materials & methods
MATERIALS AND METHODS

Experimental animals and housing conditions

Male Wistar strain albino rats (150–175 gm; obtained from Sri Venkateswara Animal Agency, Bangalore, India) were housed and maintained in clean polypropylene cages in the Departmental animal house at 26±2°C and relative humidity 44–56%, light and dark cycles of 10 and 14 h, respectively, for one week before and during the experiments. Animals were provided with standard rodent pellet diet (Sri Sai Durga Animal Feeds, Bangalore, India) and water ad libitum. The experimental protocol was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and approved by the animal ethical committee of Department of Zoology, Sri Venkateswara University, Tirupati, India (Approval No. IAEC. No.6/(i)/a/CPCSEA/ TV-KCM/SVU).

Plant material

The fresh mature leaves of Acalypha indica were collected from Tirumala hills (Chittoor district, Andhra Pradesh, India). The plant material was authenticated by Taxonomist Dr. K.Madhava Chetty (Member IAAT-No.357) at Sri Venkateswara University, Tirupati, India and a voucher specimen (Ref.No.SVUBH/1012) was deposited Universitie's Herbarium Centre for future reference.

Drugs and chemicals

Amlodipine besylate (AML)

Amlodipine besylate (10 mg tablets, Ranbaxy Laboratory, Gurgaon, India) was purchased from Hetero Pharmacy, Tirupati. It is the besylate salt of amlodipine, a long-acting calcium channel blocker. For the present study the 9 mg/kg body weight dose was fixed based on the existing literature (Umemoto et al., 2004)
**Formula**
C_{20}H_{25}ClN_{2}O_{5}·C_{6}H_{6}O_{3}

**Molecular weight**
567.1 gm/mol

**Systematic (IUPAC) name**
3-Ethyl-5-methyl (±)-2-[(2-aminoethoxy) methyl]-4-(2-chloro-phenyl)-1,4-dihydro-6-methyl-3, 5 pyridine dicarboxylate, monobenzenesulphonate.

**Structure**

![Structure Diagram](image)

**Mechanism of action**
Amlodipine is a dihydropyridine calcium antagonist (calcium ion antagonist or slow channel blocker) that inhibits the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle. Experimental data suggest that amlodipine binds to both dihydropyridine and nondihydropyridine binding sites. The contractile processes of cardiac muscle and vascular smooth muscle are dependent upon the movement of extracellular calcium ions into these cells through specific ion channels. Amlodipine inhibits calcium ion influx across cell membranes selectively, with a greater effect on vascular smooth muscle cells than on cardiac muscle cells. Negative inotropic effects can be detected in vitro but such effects have not been seen in intact animals at therapeutic doses.

**Isoproterenol hydrochloride (ISO)**
Isoproterenol hydrochloride was purchased from Aldrich Sigma, St. Louis, USA. Isoproterenol is a synthetic catecholamine that stimulates both β1 and β2
adrenergic receptors. The drug affects the heart by increasing inotropic and chronotropic activity. For the present study the 85 mg/kg body weight dose was fixed based on the existing literature (Senthil et al., 2007).

**Materials and Methods**

Structure

![Chemical Structure](image)

**Systematic (IUPAC) name**

(R)-3,4-Dihydroxy-α-(isopropylaminomethyl)benzyl alcohol hydrochloride.

**Formula**

C₁₁H₁₇NO₃·HCl

**Molecular weight**

247.72 gm/mol

**Mechanism of action**

Myocardial ischemia (MI) induced by isoproterenol (ISO), a β-adrenergic agonist, shows many metabolic and morphologic aberrations in the myocardium of experimental animals similar to those observed in human MI. ISO, a synthetic catecholamine causes severe stress in the myocardium, resulting in infarct like necrosis of the heart muscles and MI in supramaximal dosages. The various mechanisms proposed to explain ISO induced damage include hypoxia due to myocardial hyperactivity and coronary hypotension, calcium overload, depletion of energy reserves and generation of highly cytotoxic free radical through autooxidation of catecholamines.

Free radicals are produced in cells by cellular metabolism and by exogenous agents. Although these changes represent individual pathological states, they are known to affect each other and thus are interpreted as complex entities. ISO produces
Materials and Methods

a number of biochemical and electrophysiological alterations which precede the histological changes in the heart. The primary disturbances of ISO induced MI has been reported to enhance adeny1 cyclase activity resulting in increased cAMP formation, which in turn lead to the higher lipid accumulation in the myocardium. Several early events, such as ultra-structural changes, histological, biochemical, electrolyte and membrane changes, have been shown to occur within 48 hr after the injection of isoproterenol. Glycogen depletion and fat deposition have been reported. Histological changes induced by excessive amounts of isoproterenol include degeneration and necrosis of myocardial fibres, accumulation of inflammatory cells, interstitial edema, lipid droplets and endocardial hemorrhage.

Biochemical alterations in ISO induced ischemia represent a complex pattern of changes in cardiac marker enzymes, lipid profile, lipid metabolising enzymes, enzymatic and nonenzymatic antioxidants levels, glycoprotein levels, decrease in ATP store and changes in electrolyte levels in the blood as well as in the myocardial tissue. Changes including those in sarcolemma, sarcoplasmic reticulum and mitochondria, are mainly mediated by SOX, which is known to result in alterations of enzyme activity and transport systems and cause disturbances in cellular homeostasis. Lipolysis is also one of the important determinants of ISO induced MI. Chronic β-AR stimulation markedly shows iNOS upregulation, CRP release and nitrative stress and that iNOS-mediated nitrative stress functions as a main interface linking chronic β-AR activation and myocardial cell apoptosis.

Other chemicals

LDH and CK-MB kits were purchased from DIALAB production und Vertrieb von chemisch-technischen Wien-Panikengasse, Austria. All other chemicals used in this study were of analytical grade and were obtained from Sigma (St. Louis, MO, USA), Fisher (Pitsburg, PA, USA), Merck (Mumbai, India), Ranbaxy (New Delhi, India), and Qualigens (Mumbai, India).

For the present work Barnstead Thermoline water purification plant was used for Nano pure water, Kubota KR 200000T centrifuge for centrifugation of the homogenates and Hitachi UV-2000 Spectrophotometer for measuring the optical density values were used for high quality results.
Extraction of flavonoid rich fraction from *Acalypha indica*

Plants collected were washed thoroughly with tap water. The leaves were separated manually, shade dried and ground into fine powder. The leaf powder was extracted with methanol (5:1, v/w) at room temperature (30±1°C) under stirring for 8 h and the extraction process was repeated till the solvent became colorless (18 h) (Petra et al., 1999). The extracted solution was filtered and concentrated in a rotary evaporator under reduced pressure (rotary vacuum flash evaporator). The concentrated extract was again exhaustively defatted by refluxing with n-hexane and benzene (15 h twice). The two fractions were negative for polyphenols. Then the defatted bulk residue was successively extracted by refluxing with ethyl acetate (15 h twice). Total polyphenols (Fukumoto and Mazza, 2000) and total flavonoids (Jia et al., 1999) were spectrophotometrically estimated in the ethyl acetate fraction which was found to contain the bulk of flavonoids (total flavonoids content 2.89 mg (quercetin equivalent) QE/gm dry weight) and this fraction was evaporated in a rotary evaporator under reduced pressure, freeze dried and used for the study.

**Powdered leaf, extracted with methanol (Soxhlet method)**

↓

**Filtered and concentrated under vaccum**

↓

**Refluxed with benzene**

↓

**Fractioned**

↓

**Extracted with ethyl acetate**

↓

**Concentrated under vaccum (28 gm)**

↓

Flavonoid determination 510 nm spectrophotometrically (2.89 mg quercetin/gm dry weight)

Acute toxicity studies

The acute toxicity of flavonoid rich AIE was determined according to guideline No. 420 of the Organisation for European Economic Cooperation (OECD)
using male Wistar strain albino rats (150 ± 25 gm). Initial doses of 100, 400, 800, 1200, 1600, and 2000 mg/kg bodyweight of AIE were administered to the respective six groups of four rats each and monitored for three weeks for mortality and general behavior. Toxic symptoms or mortality were observed till the end of the study with doses of 1600-2000 mg/kg bodyweight. The lethal dose (LD₅₀) was determined as 800 mg/kg bodyweight. Hence, the experimental dose was selected as one fourth (200 mg/kg bodyweight) of the LD₅₀. Moreover, previous experimental studies also reported that the doses below 200 mg/kg of AIE per day are more efficacious and without any recognised side effects (Suri et al., 2004).

**Induction of myocardial ischemia to rats**

Isoproterenol (85 mg/kg) dissolved in saline was subcutaneously injected to rats at an interval of 24 h for 2 days. ISO-induced cardiotoxicity was confirmed by elevated activities of LDH and IMA in rats.

**Experimental design**

In this study, a total of 30 rats were divided into five groups (n = 6 in each group); the experimental groups are summarized below:

**Group I (n = 6):** Received normal saline (0.5 mL/kg bodyweight, i.p) for 30 days as the control, designated as CON.

**Group II (n = 6):** Received flavonoid rich *Acalypha indica* extract (AIE) (200 mg/kg bodyweight, by gavage) alone for 30 days, designated as CON+AIE.

**Group III (n = 6):** Received isoproterenol (85 mg/kg bodyweight, i.p) dissolved in saline at an interval of 24 h for 2 days (on 29th and 30th day), designated as ISO.

**Group IV (n = 6):** Received AIE (200 mg/kg bodyweight, by gavage) and then injected with ISO (85 mg/kg body weight) at an interval of 24 h for 2 days (on 29th and 30th day), designated as ISO+AIE.

**Group V (n = 6):** Received amlodipine (9.0 mg/kg bodyweight, by gavage) and then injected with ISO (85 mg/kg body weight, i.p) at an interval of 24 h for 2 days (on 29th and 30th day), designated as ISO+AML.

All rats were fasted overnight but had free access to water at the last administration of the drug. Twelve hours after the second dose of ISO injection (on 30th day), rats were killed under anesthesia using 85 mg/kg bodyweight ketamine and
Materials and Methods

95 mg/kg bodyweight xylazine (i.p). Blood samples were collected from the retro-orbital plexus in centrifuge tubes in a heparinised tube centrifuged at 2000 rpm for 10 min. The separated plasma was used for lipid estimations.

Some portion of the blood samples were allowed to clot for 30 min at room temperature and then centrifuged at 3500 rpm for 15 min. The clear, non heamolysed supernatant serum samples thus obtained were stored at -20°C until IMA assays were carried out. Heart tissues were excised immediately and rinsed in ice-chilled saline.

Methodological considerations

Estimation of blood glucose

Blood glucose was determined using a glucometer, Accu Chek Sensor set, Roche Diagnostics (Mannheim, Germany). Accu Chek sensor is a blood glucose monitoring system based on biosensor technology. The system consists of a meter and dry reagent test strips, which people with diabetes or health care professionals use for blood glucose test. The test strips used in this evaluation are calibrated to indicate plasma glucose values. The meter is turned on by the insertion of these strips.

Principle

Glucose dehydrogenase converts glucose to gluconolactone. The coenzyme in the reaction is pyrroloquinolone quinone (PQQ). A current proportional to glucose concentration is produced by means of an electron-transporter (ferro/ferricyanid) and a redox-process. It is provided with a digital converter which reads this value and displays concentration numerically.

Evaluation of hypertrophic index

The weight of the whole heart and the body weight were weighed. The ratio of heart weight to body weight (HW/BW) was then calculated. These measurements are generally used for the assessment of hypertrophic index.

Extraction of plasma and myocardial lipids

Plasma and tissue lipids were extracted by the method of Folch et al., (1957). To a known volume of plasma or tissue homogenate, 10.0 mL of chloroform-methanol (2:1, v/v) mixture was added and mixed well for 30 min and was filtered through Whatmann filter paper (No.1) into a separating funnel. The filtrate was mixed
with 0.2 mL of physiological saline and the mixture was kept undisturbed overnight. The lower phase containing the lipid was drained off into preweighed beakers. The upper phase was reextracted with more of chloroform–methanol mixture; the extracts were pooled and evaporated under vacuum at room temperature. The lipid extract was redissolved in 3.0 mL of chloroform–methanol (2:1) mixture and aliquots were taken for the estimation of lipids.

**Determination of plasma and myocardial lipids**

**Triglyceride (TG)**

Plasma/tissue triglyceride levels were measured by the GPO-POD method using ethyl-N-(3-sulfopropyl)-m-anisidine sodium (ESPAS) (Lifechem kits, Kamineni Life Sciences, Pvt Ltd, Hyderabad). This method is based on enzymatic reactions that produce colour which can be read at 546 nm.

**Principle**

\[
\text{Triglyceride} + 3\text{H}_2\text{O} \xrightarrow{\text{Lipase}} \text{Glycerol} + 3 \text{fatty acids}
\]

\[
\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol Kinase}} \text{Glycerol-3-PO}_4 + \text{ADP}
\]

\[
\text{Glycerol-3-PO}_4 + \text{O}_2 \xrightarrow{\text{Glycerol Phosphate Oxidase}} \text{Dihydroxyacetone-PO}_4 + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + 4\text{-Aminoantipyrine} + \text{ESPAS} \xrightarrow{\text{Peroxidase}} \text{Purplish Brown Quinoneimine complex} + 4\text{H}_2\text{O} + \text{HCl}
\]

Triglycerides are hydrolysed to glycerol and free fatty acids by lipases. In the presence of ATP and glycerol kinase, glycerol is converted to glycerol-3-phosphate which is then oxidized by glycerol phosphate oxidase (GPO) to yield hydrogen peroxide. Peroxidase catalyses the conversion of \(\text{H}_2\text{O}_2\), 4-aminoantipyrine (4-AAP) and ESPAS to a coloured quinoneimine complex measurable at 546 nm.

The values were expressed as mg/dL-plasma & mg/gm-tissue.

**Total cholesterol (TC)**

Plasma/tissue total cholesterol levels were measured by the CHOD-POD method (Kamineni Life Sciences, Pvt, Hyderabad, India). This method is based on enzymatic reactions that produce color which can be read at 505 nm.
Principle

Cholesterol esters are hydrolysed to cholesterol and free fatty acids by the enzyme cholesterol esterase. This free cholesterol gets oxidized in the presence of cholesterol oxidase to liberate cholesterol-4-en-3-one and peroxide. The indicator quinoneimine is formed from $H_2O_2$ and 4-AAP in the presence of phenol and peroxidase. The intensity of this coloured complex is measured at 505 nm and is directly proportional to the cholesterol concentration present in the sample.

The values were expressed as mg/dL-plasma & mg/gm-tissue.

Estimation of cholesterol in lipoprotein fractions

High Density Lipoprotein—Cholesterol (HDL-C)

Plasma HDL-C levels were measured by the CHOD-POD method (Kamineni Life Sciences, Pvt, Hyderabad, India). The method is based on enzymatic reactions that produce color. This color can be read by colorimeter at 505 nm.

The values were expressed as mg/dl.

Very Low Density Lipoprotein—Cholesterol (VLDL-C)

VLDL-C was calculated using the following equation Friedwald et al., (1972).

$$VLDL-C = TG/5$$

The values were expressed as mg/dL.

Low Density Lipoprotein—Cholesterol (LDL-C)

LDL-C was calculated using the following equation.

$$LDL-C = TC-(HDL-C+VLDL-C)$$

The values were expressed as mg/dL.

Determination of phospholipids (PL)

Plasma/tissue phospholipid content was determined by the method of Zilversmit and Davis (1950). The principle involve the conversion of organic
phosphorus to inorganic phosphorus which reacts with ammonium molybdate to form phosphomolybdic acid. This on treatment with amino napthol sulphonic acid (ANSA) forms a stable blue colour, which is read at 640 nm. To 0.1 mL of lipid extract (plasma/tissue), added 1 mL of 5 N H$_2$SO$_4$ and 1 mL of concentrated nitric acid and digested to a colourless solution. The phosphorus content in the extract was determined by the method of Fiske and Subba Row (1925). The values were expressed as mg/dL-plasma & mg/gm-tissue.

**Determination of free fatty acids (FFA)**

Plasma/tissue FFAs were estimated by the method of Falholt et al., (1973).

**Principle**

In the presence of phosphate buffer, the lipid extract was shaken with a higher copper reagent (pH 8.1). The FFA(s) in the copper solution was determined calorimetrically with diphenyl carbazide.

**Procedure**

0.1 mL of lipid extract (plasma/tissue) was evaporated to dryness. 1 mL of phosphate buffer, 6 mL of extraction solvent and 2.5 mL of copper reagent were added. All the tubes were shaken vigorously. 200 mg of activated silicic acid was added and left aside for 30 min. The tubes were centrifuged and 3 mL of the copper layer was transferred to another tube containing 0.5 mL of diphenyl carbazide and mixed carefully. The absorbance was read at 550 nm immediately.

The values were expressed as mg/dL-plasma & mg/gm-tissue.

**Assay of cardiac injury enzymes and ischemic markers**

**Estimation of lactate dehydrogenase (LDH)**

Plasma lactate dehydrogenase (LDH) levels were measured by using method of Stein (1998) using assay kit purchased from DIALAB Produktion und Vertrieb von chemisch - technischen, Austria.

**Principle**

Pyruvate + NADH + H$^+$ $\xrightarrow{LDH}$ Lactate + NAD$^+$

**Reagents**

**Components of Reagent 1:** Pyruvate, Phosphate
Components of Reagent 2: NADH, Good’s Buffer (pH 9.6)

Test procedure

Reagents and samples were brought to room temperature

<table>
<thead>
<tr>
<th>Substrate Start</th>
<th>25°C - 30°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipette into test tubes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>20 µl</td>
<td>10 µl</td>
</tr>
<tr>
<td>Reagent 1</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Reagent 2</td>
<td>250 µl</td>
<td>250 µl</td>
</tr>
</tbody>
</table>

Mixed read initial absorbance after one minute at 37°C, and read absorbance again after exactly 1, 2 and 3 minutes at 37°C

\[ \Delta A/\text{min} = (\Delta A/\text{min sample}) - (\Delta A/\text{min blank}) \]

<table>
<thead>
<tr>
<th>Sample Start</th>
<th>Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipette into test tubes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>20 µl</td>
<td>10 µl</td>
</tr>
<tr>
<td>Working reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
</tbody>
</table>

Mixed read initial absorbance after one minute at 37°C, and read absorbance again after exactly 1, 2 and 3 minutes at 37°C

\[ \Delta A/\text{min} = (\Delta A/\text{min sample}) - (\Delta A/\text{min blank}) \]

Calculation

LDH (IU/L) = \( \Delta A/\text{min} \times \text{Factor} \)

Factors (37°C)

<table>
<thead>
<tr>
<th></th>
<th>25°C - 30°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor for: 340 nm:</td>
<td>8095</td>
<td>16030</td>
</tr>
<tr>
<td>Factor for: 334 nm:</td>
<td>8250</td>
<td>16345</td>
</tr>
<tr>
<td>Factor for: 365 nm:</td>
<td>15000</td>
<td>29705</td>
</tr>
</tbody>
</table>

The lowest detection limit of the assay was <240 IU/L - <480 IU/L.

The inter assay and intra assay coefficients of variations was 2.13% and 3.86% respectively.

Estimation of creatine kinase-MB

Following the procedure described in the assay kit (purchased from DIALAB Produktion und Vertrieb von chemisch-technischen, Austria), the concentrations of CK-MB was determined in the plasma.
Principle

Creatine kinase is a dimer. Its monomeric subunits are designated M (muscle) and B (brain, nerve cells). The subunits combine to form three isoenzymes namely CK-BB, CK-MB, CK-MM. M subunits of CK-MM and CK-MB are inactivated by reaction with anti-M antibody (immunoinhibition). The remaining B-subunit is measured enzymatically.

Reagents

Components of reagent 1 and reagent 2

Imidazole pH (6.7); Creatine phosphate; Glucose; N-acetylcystein; EDTA; NADP; AMP; Diadenosinpentaphosphate; Glucose-6-phosphate dehydrogenase; Hexokinase; CK-M inhibiting polyclonal antibodies.

Test procedure

Reagents and samples were brought to room temperature

<table>
<thead>
<tr>
<th>Substrate Start</th>
<th>Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipette into test tubes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>50 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>50 µl</td>
<td>-</td>
</tr>
<tr>
<td>Reagent 1</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Mixed and incubated for approximately three minutes. Then added</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reagent 2</td>
<td>250 µl</td>
<td>250 µl</td>
</tr>
</tbody>
</table>

Mixed read initial absorbance after two minutes at 37°C, and read absorbance again after exactly 1, 2, 3, 4, 5 minutes at 37°C

\[ \Delta A/min = (\Delta A/min \text{ sample}) - (\Delta A/min \text{ blank}) \]

<table>
<thead>
<tr>
<th>Sample Start</th>
<th>Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipette into test tubes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>40 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>40 µl</td>
<td>-</td>
</tr>
<tr>
<td>Working reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>(four parts of reagent 1 + one part of reagent 2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mixed read initial absorbance after two minutes at 37°C, and read absorbance again after exactly 1, 2, 3, 4, 5 minutes at 37°C

\[ \Delta A/min = (\Delta A/min \text{ sample}) - (\Delta A/min \text{ blank}) \]

Calculation

CK-MB (IU/L) = \( \Delta A/min \times \text{Factor} \)
Factors (37°C)
Factor for: 340 nm = 8254; Factor for: 334 nm = 8414

The lowest detection limit of the assay was < 24 IU/L.
The inter assay and intra assay coefficients of variations was 0.85% and 1.40% respectively.

Tools for evaluation of myocardial ischemic risk
Assay of ischemia modified albumin (IMA)

Serum IMA was measured by colorimetric albumin cobalt binding (ACB) assay based on biochemical properties of albumin to bind exogenous cobalt as previously described (Arzu et al., 2008), with certain modifications.

Principle
The colorimetric assay format quantitatively measures with unbound cobalt remaining after cobalt–albumin binding has occurred. Thus, with reduced cobalt albumin binding, there is more unbound cobalt, resulting in elevated assay levels.

Procedure
Briefly, 95 µl of serum is pipette to the reaction cuvette, and 25 µl of 4.2 mM cobalt chloride (CoCl₂.6H₂O) in H₂O is added 25 seconds later. The sample/cobalt mixture is incubated for 275 seconds to allow binding of cobalt to albumin then a blank read optical measurement is made at 500 nm. A total of 25 µl of 3.2 mM dithiothreitol (DTT) in H₂O is added 25 seconds later. DTT reacts with unbound (non N-terminal sequestered) cobalt to form a colored product. The final reaction mixture is incubated for an additional time of 100 seconds and read at 470 nm. All incubations are at 37 °C. Total assay time once the sample is pipette is 7.5 min. IMA results were expressed in absorbance units (ABSU).

Intra-assay and inter-assay coefficients of variation for IMA were 4.3% and 5.2%, respectively.

Calculations
Using a spectrophotometer at 470 nm, color development with DTT was compared to a serum-cobalt blank without DTT and reported in absorbance units (ABSU).

IMA (ABSU) = \text{Absorbance of test-absorbance of blank}
Measurement of non-high density lipoprotein–cholesterol (non-HDL-C)

non-HDL-C is calculated as total cholesterol minus HDL
non-HDL-C=TC-HDL-C

The values were expressed as mg/dL.

Measurement of low density lipoprotein–cholesterol/high density lipoprotein–cholesterol

Ratio to LDL-C to HDL- was also computed as LDL-C/HDL-C.

Measurement of atherogenic index of plasma (AIP)

Atherogenic index of plasma (AIP), calculated as log (TG/HDL-C).

Electrocardiographic recordings

The ST segment alterations were recorded using ECG according to the method described by activities of Patrick and Fakhreddin (1999). After 12 hr of the second injection of either isoproterenol or vehicle, ECG was recorded under light ketamine anesthesia through needle electrodes (Lead II) using My Card RC3 data acquisition system (Marks Electronics, Chennai, India). The data on changes in ST interval were recorded on chart recorder.

Figure 5: Electrode placement for the rat ECG.
Oxidative stress and antioxidants milieu

Isolation of heart mitochondria

Heart mitochondria were isolated by the method of Takasawa et al., (1993). The heart tissue was put into ice-cold 50 mM Tris–HCl (pH 7.4) containing 0.25 M sucrose and homogenized. The homogenates were centrifuged at 700 x g for 20 min and then the supernatants obtained were centrifuged at 9,000 x g for 15 min. Then, the pellets were washed with 10 mM Tris–HCl (pH 7.8) containing 0.25 M sucrose and finally resuspended in the same buffer and the aliquots were used for assaying the enzyme activity.

Estimation of superoxide dismutase (SOD) (E.C. 1.15.1.1) activity

SOD activity was determined by using the epinephrine assay of Misra and Fridovich (1972). At alkaline pH, superoxide anion \( \text{O}_2^- \) causes the autooxidation of epinephrine to adenochrome. In this reaction, SOD decreases the adenochrome formation. One unit of SOD is defined as the amount of extract that inhibits the rate of adenochrome formation by 50%. To 0.5 mL of enzyme source, 1.5 mL of carbonate
buffer (pH 10.2) and 0.5 mL of 0.4 M ethylenediaminetetraacetic acid (EDTA) solutions were added. The reaction was initiated by the addition of 0.4 mL of ephinephrine (3 mM) and change in the absorbance of this mixture was measured at 10 sec intervals for 1 min in a spectrophotometer (Hitachi UV-2000) at 480 nm. Finally the enzyme activity was expressed in units/mg protein.

**Estimation of catalase (E.C. 1.11.1.6) activity**

Catalase activity in the heart tissues of rats was assayed by the method of Chance and Maehly (1955). The reaction reagent composed of CAT activities contained 2.5 mL of 50 mM Na₂SO₄ (pH 5.0), 0.4 mL of 5.9 mM H₂O₂ and 0.1 mL sample. The decomposition of H₂O₂ was followed directly by measuring the decrease in absorbance at 240 nm, at regular intervals of 10 sec for 1 min in a spectrophotometer (Hitachi UV-2000). The activity was finally expressed as μmoles of H₂O₂ metabolized/mg protein/min.

**Estimation of glutathione peroxidase (E.C. 1.11.1.9) activity**

Glutathione peroxidase activity in the heart tissues of rats was assayed by the method of Rotruck et al., (1973). Briefly, reaction mixture contained 0.2 mL of 0.4 M Tris-HCl buffer pH 7.0, 0.1 mL of 10 mM sodium azide, 0.2 mL of tissue homogenate, 0.2 mL glutathione, 0.1 mL of 0.2 mM H₂O₂. The contents were incubated at 37°C for 10 min. The reaction was arrested by 0.4 mL of 10% TCA, and centrifuged. Supernatant was assayed for glutathione content by using Ellmans reagent (19.8 mg of 5,5-dithiobisnitro benzoic acid (DTNB) in 100 mL of 0.1% sodium nitrate). This mixture was read at 412 nm in spectrophotometer (Hitachi UV-2000). The enzyme activity was expressed in μmoles of GSH consumed/mg protein/min.

**Estimation of glutathione reductase (E.C. 1.6.4.2) activity**

Glutathione reductase was assayed by the method of Staal et al., (1969). The assay mixture containing 0.2 mL of extract, 1.5 mL of sodium phosphate buffer, 0.5 mL of EDTA, 0.2 mL of oxidized glutathione and 0.1 mL of NADPH were added. The decrease in optical density at 340 nm was then monitored for 2 min at 30 seconds interval in a spectrophotometer against a reagent blank. The enzyme activity is expressed as μmoles of NADPH oxidised/min/mg protein.
Estimation of glutathione activity

Glutathione content was determined according to the method of Theodorus et al., (1981). Protein in heart mitochondria extract is precipitated with 1 mL of 5% sulfosalicylic acid (w/v) and the contents were centrifuged at 5000 x g for 15 min at 4°C. The resulting supernatant was used as the enzyme source. The reaction mixture in a total volume of 2.5 mL contained 2.0 mL of 0.1 M potassium phosphate buffer, 0.005 mL of NADPH (4 mg/mL of 0.5% NaHCO₃), 0.02 mL of DTNB (1.5 mg/mL), 0.02 mL of glutathione reductase (6 units/mL) and require amount of tissue source. The reaction was initiating by adding 0.41 mL of enzyme source and change in absorbance was recorded at 425 nm against the reagent blank. The glutathione content was expressed in μmoles/gram wet weight of the tissue.

Lipid peroxidation

The level of lipid peroxidation in the tissues was measured in terms of malondialdehyde (MDA) content and determined by using thiobarbituric acid (TBA) reagent. The reactivity of TBA was determined using the method of Hiroshi et al., (1979) with minor modifications. 1 mL of the homogenate was added to 2.5 mL of 20% trichloroacetic acid (TCA). The mixture was centrifuged at 3,500 rpm for 10 min at 4°C. The pellet was then dissolved in 0.05 M sulphuric acid and 3 mL of 2 M thiobarbituric acid was added to it. The test tubes were incubated in boiling water bath for 30 min. The contents were cooled and the color was extracted into 4 mL of n-butanol. The colour was read at 530 nm using a spectrophotometer against the blank. The results were expressed as μmoles of MDA formed/gm weight of tissue.

Validity of experimental procedures

For all the enzyme studies in the present investigation, the assays were standardized by conducting preliminary test to determine the optimal pH, temperature, enzyme and substrate concentration and these optimal conditions were subsequently followed for each enzyme assay.

Aliquots for assay

Aliquots were selected such that initial rates were approximated as nearly as possible yet providing sufficient product to fall in a convenient range of spectrophotometric measurement.
Enzyme units

Enzyme activities were expressed in standard units i.e., $\mu$ moles of product formed or substrate cleaved/mg protein/min.

Substrate requirement

All the enzyme assays were done under the conditions following zero order kinetics unless otherwise stated.

Beer-Lambert Law

All the products of the reactions were measured by the spectrophotometric procedures in which the optical density (absorbance) of the resulting colored complex was proportional to the concentrations of the reaction products.

Enzyme nomenclature

The nomenclature of enzymes used in the present study is according to the report of the commission of the "International union of Biochemistry" (IUB).

Assay of dehydrogenases using INT

INT: 2-(p-iodophenyl)-3(p-nitrophenyl)-5-phenyltetrazolium chloride

Tetrazolium salts are unique class of oxidation-reduction indicators in the study of dehydrogenases. The advantages of using tetrazolium salts as electron acceptors are:

- The tetrazolium salts give a stable color or reduction.
- They are highly soluble in aqueous solutions.
- They can be reduced both aerobically and anaerobically.
- They have high redox potential which makes the reduction easier.
- They are freely permeable through membranes.

The first developed tetrazolium salt was triphenyl tertazolium chloride (TTC). Following the application of TTC, new tetrazolium salts were developed.

Various tetrazolium salts receive electrons from various sites of electron transport system (Oda et al., 1958; Nachlas et al., 1960), which is due to the inherent difference in the redox potentials of various tetrazolium salts. The phenyl ring was observed to increase its redox potential.
Myocardial tissue histology

The hearts were fixed in 10% formalin solution and embedded in paraffin. Sections measuring 5 μm were made and stained with hematoxylin and eosin (H&E) and observed microscopically. The severity of MI was noted for each specimen. The pathologist was blinded to the treatment. The sections were evaluated under light microscope (model CX31, Olympus, Tokyo, Japan) with a digital camera for inflammation, interstitial fibrosis, myocardial disorganisation, interstitial edema necrosis, nuclear pyknosis, hypertrophy, scar formation and macrophage activity, lipid droplets, vacuoles, swollen mitochondria with disorganized and dissolved cristae, glycogen granules and loss of striations with nuclear changes.

Statistical analysis

Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Duncan’s multiple range test (DMRT) using statistical package for the Social Science (SPSS) software, version 11.5. Results were expressed as mean±S.D for 6 rats in each group. P values < 0.05 were considered significant.