted to 7.5 with 1M hydrochloric acid. The resulting solution was diluted to 1000ml with distilled water.
2. Calcium chloride solution (50mM): 5.55gms of calcium chloride was dissolved in 400ml of distilled water and the final volume was made upto 1000ml with distilled water.
3. Adenosine triphosphate (ATP) solution (10mM): 0.551gm of ATP was dissolved in 4ml of distilled water and the final volume was made upto 100ml with distilled water.
4. Trichloroacetic acid (10% w/v): 10gms of trichloroacetic acid was dissolved in sufficient quantity of distilled water and the final volume was made up to 100ml with distilled water.

Procedure:

The incubation mixture contained 0.1ml each of tris-hydrochloride buffer, calcium chloride, ATP and homogenate in a test tube. The mixture was incubated at 37°C for 15 minutes. The reaction was arrested by addition of 1.0ml of 10% TCA, mixed well and centrifuged. The phosphorus content of the supernatant was estimated as described in section 3.2.6.4. The enzyme activity was expressed as μM of inorganic phosphorus liberated/mg protein/min.

4.2.7.3. Magnesium dependent adenosine triphosphatase (Mg^{2+}ATPase)

Mg^{2+}ATPase were assayed according to the method described by Ohinishi *et al.* (1982).

Reagents:

1. Tris hydrochloride buffer (374mM, pH 7.6): 45.24gms of tris was dissolved in 900ml of distilled water and the pH was adjusted to 7.6 with 1M

hydrochloric acid. The resulting solution was diluted to 1000ml with distilled water.

2. Magnesium chloride solution (25mM): 6.16gms of magnesium chloride was dissolved in 400ml of distilled water and the final volume was made upto 1000ml with distilled water.
3. Adenosine triphosphate (ATP) solution (10mM): 0.551gm of ATP was dissolved in 4ml of distilled water and the final volume was made upto 100ml with distilled water.
4. Trichloroacetic acid (10% w/v): 10gms of trichloroacetic acid was dissolved in sufficient quantity of distilled water and the final volume was made upto 100ml with distilled water.

Procedure:

The incubation mixture contained 0.1ml each of tris-hydrochloride buffer, magnesium chloride, ATP and homogenate in a test tube. The mixture was incubated at 37°C for 15 minutes. The reaction was arrested by addition of 1.0ml of 10% TCA, mixed well and centrifuged. The phosphorus content of the supernatant was estimated as described in section 3.2.6.4. The enzyme activity was expressed as μM of inorganic phosphorus liberated/mg protein/min.

4.2.7.4. Determination of Inorganic Phosphorus (P_i)

Inorganic Phosphorus (P_i) was estimated by the method described by Fiske and Subbarow (1925).

Reagents:

1. Ammonium molybdate reagent (2.5% w/v): 2.5 gm of ammonium molybdate was dissolved upto 100ml with 3M sulphuric acid.
2. 1-Amino 2-naphthol 4-sulphonic acid (ANSA) reagent: (a) 0.25% w/v of ANSA reagent in 15% w/v of sodium metabisulphite and 20% w/v of sodium sulphite. (b) 15gms of sodium metabisulphite was dissolved in 100ml of

distilled water. (c) Accurately weighed 20gms of sodium sulphite was dissolved in 100ml of distilled water. 250mg of ANSA was dissolved in 97.5ml of 15% w/v of sodium metabisulphite and 2.5ml of 20% w/v of sodium sulphite, mixed well and stored at room temperature.

3. Standard Phosphorus: 35.1mg of potassium dihydrogen orthophosphate was dissolved in 100ml of distilled water. This contained 80µg of phosphorus/ml.

Procedure:

1ml of the supernatant was taken and the volume was made upto 5.0ml with distilled water. To this, 1ml of 2.5% ammonium molybdate reagent and 0.5ml of ANSA reagent was added. The colour developed in 20 minutes was read using blank containing water instead of sample at 620nm. A standard graph was prepared taking different concentrations of standard phosphorus (16-80µg). The values were expressed as µM of inorganic phosphorus liberated/mg protein/min.

4.2.7.5. Estimation of Na^+, K^+ and Ca^{++}

The concentration of Na^+ and K^+ were estimated using commertial kits (Monozyme India Ltd, Secunderabad). Ca^{++} in the heart homogenate was measured by the O-cresophthalein complexone method using a reagent kit (Span Diagnostic Ltd, Gujarat, India). In this method O-cresophthalein complexone binds to calcium tightly in alkaline solution to form a deeply colored complex, owing to lactone formation in the phthalein part of molecule (Devika and Prince, 2007).

4.2.7.6. Estimation of tissue Protein

The method of Lowry *et al.* (1951) was used for the estimation of total protein.

Reagents:

1. Sodium hydroxide (0.1M): 4gms of sodium hydroxide was dissolved in 400 ml of distilled water and the final volume was made up to 1000 ml with distilled water.

2. Lowry C reagent: (a) Copper sulphate in 1% sodium potassium tartarate (1% w/v) 0.5gm of copper sulphate was dissolved in 1% sodium potassium tartarate (Prepared by dissolving 1gm of sodium potassium tartarate in 100 ml of distilled water). (b) Sodium carbonate in 0.1M sodium hydroxide (2% w/v) 2gms of sodium carbonate was dissolved in 100 ml of 0.1M sodium hydroxide. 2ml of solution (a) was mixed with 100 ml of solution (b) just before use.
3. Standard Protein (Bovine serum albumin): 20mg of bovine serum albumin was dissolved in 80 ml of distilled water and few drops of sodium hydroxide were added to aid complete dissolution of bovine serum albumin and to avoid frothing. Final volume was made up to 100 ml with distilled water and stored overnight in a refrigerator.
4. Folin's phenol reagent: Folin's phenol reagent was diluted with distilled water in the ratio of 1:2. (i.e. 1ml of Folin's phenol reagent was mixed with 2ml of distilled water).

Procedure:

Diluted membrane fraction aliquots (0.1ml) were taken in test tubes. To this, 0.8ml of 0.1M sodium hydroxide and 5ml of Lowry C reagent was added and the solution was allowed to stand for 15 minutes. Then 0.5ml of 1N Folin's phenol reagent was added and the contents were mixed well on a vortex mixer. Colour developed was measured at 640nm against reagent blank containing distilled water instead of sample.

Different concentrations (40-200μg) of standard protein (Bovine serum albumin) were taken and processed as above for standard graph. The values were expressed as mg of protein/ gm of wet tissue (mg/ gm).

4.2.8. Separation of Lactate dehydrogenase (LDH) isoenzymes by agarose gel electrophoresis (McKenzie and Henderson, 1983).

Reagents:

1. 1% Agarose
2. Electrophoretic buffer-tris-glycine buffer (0.1M) pH 8.3
3. Staining solution: contained 1.0ml of 1.0M lithium lactate, 1.0ml of 0.1M sodium chloride, 1.0ml of 5.0mM magnesium chloride, 2.5 ml of 0.1% (w/v) nitro blue tetrazolium (NBT), 0.25ml of 0.1% phenazine methosulphate, 2.5ml of 0.5M phosphate buffer, pH 7.4 and 10mg of NAD in a total volume of 10ml

Procedure:

LDH isoenzymes were separated and quantified by agarose gel electrophoresis. Agarose gel (1%) was prepared and applied immediately to the glass slide. After the agarose gel had set properly, serum samples were applied into wells and separated by electrophoresis. After the run, the gels were removed and stained by using staining solution. The gels were incubated with the staining solution at 37°C in the dark for suitable period (approx. 30min). The separated LDH isoenzymes appeared as purple bands. The gel was washed with 7.5% acetic acid, preserved in 5% acetic acid. The photographs were taken using AlphaEase Fc Imaging system, USA.

4.2.9. Lipid Profile

4.2.9.1. Extraction of Lipids from heart tissue

From the sample of heart tissue homogenate lipids were extracted by the method of Folch *et al* (1957). To a known volume of tissue homogenate,10ml of chloroform methanol mixture was added and mixed well for 30min using shaker and was filtered through What-man filter paper (No.42) into a separating fennel. The filterate was mixed with 0.2ml of physiological saline and the mixture was kept overnight undisturbed. The lower phase containing lipid was drained off into pre-weighted beaker. The upper phase was re-extracted with more of chloroform-

methanol mixture and the extract were pooled and evaporated under vaccum at room temperature. The lipid extract was re-dissolved in 3ml of chloroform methanol (2:1) mixture and the aliquots collected. The aliquots were named as LEA (Lipid extract Aliquots).

4.2.9.2. Estimation of Total cholesterol

Quantitative estimation of TC in serum and tissue LEA was performed as per instructions provided for ENZOPAK kit using UV Spectrophotometer at 505nm.

4.2.9.3. Estimation of Triglyceride

Quantitative estimation of TG in serum and tissue LEA was performed as per instructions provided for ENZOPAK kit using UV Spectrophotometer at 505 nm.

4.2.9.4. Estimation of HDL

Quantitative estimation of HDL in serum was performed as per instructions provided for ENZOPAK kit (PTA method) using UV Spectrophotometer at 505 nm.

4.2.9.5. Estimation of LDL and VLDL

LDL and VLDL were calculated as per Friedewald's equation.

LDL = Total Cholesterol-(HDL+VLDL)

VLDL= TG/5

4.2.9.6. Estimation of Phospholipids

Tissue PL was estimated as 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% ammonium molybdate in 3N H_2SO_4

3. Amino naptholsulphonilic acid (ANSA)
4. Phosphorus stock standard: 35.1mg of potassium dihydrogen phosphate was dissolved in 100ml of distilled water.

Procedure:

To 0.5ml of LEA, 1ml of perchloric acid was added and digested on sand bath until the mixture become colourless. It was then made up to a known volume (3ml) and a suitable aliquote (0.5ml) was taken and diluted with water to 4.5ml.Then 0.5ml of ammonium molybdate and 0.2ml of ANSA was added. The contents were mixed well. The blue colour developed was read after 10min at 620nm using red filter. The phosphorus content was multiplied by a factor 25 to get total phospholipids content and expressed as mg/g wet tissue for tissue and mg/dl for serum.

4.2.9.7. Estimation of Free Fatty acids (FFA)

FFA was estimated as per the method by Horn and Menahan 1981 and modified by Itaya 1977.

Reagents:

1. Chloroform (200): Heptane (150): methanol (7) mixture (v/v)
2. Copper nitrate-triethanolamine reagent (Cu-TEA)
3. Diethyl dithiocarbamate solution (Colour reagent)
4. Activated silicic acid
5. 20mg palmitic acid was dissolved in 100ml of Chloroform: Heptane: methanol mixture-standard solution

Procedure:

About 0.5ml of LAE was added to 5.5ml of CHM (Chloroform heptane methanol) solvent. After adding 200mg of activated silicic acid the content were shaken well and centrifuged. Two ml of Cu-TEA (Copper triethanolamin) reagent was added and mixed well. The tubes were centrifuged to separate the two phases and 2ml of

upper phase from each tube was transferred to another set of tubes. To all these tubes 1ml of 0.1% DDC (Diethyl dithio carbamate) colour reagent was added. The colour intensity was measured at 430nm using blue filter. It was expressed as mg/g wet tissue for tissue and mg/dl for serum.

4.2.10. Lipid metabolizing enzymes

4.2.10.1. Estimation of tissue Lecithin cholesterol acyl transferase (LCAT) activity (Hitz *et al*, 1983)

Reagents:

1. Dextran sulphate (0.2%): 200mg of dextran sulphate was dissolved in 100ml of physiological saline
2. Isopropanol
3. Acetone
4. Digitonin: 5mg of digitonin was dissolved in 1ml of 50%ethanol v/v
5. Substrate: A pool of control plasma was warmed at 56ºC for 30 min to inactivate the LCAT present in the plasma. The plasma was then incubated at 4ºC for 15min with 0.2% dextran sulphate. This was followed by centrifugation for 10min at 1750g. The supernatant rich in HDL was used as the substrate for the enzyme assay.
6. Test Plasma: To 1ml of plasma, 0.5ml of dextran sulphate was added. After incubation at 4ºC for 15min, it was centrifuged at 1750g for 10min. The supernatant was used for the assay of the enzyme
7. Ferric chloride uranyl acetate reagent
8. Sulphuric acid ferrous sulphate reagent

Procedure:

The incubation mixture contained 0.6ml of the substrate and 0.6ml of plasma. 0.4ml of this mixture was immediately transferred to the tube containing 1ml of isopropanol to arrest the reaction. This gave the cholesterol present at the

beginning of the experiment. Incubation at 27ºC was continued for 90min. 0.4ml of mixture was again pipette out and the reaction was arrested similarly as earlier. The resultant mixture was centrifuged. To the supernatant, 2ml of acetone and 1ml of digitonin were added. It was left aside for 60min. The mixture is centrifuged and the supernatant was decanted. Precipitate at the bottom was process for cholesterol estimation as per Parekh and Jung 1970. To 0.5ml of LEA, 2.5ml of ferric chloride-uranyl acetate reagent was added. Then, 2ml of sulfuric acid, ferrous sulphate reagent was added and mixed well. After 20 min of incubation at room temperature the Optical density was measured at 560nm using green filter. LCAT activity is expressed as μmoles of cholesterol esterified/hr/gm of protein.

4.2.10.2. Estimation of Tissue Cholesterol ester synthase (CES)

The activity of tissue cholesterol ester synthase was estimated as reported by Kothari *et al*, 1973.

Reagents:

1. Acetate buffer (0.1 M, pH 4.0): 1.8mg of sodium acetate and 0.05 ml of acetic acid were dissolved in 1.8ml and 8.2ml of distilled water respectively, both solutions were mixed.
2. Acetone ethanol mixture 1:1(v/v)
3. Acetone ether mixture 1:2 (v/v)
4. Acetic acid (10%)
5. Digitonin: 5mg of digitonin in 1ml of 50% ethanol
6. Substrate: 35.4mg of cholesterol and 63.87mg of oleic acid were dissolved in 1.8ml of chloroform. To this 103.6 mg of sodium taurocholate and 32.1mg of ammonium chloride in 7.5 ml of acetate buffer were added and homonogenised.
7. Ferric chloride-uranyl acetate reagent
8. Sulphuric acid-ferrous sulphate reagent

Procedure:

Tissue homogenate is prepared in acetate buffer (10%).The incubation mixture containing 0.5ml of acetate buffer, 0.2ml substrate and 0.2ml tissue homogenate was incubated at 37C for 6 hrs with occasional shaking. The reaction was arrested by the addition of 5ml of acetone ethanol mixture and centrifuge. 1ml of digitonin was added to the supernatant, followed by two drops of acetic acid (10%).the contents were closed and kept in a dark chamber for 16 hours. The ppt after centrifugation was washed twice with acetone ether mixture and finally with dry ether. The cholesterol content was estimated as earlier. CES activity is expressed as micromole of cholesterol etherified/hr/mg protein.

4.2.10.3. Estimation of Tissue Lipoprotein Lipase (LPL)

LPL was estimated as describe by Schmidt, 1947

Reagents:

1. Olive oil emulsion; 5g olive oil was mixed with 5g gum Arabic and 95ml of saline.
2. Sodium deoxy cholate: 10mM
3. Triethanolamine (TEA) buffer: 1M pH 8.5
4. Copper reagent: prepared by dissolving 6.45g copper nitrate and 18.6g TEA in 100ml water. The pH was adjusted to 7.5 with 5N sodium hydroxide solution. The final volume was made upto 200ml.
5. Diethyl dithio cardamate (DDC): 0.25% in isopropanol
6. Standard steric acid: 50μM in chloroform

Procedure:

To 0.4ml of TEA buffer, 0.5ml of olive oil emulsion, 0.05ml deoxycholate solution and 0.1ml of tissue homogenate were added. Blank tubes contained 0.1ml of saline instead of tissue homogenate. Incubated the tubes at 20°c for 10min with constant shaking. The tubes were dipped in a boiling water bath to arrest the reaction. Add

5ml of chloroform and 2.5ml of copper reagent and mixed thoroughly for 20min. After centrifugation 2ml of the chloroform layer was mixed with 2.5ml of DDC and the colour developed was immediately read at 430nm in a UV spectrophotometer. The enzyme activity was expressed as μmoles of free fatty acids liberated/hr/mg protein.

4.2.11. Measurement of Myeloperoxidase (MPO) activity

MPO was extracted and the activity was measured using a modified spectroscopic method described by Bradley *et al* (1982) which is as follows.

Reagents:

1. Potassium phosphate buffer (50mM pH 6.0): Dissolve 3.402gm KH_2PO_4 in 450ml, adjust to pH 6.0 and make up to 500ml.
2. Extraction Buffer: Dissolve 0.5gm HTAB (hexadecyl trimethyl ammonium bromide) in 100ml phosphate buffer.
3. Substrate solution (0.167mg/ml dianisidine):Dissolve 16.7mg o-dianisidine in 100ml phosphate buffer. If there is a precipitate in solution filter using 0.2μm filter. Cover in alfoil as this substrate is light sensitive and store at 4°C. Solution lasts approximately 1 week. Discard if solution becomes slightly brownish. On the day of the assay, warm solution up to room temperature and add hydrogen peroxide (100μl of 0.5% per 100ml substrate solution) approx 30 mins before use.

Procedure:

Tissue samples (heart) were homogenized in ice cold 50mM potassium phosphate buffer pH-6 containing 0.5% HTAB. The homogenate was freeze thawed three times then centrifuged at 11000 x g for 20 min at 4°C. The supernatant (34 μl) was mixed with the same phosphate buffer (986 μl) which containing 0.167 mg/ml ortho-dianisidine dihydrochloride (Sigma) and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was recorded by spectrophotometer. One unit of

MPO activity was defined as that consuming 1nmole of peroxide per minute at 22° C. The results were expressed as units/mg of protein.

4.2.12. Measurement of tissue nitrite level

The nitrite was estimated by the method of Guevara et al, 1998.

Reagents:

1. Sulphosalicyclic acid
2. Tris-HCL Buffer
3. Griess reagent: prepare 100ml of Griess reagent by mixing sulfanilamide (1% w/v), naphthylethylenediamine dihydrochloride (0.1%w/v) and ortho-phosphoric acid (2.5%v/v)

Procedure:

To 1 ml of tissue homogenate, add 1 ml of Griess reagent and incubate it for 15min at 37°C. Read the absorbance at 540nm against a Griess reagent blank. Sodium nitrite solution was used as the standard. The amount of nitrite present in the samples was estimated from the standard curves obtained.

4.2.13. Measurement of C-reactive Proteins

Quantitative estimation of CRP in serum was performed as per instructions provided for by SPINREACT kit (Latex turbidimetry method). Sample is allowed to react with a buffer and anti C-reactive latex. The formation of the antibody antigen complex during the reaction results in an increase in turbidity and extent of turbidity is measured as the amount of the light absorbed at 540nm.

Reagents and procedure

The kit comprised, assay buffer (Tris buffer 20mmol/L), Sodium azide 0.95g/L and antibody latex reagent (latex particles coated with antibody to C-reactive protein). To 10µl of the serum, 250 µl of working reagent (1ml of latex reagent + 9ml

distilled water) were added and the absorbance was read at 540nm, CRP concentration of the sample was determined from the standard curve.

4.2.14. Histopathology study

4.2.14.1. Haemotoxyline and eosin staining

After the treatment period, the animals were sacrificed and the organs were excised, blotted free of blood and tissue fluids and preserved in 10% v/v formal saline solution. The specimens were given for further processing to Baroda Pathological Laboratory, Vadodara where routine procedure for sectioning, staining and mounting was observed by the laboratory personnel. Briefly, after a week the tissues were washed thoroughly in repeated changes of 70% alcohol and then dehydrated in ascending grades of alcohol (70-100%). After dehydration, the tissues were cleaned in xylene and embedded in paraffin wax.

Sections of 5μ thickness were cut on a microtome and taken on glass slides coated with albumin. The sections were deparaffinated in xylene and downgraded through 100, 90, 70, 50, 30% alcohol and then finally in water. The haematoxylin-stained sections were stained with eosin for 2 minutes and were then quickly passed through ascending grades of alcohol, cleaned in xylene and mounted on Canada balsam.

The stained sections were examined under Olympus (Magnus MLX series) India Pvt Ltd. Photomicroscope and photographed (10X). The pathologist was blinded to the treatment. Analyzes of the heart sections were analyzed as described elsewhere (Acikel et al. 2005). Briefly, the findings were graded as no changes (A); (+) mild (focal myocyte damage or small multifocal degeneration with slight degree of inflammatory process); (++) moderate (extensive myofibrilary degeneration and/or diffuse inflammatory process); (+++) marked (necrosis with diffuse inflammatory process).

4.2.14.2. Masson's trichrome staining

Heart tissue sections were obtained and fixed in 10% formalin and embedded in paraffin wax. Sections were cut at 4μm in thickness. The sections were stained by Masson's trichrome method for collagen fibres as describe in John 2008a.

Reagents:

1. Solution a: Acid fuchsin (0.5g), Glacial acetic acid (0.5ml), Distilled water (100ml)
2. Solution b: Phosphomolybdic acid (1g), Distilled water (100ml)
3. Solution c: Methyl blue (2g), Glacial acetic acid (2.5ml), Distilled water (100ml)

Procedure:

Deparaffinize the sections and bring to water. Mercury pigments were removed by iodine, sodium thiosulphate sequence, and then wash under tap water. The section was then stained by Celestine blue-hematoxyline method which stains nuclei. It was then differentiated with 1% acid alcohol, washed well in tap water again stained in acid fuchsin solution a and kept for 5 minutes. Section was rinsed in distilled water and treated with phosphomolybdic acid solution b and kept for 5 min. it was then stained with methyl blue solution c, kept for 2-5min followed by rinsed with distilled water. It was then treated with 1% acetic acid and kept for 2 min and dehydrated through alcohols. The section was clean in xylene and mount in permanent mounting medium. The stained sections were examined under Olympus (Magnus MLX series) India Pvt Ltd. Photomicroscope and photographed (40X)

Results

Nuclei	:	*Black*
Cytoplasm, muscle and erythrocytes:	:	*Red*
Collagen	:	*Blue/green*

4.2.14.3. Periodic acid Schiff's staining

PAS staining was carried out as described in John 2008b.

Reagent:

1. Periodic acid solution: 1g of periodic acid in 100ml distilled water
2. Schiff reagent: Dissolve 1g of basic fuchsin and 1.9g of sodium metabisulfite in 100ml of 0.15N HCL. Shake the solution at intervals or on a mechanical shaker for 2hr. Add 500mg of activated charcoal and shake for 1-2 min. Filter the solution and store at 4°C

Procedure:

Heart tissue sections of 4.5µm size were cut using microtome from paraffin-embedded tissue blocks and placed on pre-coated slides with poly-L-lysin or egg albumin and then incubated at 56 ºC for overnight. Sections were deparaffinzed in xylene, using three changes of fresh xylene,10min each and further rehydrated gradually through descending grades of alcohol (95, 90, 70, 50, 30%), 5 min each. Finally the sections were hydrated in distilled water. Selected sections were oxidized with 2% periodic acid for 5min then rinsed in distilled water. To the section, Schiff's reagent was added and kept in dark for 20minutes. The sections were then rinsed in distilled water for 10min. and counterstained with haematoxyline for 5min and then washed in running tap water for 5min or distilled water. Then dehydrated gradually in ascending grades of alcohol (70, 75 and 100%) 5min each. Then the sections were mounted in DPX (mount) with cover slips and allow drying. The stained sections were examined under Olympus (Magnus MLX series) India Pvt Ltd. Photomicroscope and photographed (10X).

Results

Various glycoproteins and glycoconjugates	:	*magenta colour*
Nuclei	:	*Blue*

4.2.15. DNA fragmentation Analysis by Gel Electrophoresis

Extraction of DNA, The protocol was followed as per provided by GeNei™, Bangalore-India.

Protocol

1. 5-10mg of heart tissue was homogenize in 2ml of DNA extraction solution provider in the kit and left it for 15-20min at room temperature.
2. centrifuged the homogenate at 10,000rpm for 15 min. and the resultant supernatant was transferred into a fresh tube. This step removes insoluble tissue fragments, partially hydrolyzed RNA and excess polysaccharides from the homogenate.
3. To the tube equal volume of 100% ethanol was added so as to precipitate DNA. Mixed well so that the DNA extraction solution and ethanol form a homogenous solution. A cloudy ppt was visible indicating the presence of DNA. Further the DNA was precipitated by centrifugation at 10,000rpm for 5min.
4. The ppt DNA was washed with 0.8-1ml of 95% ethanol. At each wash the DNA was suspended in ethanol by inverting the tubes 3-6 times and the ethanol was removed by decantation. Final wash was given with 0.8-1ml of 70%ethanol.
5. The final DNA pellet was dissolved in nuclease free water (Bangalore GeNei™) by slowly passing the pellet through a pipette. The solution was stored at 4ºC for 48hrs so as to increase the yields of DNA. The solution was finally stored at -20 ºC until used.

Approximately 8 µg of DNA was loaded in each lane and run at 70 V on a 0.8% agarose gel and stained with ethidium bromide (0.5 µg/ml) to separate DNA. Since extensive DNA fragmentation is an important characteristic of apoptosis, visualization of DNA breaks could greatly facilitate the identification of apoptotic cells.

4.2.16. Macroscopic enzyme mapping (TTC Staining)

The triphenyl tetrazolium chloride (TTC) test, used for the macroscopic enzyme mapping of infarcted myocardium was done according to the method of Lie *et al* (1975). The heart was washed rapidly in cold water to remove excess blood, taking care not to macerate the tissue. The excess epicardial fat was lightly trimmed off. The heart was transversely cut across the left ventricle to obtain slices no more that 0.1cm in thickness.

The heart slices were placed in the covered, darkened glass dish containing pre warmed (1%) TTC solution in phosphate buffer and the dish was incubated between 37-40ºC for 30-45min. the heart slices were turned over once or twice to make certain that it remains immersed and covered by 1cm of the TTC solution. At the end of incubation period, the heart slices was placed in 10% formalin solution which enhances the colour contrast developed. The % infarction was measured using Image J Software system.

The expected reaction of TTC test was as follows: normal myocardium (succinate dehydrogenase or LDH enzyme active) turned to bright red, ischemic myocardium (succinate dehydrogenase or LDH enzyme deficient) turned to pale grey or grayish yellow or uncolored and fibrous scars turned to white.

4.2.17. Caspase-3 protease level

The levels of caspase-3 Protease was estimated as per the instruction provided by BioVision (Caspase-3/CPP32 Colorimetric Assay Kit, USA). The kit contains

Cell lysis buffer	:	100ml
2X reaction buffer	:	8ml
DEVD-pNA (paranitroaniline) (4mM)	:	0.5ml
DTT (1M)	:	0.4ml
Dilution buffer	:	100ml

Procedure:

1. Tissue sample was homogenized in lysis buffer (1X volume of tissue+3X volume of lysis buffer) so as to get tissue lysate.
2. Lysate was centrifuge for 1min in a microcentrifuge (10,000xg).
3. The supernatant (Cytosolic extract) was transfer to a fresh tube and put on ice bath for immediate assay.
4. 50µl of 2X reaction buffer (containing 10mM DTT) was added to each sample.
5. 5 µl of the 4mM DEVD-pNA substrate (200 µM final conc.) was added to each sample and then incubated at 37C for 2hr.
6. The reading was taken at 405nm in a microtiter plat reader (BIO RAD, Model 680XR microplate reader).

4.3. *IN VITRO* ANTIOXIDANT STUDIES

To ascertain free radical scavenging property of green tea extract (GTE), vitamin E, Lycopene, LSFJ and PGFE, *in vitro* methods were performed in four systems, namely radical scavenging activity by DPPH reduction (DPPH assay), superoxide radical scavenging activity in riboflavin/light/NBT system, nitric oxide scavenging activity and lipid peroxidation in rat liver homogenate.

4.3.1. DPPH radical scavenging assay

Principle:

DPPH assay was used as a rapid method to provide an evaluation of antioxidant activity due to free radical scavenging. Antiradical activity was measured by a decrease in absorbance at 516 nm of a methanolic solution of coloured DPPH brought about by the sample. 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) a purple coloured stable free radical is reduced into the yellow coloured diphenylpicryl hydrazine (Vani *et al.*, 1997).

Reagents:

1. *DPPH stock solution:* 1.3 mg of 1, 1-diphenyl-2-picryl hydrazyl (DPPH) was dissolved in 1ml of methanol.

Procedure:

100 µl of suitably diluted stock solution of either aqueous or methanolic solutions of the drugs were mixed with 3ml of methanol. 75µl of DPPH solution was added and decrease in the absorbance was noted after 15 minutes at 516nm against methanol as blank. The obtained data was used to determine the ability of the sample to scavenge the DPPH free radicals. The results were expressed as the mean ± SEM of three replicates. Percentage (%) inhibition was calculated as: - [(absorbance of control – absorbance of test sample) / absorbance of control] X 100.

4.3.2. Assay for superoxide radical scavenging activity

Principle:

The assay was based on the capacity of the methanolic or aqueous solutions of drugs to inhibit formazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system (Beauchamp and Fridovich, 1971).

Reagents:

1. *Phosphate Buffer (50mM, pH 7.6):* (a) 2.72 gm of potassium dihydrogen orthophosphate (KH_2PO_4) was dissolved in 60 ml of distilled water. The volume was then made up to 100 ml with distilled water. (b) 800mg of sodium hydroxide (NaOH) was dissolved in 60 ml of distilled water. The volume was then made up to 100 ml with distilled water. 50 ml of (a) was mixed with 42.4ml of (b). The pH of the mixture was adjusted to 7.6 with 1N hydrochloric acid (HCl). The volume was then made up to 200 ml with distilled water.

2. *Riboflavin:* 5mg of riboflavin was dissolved in 25ml of phosphate buffer (50mM, pH 7.6).
3. *EDTA (12mM):* 402 mg of EDTA was dissolved in 5ml of buffer. The volume was then made up to 10 ml with phosphate buffer.
4. *Nitro blue tetrazolium (NBT) (0.1%):* 5mg of NBT was dissolved in 2ml of buffer. The volume was then made up to 5ml with phosphate buffer.

Procedure:

The reaction mixture contained 2.5ml buffer, 100µl riboflavin solution, 200µl of EDTA solution, 10-100µl of diluted methanolic or aqueous solutions of drugs and 100µl of NBT solution, added in the mentioned sequence. Reaction was started by illuminating the reaction mixture in UV light for 5 minutes. Immediately after illumination the absorbance was measured at 290nm. The percent inhibition by sample exposure was determined by comparison with a methanol-treated control group. Percentage (%) inhibition was calculated as: - [(absorbance of control – absorbance of test sample) / absorbance of control] X 100.

4.3.3. Assay for nitric oxide scavenging activity

The assay is based on the principle that sodium nitroprusside in aqueous solution at physiologic pH liberates nitric oxide at steady rate which is converted to nitrite that can be measured by reaction with Griess reagent. The agents having nitric oxide scavenging activity will reduce the nitrite formation. (Sreejayan and Rao, 1997)

Reagents:

1. *Phosphate buffered saline (PBS)* pH *7.4* : 1.38 gm of disodium ethylenediaminetetraacetic acid, 0.19 gm of potassium dihydrogen phosphate and 8 gm of sodium chloride was dissolved in 900 ml of distilled water and adjusted pH using dilute hydrochloric acid. The volume was adjusted to 1000 ml using distilled water.

2. *Griess reagent* : 1gm of sulphanilamide, 2gm phosphoric acid and 0.1 gm naphthylenediamin dihydrochloride was dissolved in 100 ml of distilled water.
3. *Sodium nitroprusside solution (5 mM):* 15 mg of Sodium nitroprusside was dissolved in 10 ml of phosphate buffered saline.

Procedure:

All the reagents were freshly prepared. All the drug solutions were added (10 to 100 µl) to 1.5 ml sodium nitroprusside solution and incubated at room temperature for 150 minutes. Blank without test but equivalent amount of either methanol or distilled water was conducted in identical manner. After incubations the solutions were removed and equal amount of Griess reagent was added. The absorbance of chromophore was measured at 546 nm. Percentage (%) inhibition was calculated as: - [(absorbance of control – absorbance of test sample) / absorbance of control] X 100.

4.3.4. Measurement of effect on lipid peroxidation in rat liver homogenate

The study was based on principle that ferric chloride in presence of ADP and ascorbic acid induces oxidative stress to liver homogenate thereby producing MDA as lipid peroxidation product. Antioxidant will minimise the formation of MDA (Sreejayan and Rao, 1993 and 1994).

Reagents:

1. *Tris buffer (pH 7.4):* 1.21gm tris was dissolved in 900 ml of distilled water and the pH was adjusted to 7.4 with 1M hydrochloric acid. The resulting solution was diluted to 1000 ml with distilled water.
2. *Trichloroacetic acid (10% w/v):* 10gms of trichloroacetic acid was dissolved in 60 ml of distilled water and the final volume was made up to 100 ml with distilled water.
3. *Adenosine diphosphate (ADP) (1.7 µM):* 1.5 mg of Adenosine diphosphate

(ADP) was dissolved in 10 ml tris buffer (pH 7.4).

4. *Ascorbate solution (500 μM):* 17.6 mg of Ascorbic acid was dissolved in 10 ml tris buffer (pH 7.4).
5. *Ferric chloride solution (100 μM):* 16.22 mg of Ferric chloride was dissolved in 100 ml tris buffer (pH 7.4).
6. Thiobarbituric acid solution: 0.67gm of thiobarbituric acid was dissolved in 50 ml of hot distilled water and the final volume was made up to 100 ml with hot distilled water.

Procedure:

Rat liver was excised and processed in the manner similar to heart as described in section to get homogenate. 1.5 ml of homogenate was mixed with 10 μl ADP solution, 100 μl ascorbic acid, 200 μl of ferric chloride solution and 10 to 100 μl of drug solutions in sequence. The volume was made to 2 ml with tris buffer. The reaction was started by incubating at 37 ^{0} C for 20 minutes. The reaction was stopped by adding 2 ml of TCA solution and the mixture was allowed to stand in an ice bath for 15 minutes. After 15 minutes, the precipitate was separated by centrifugation and 2.0 ml of clear supernatant solution was mixed with 2.0 ml of freshly prepared thiobarbituric acid (TBA). The resulting solution was heated in a boiling water bath for 10 minutes. It was then immediately cooled in an ice bath for 5 minutes. The colour developed was measured at 532nm against reagent blank.

4.5. Statistical Analysis

Results of all the above estimations have been indicated in terms of mean ± SEM. Difference between the groups was statistically determined by analysis of variance (ANOVA) with Bonferroni multiple comparisons test using GraphPad InStat version 5.00, GraphPad Software, California USA. The level of significance was set at $P<0.05$.