Methodology
1. List of chemicals and manufacturers

*Caymann chemicals, USA*
Maslinic acid (MA)

*Sigma chemicals Co. St. Louis, Mo, USA*
Ammonium molybdate
Aminonaphthosulphonic acid (ANSA)
Bovine serum albumin (BSA)
Isoproterenol (IPL)
Thiobarbituric acid (TBA)
Trichloro acetic acid (TCA)

*Sisco Research Laboratories Private Ltd., Maharashtra, India*
1-Amino-2-naphtho-4-sulphonic acid
Acetonitrile
DL-Aspartic acid
Gallic acid (GA)
Glutathione reduced (GSH)
Glutathione oxidised (GSSH)
α-Ketoglutaric acid
Pyrogallol
Sodium pyruvate
Triethanolamine hydrochloride

Qualigens, Maharashtra, India
Ammonium sulphate
Cholesterol
Disodium dihydrogen ortho phosphate
Dipotassium hydrogen ortho phosphate
Ethylene diamine tetra acetic acid disodium salt (EDTANa2)
Folin & Ciocalteu reagent (2N)
Hydrogen peroxide
Magnesium sulphate
Potassium dihydrogen ortho phosphate
Sodium carbonate
Sodium dihydrogen ortho phosphate
Trichloroacetic acid
Trisodiumcitrate

Sd-fine Chemicals, Maharashtra, India
2,4-Dinitro phenyl hydrazine (DNPH)
Ortho phosphoric acid

Merck AG, Darmstadt, Germany
Sodium-Carboxymethyl cellulose (Na-CMC)
Sodium disulphite
Sodium sulphite
Potassium sodium tartarate
All other chemicals used for the estimations were of analytical grade (AR).
2. Extract preparation

Fresh fruits of *T. Pallida* were collected and washed with distilled water. The dried and coarsely powdered fruits were packed in a soxhlet apparatus and subjected to extraction with absolute ethyl alcohol. The extracted material was evaporated to dryness under reduced pressure at 45°C. The dried ethanolic extract was suspended in distilled water and used for oral treatment of rats.

3. Qualitative HPLC and MS analysis of TpFE

**Sample preparation:**

The TpFE sample was used for qualitative HPLC fingerprint study. GA is used as standard biomarker. GA in the sample was identified by comparison of its retention time (t<sub>R</sub>) and molecular mass with the standard GA. 1 mg of both GA standard and TpFE test sample were dissolved in 1 ml of 50% acetonitrile. 5 µl of both GA and TpFE were injected to LCMS.

**Instrumentation:**

The LCMS system, Agilent 1200 HPLC & Q-TOF 6500 Series was used. The output signal was monitored and processed using Intel ® Core (TM) 2 Duo computer (HP xw 4600 Workstation). HPLC was equipped with binary gradient pump, auto sampler, thermostatted column compartment, variable wavelength detector, auto sampler thermostatted (G 1330B), computer with windows based mass hunter software version B.02.01 (B2116.20).

**Chromatographic Technique:**

LUNA C18 5µm column (4.6x250mm) was used in this study. The mobile phase solvents were water (acidified with 0.1% formic acid) (A) and acetonitrile (B) using the follow gradient: 75% A for 2 min, 70% A in 8 min to 75% A for 10min. The solvents were ultrasonic bath for 30 min and filtered through a 0.45µ membrane filter before use. The flow rate of the mobile phase was maintained at 0.5 mL/min. the column temperature was maintained at room temperature, the detection of the samples was carried out at 280nm by UV detector and PDA detection at 280nm.

**Mass Conditions:**

Ion source – ESI, Acquisition Mode-MS, Mass Range-100-500, Mode-negative mode, Gas Temp-325\(^\circ\)C, Gas flow (l/min)-5, Nebulizer-30 Psi, Vcap-3500, Fragmentor-175, and Skimme-65.

4. Acute toxicity studies

The acute toxicity study of TpFE was carried out according to the limit test described by the Office of Prevention, Pesticide and Toxic substance (OPPTS) guidelines (http://www.epa.gov/oppts/home/guideline.htm). Test dose of 2 and 5 g kg\(^{-1}\) body weight (bw) were given to the mice. The extract was found to be safe at both doses orally. Hence, 1/10\(^{th}\) and 1/50\(^{th}\) of the maximum safe dose corresponding to 500 and 100 mg kg\(^{-1}\) bw orally were selected as high and low dose, respectively. A medium dose 300 mg kg\(^{-1}\) bw also selected to check for multiple doses.

5. Experimental animals

Male albino rats of Wistar strain weighing between 100 and 120g were obtained from National Institute of Nutrition (NIN), Hyderabad, India. The animals were housed at 24 ± 2 \(^\circ\)C in a well-ventilated animal house under 12/12 h light and dark cycle. Animals were maintained under standard conditions in an animal house. They were acclimatized to animal house conditions. Commercial pellet diet (NIN, Hyderabad) and water provided ad libitum. This study was carried out following approval from the ethical committee on the use and care of animals of the Sri Krishnadevaraya University, Anantapur. Local Institutional Animal Ethical Committee of our University, obtained ethical clearance for conducting experiments on animals from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (Regd. no. 470/01/a/CPCSEA, dt.24\(^{th}\) Aug 2001).

6. Experimental Design

Two batches of animals were used to analyze the effect of TpFE and the active compound MA. GA was used as the cardio-protective positive control.
**Batch 1:**

The animals were divided into 7 groups and each group consisted of 8 rats to determine the effect of TpFE.

- **Group 1:** Control rats
- **Group 2:** Rats daily fed orally with TpFE (500 mg kg\(^{-1}\)) for 30 days.
- **Group 3:** Rats received ISO (85 mg kg\(^{-1}\), s.c.) on the 29\(^{th}\) and 30\(^{th}\) day.
- **Group 4:** Rats daily fed orally with TpFE (100 mg kg\(^{-1}\)) for 30 days and in addition received ISO (85 mg kg\(^{-1}\), s.c.) on the 29\(^{th}\) and 30\(^{th}\) day.
- **Group 5:** Rats daily fed orally with TpFE (300 mg kg\(^{-1}\)) for 30 days and in addition received ISO (85 mg kg\(^{-1}\) s.c.) on the 29\(^{th}\) and 30\(^{th}\) day.
- **Group 6:** Rats daily fed orally with TpFE (500 mg kg\(^{-1}\)) for 30 days and in addition received ISO (85 mg kg\(^{-1}\) s.c.) on the 29\(^{th}\) and 30\(^{th}\) day.
- **Group 7:** Rats daily fed orally with 15 mg kg\(^{-1}\) bw GA for 30 days and in addition received 85 mg kg\(^{-1}\) bw ISO subcutaneously on the 29\(^{th}\) and 30\(^{th}\) day.

**Batch 2:**

The animals were divided into 5 groups of eight rats in each as follows to determine the effect of Maslinic acid.

- **Group 1:** Control rats
- **Group 2:** Rats daily fed orally with MA (15 mg/kg) for 7 days
- **Group 3:** Rats received ISO (85 mg kg\(^{-1}\), s.c.) on the 6\(^{th}\) and 7\(^{th}\) day.
- **Group 4:** Rats daily fed orally with MA (15 mg kg\(^{-1}\)) for 7 days and in addition received ISO (85 mg kg\(^{-1}\), s.c.) on the 8\(^{th}\) and 9\(^{th}\) day.
- **Group 5:** Rats daily fed orally with GA (15 mg kg\(^{-1}\)) for 7 days and in addition received ISO (85 mg kg\(^{-1}\), s.c.) on the 8\(^{th}\) and 9\(^{th}\) day.
7. Experimental procedure

MA was dissolved in Na-CMC and GA was dissolved in saline for the oral treatment of animals. ISO (85 mg kg\(^{-1}\) bw) was dissolved in distilled water and injected subcutaneously to rats at an interval of 24 h for 2 days. Twelve hours after the second injection of ISO, rats were sacrificed by cervical dislocation. Animals were made to fast 12 h before sacrificing. For the analysis of various biochemical parameters, heart tissues of animals were suspended in 0.15M potassium chloride in polypropylene containers and serum samples were preserved in eppendorf tubes, sealed with parafilm, labeled carefully and preserved at -20°C until assays were carried out.

Blood was collected in eppendorf tubes from heart puncture of sacrificed rats after second dose of ISO administration. The blood collected without adding anticoagulant was allowed to clot for 30 min and serum was separated by centrifugation at 3000 rpm for 10 min.

After the excision of heart tissue, immediately rinsed in ice chilled saline and the whole heart weight was determined. 200 mg of the heart tissue was homogenized with 5 ml of 0.1 M Tris-Hcl (pH 7.4) solution. The homogenate was centrifuged at 3000 rpm for 5 min and the supernatant was used for the analysis of various biochemical parameters.

8. Biochemical estimations

Cardiac Marker Enzymes

9. Creatine kinase (CK)

The activity of CK was estimated by the method of Teitz (1995) by using Robonik Diagnostic Kit.

Principle: CK catalyses the conversion of creatine phosphate and ADP to creatine and ATP. The ATP and glucose are converted to ADP and glucose-6-phosphate by hexokinase (HK). Glucose-6-phosphate dehydrogenase (G-6-PDH) oxidizes D-glucose-6-phosphate and reduces the nicotinamide adenine dinucleotide (NAD). The
rate of NADH formation, measured at 340 nm, is directly proportional to serum CK activity.

**Procedure:** 1 ml working reagent was added to 50 μl of test sample, mixed and incubated at 37°C for 1 min. After incubation, change in the optical density was measured for 3 min at an interval of 1 min against reagent blank at 340nm. The activity of creatine kinase expressed as U/L.

10. Creatine kinase-MB (CK-MB)
The activity of CK-MB was estimated by the method of Teitz (1995) by using Robonik Diagnostic Kit.

**Principle:** Creatine kinase exists in 3 cytoplasmic forms: CK-MB (in cardiac muscle only), CK-MM (in striated muscle) and CK-BB (especially in brain). Determination of CK in serum is used for the diagnosis and the follow-up of cardiac muscle damages. In myocardial infarction, the activity of total CK and CK-MB increase quickly until reaching a peak 10-24 h after the onset of the infarction. CK-MB reagent contains an antibody inhibiting specifically CK-M subunits (i.e. 100% of CK-MM and 50% of CK-MB isoenzymes). The remaining activity, corresponding to CK-B fraction activity, is measured according to the IFCC reference method for measuring CK activity. CK-MB activity is then obtained by multiple by 2 the remaining activity.

**Procedure:** 1 ml working reagent was added to 40 μl of test sample, mixed and incubated at 37°C for 5 min. After incubation, change in the optical density was measured for 3 min at an interval of 1 min against reagent blank at 340nm. The activity of creatine kinase expressed as U/L.

11. Lactate Dehydrogenase: (LDH)
The activity of the enzyme LDH was estimated by the method of Wie Bhaar et al. (1975) by using Agappe Diagnostic Kit.

**Principle:** The enzyme LDH is distributed in tissues particularly in heart, muscle and kidney. LDH catalyzes the oxidation of lactate to pyruvate in the presence of NAD
which is subsequently reduced to NADH. The rate of NADH formation measured at 340 nm is directly proportional to LDH activity.

**Procedure:** 1 ml working reagent was added to 10 μl of test sample, mixed and incubated at 37°C for 1 min. After incubation, change in the optical density was measured for 3 min at an interval of 1 min against reagent blank at 340 nm. The activity of LDH was expressed as U/L.

12. Alanine transaminase (ALT)

The activity of the enzyme alanine transaminase was estimated by the method of Teitz (1976) by using Aspen Diagnostic Kit.

**Principle:** The enzyme alanine transaminase or alanine aminotransferase (ALT) is also called as serum glutamate pyruvate transaminase (SGPT) which is widely distributed in a variety of tissue sources. The major source of ALT is of hepatic origin, and elevated levels are found in hepatitis, obstructive jaundice and in myocardial infarction. The enzymatic reaction sequence in the assay is as follows.

\[
\alpha \text{-keto glutarate} + \text{L-alanine} \leftrightarrow \text{L-glutamate} + \text{pyruvate}. 
\]

**Procedure:** To the 100 μl of test sample, 1ml of reconstituted regent was added, mixed and incubated at 37°C for 1 minute. After incubation change in the optical density was measured upto 3 min at an interval of 1 min against reagent blank at 340nm. The activity of glutamate pyruvate transaminase was expressed as U/L of serum.

13. Aspartate transaminase (AST)

The activity of the enzyme alanine transaminase was estimated by the method of Teitz (1976) by using Aspen Diagnostic Kit.

**Principle:** Aspartate transaminase or aspartate aminotransferase (AST) also called as serum glutamate oxaloacetate transaminase (SGOT) is a tissue enzyme that catalyses the transfer of an amino and keto group between \(\alpha\)-keto acids. It is widely distributed
in tissues mainly heart, liver, muscle and kidney, and injury to these tissues results in the release of SGOT into the circulation.

\[
\alpha\text{-keto glutarate} + L\text{-aspartate} \leftrightarrow L\text{-glutamate} + \text{oxaloacetate} \rightarrow \text{Pyruvate}
\]

**Procedure:** To the 100 µl of test sample, 1ml of reconstituted reagent was added, mixed and incubated at 37°C for 1 min. After incubation, change in the optical density was measured for 3 min at an interval of 1 min against reagent blank at 340nm. The activity of glutamate oxaloacetate transaminase was expressed as U/L.

### 14. Alkaline Phosphatase

The activity of the enzyme alkaline phosphate was estimated by the method of Teitz (1995) by using Robonik Diagnostic Kit.

**Principle:** Alkaline phosphatase catalyzes the hydrolysis of P-Nitrophenyl phosphate to ortho-phosphoric acid and P-Nitrophenol. P-Nitrophenol has maximum absorbance at 405 nm. The rate of increased absorbance at 405 nm is proportional to the enzyme activity.

**Procedure:** This is based on kinetic method. 20 µl of test sample was added to 1.0 ml of reagent, mixed well and incubated for 1 min at 37°C, after 1 min absorbance was taken at 405 nm. The activity of alkaline phosphatase was expressed as U/L.

### 15. γ-Glutamyl transferase

The activity of γ-glutamyl transpeptidase was estimated according to the method of Young (1995) by using Robonik Diagnostic Kit.

**Principle:** Gamma glutamyl transferase (γGT) catalyses the transfer of the gamma glutamyl group from gamma glutamyl peptides to suitable acceptors. In the reaction rate analysis method, the artificial substrate γ-glutamyl-p-nitroanilide is used.

**Procedure**
50 of test sample was added to 1 ml of reagent, mixed well and incubated for 1 min. After incubation for at 37° C, the samples were measured at 405 nm. The activity of γ-glutamyl transferase was expressed as U/L.

16. Lipid Peroxidation
Lipid peroxidative extent was measured by the formation of Malondialdehyde (MDA) by using the method of Okhawa et al., (1979).

**Principle:** Malondialdehyde formed from the break down of polyunsaturated fatty acids, serves as a convenient index for determining the extent of the peroxidation reaction. Malondialdehyde has been identified as the product of lipid peroxidation that reacts with TBA to give a chromogen absorbing at 535 nm.

**Procedure:** 1 ml of test sample was mixed with 600 µl of distilled water and 200 µl of 8.1% (w/v) sodium dodecyl sulfate (SDS), vortexed, and incubated at room temperature for 5 min. After adding 1.5 ml of 20% (w/v) acetic acid (pH 3.5) and 1.5 ml of 0.8% (w/v) TBA, the mixture was heated at 95°C for 60 min. After cooling 1.0 ml of distilled water, 5.0 ml of a mixture of n-butanol and pyridine (15:1 v/v) were added and vortexed. Then after centrifugation at 800g for 15 min the absorbance of the upper layer was measured at 532 nm using 1,1,3,3 tetraethoxypropane as the standard.

Serum biochemical analysis

**Lipid Profile**

Total cholesterol and triglycerides were estimated using Accurex enzymatic diagnostic kit and HDL-C was estimated by using Autozyme cholesterol diagnostic kit.

17. Total Cholesterol (TC)

**Principle:** Cholesterol esterase hydrolyses cholesterol esters into free cholesterol and fatty acids. Cholesterol oxidase convert cholesterol to cholest-4-en-3-one and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide oxidatively couples with 4 – aminoantipyrine and phenol to produce red quinoneimine dye which has
absorbance maximum at 510 nm. The intensity of the red colour is proportional to the amount of total cholesterol (Allain, 1974).

**Procedure:** To 0.01 ml of serum, 1.0 ml of the reagent was added, mixed and incubated at 37°C for 10 min. Cholesterol standard and water blank were also treated in a similar manner. After incubation, absorbance was read at 510 nm and values are expressed as mg/dL.

18. **Triglycerides (TG)**

**Principle:** Glycerol released from hydrolysis of triglycerides by lipoprotein lipase is converted by glycerol kinase into glycerol-3-phosphate, which is oxidized by glycerol phosphate oxidase to dihydroxyacetone phosphate and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide oxidizes phenolic chromogen to a red coloured compound which is measured at 510 nm (Fossati et al., 1982).

**Procedure:** To 0.01 ml of serum, 1.0 ml of the reagent was added, mixed and incubated at 37°C for 10 min. Triglyceride standard and water blank were also treated in a similar manner. After incubation, absorbance of the standard and serum was read at 510 nm against and values are expressed as mg/dL.

19. **High density lipoprotein cholesterol (HDL-C)**

**Principle:** Phosphotungstate/Mg²⁺ precipitate chylomicrons, Low density lipoprotein (LDL) fractions. High density lipoprotein (HDL) fraction remains unaffected in supernatant. Cholesterol content of HDL fraction is assayed using Autozyme cholesterol diagnostic kit (Assmann, 1983).

**Procedure:** To 0.2 ml of serum, 0.2 ml of precipitant reagent was added, mixed and centrifuged at 4,000 rpm for 10 min to obtain a clear supernatant. To 0.05 ml of supernatant, 1.0 ml of Autozyme HDL cholesterol working solution was added, incubated for 10 min at 37°C and colour developed was read at 510 nm against a blank and a standard (50 mg%) was run simultaneously. Values are expressed as mg/dL.
20. **Very Low Density Lipoprotein cholesterol (VLDL-C) and Low Density Lipoprotein cholesterol (LDL-C)**

VLDL and LDL were calculated using the Friedewald et al (1972) formulae as follows:

\[
\text{VLDL-C} = \frac{\text{TG}}{5} \\
\text{LDL-C} = \text{TC} - (\frac{\text{TG}}{5} + \text{HDL-C})
\]

21. **Sodium (Na\(^+\))**

The content of sodium was estimated by the method of Trinder (1951) by using Excel Diagnostic Kit.

**Principle:** The sodium and the proteins are precipitated simultaneously by means of a reagent containing magnesium uranyl acetate containing alcohol. The precipitate is separated by centrifugation. The content of sodium is calculated from the loss in the concentration of magnesium uranyl acetate in the reagent solution in comparison to a standard sodium solution treated similarly. The residual amount of magnesium uranyl acetate is estimated by forming brown (dark) ferrous uranyl acetate which is read in a colorimeter.

**Procedure:** 1 ml of sodium precipitating reagent was taken into two test tubes labelled as standard and test. 0.02 ml of standard sodium was added to the first tube and 0.02 ml of serum was added to the second tube. The tubes were mixed well and incubated for 5 min at room temperature then centrifuged for 1 min at 3000 rpm. 0.05 ml of the supernatant was separated into another set of test tubes. 3 ml of distilled water and 0.2 ml of sodium color reagent were added and mixed well and incubated for 5 min at room temperature. Then the absorbance was measured at 540 nm.

22. **Potassium (K\(^+\))**

The content of sodium was estimated by the method of Trinder (1951) by using Excel Diagnostic Kit.
Principle: Potassium can be determined by a number of different methods. It can be directly estimated by flame photometry, colorimetry. It can also be measured by the use of ion selective electrode. The method is based on the measurement of turbidity of the reaction mixture containing sodium tetraphenyl boron, alkaline EDTA, formaldehyde and sample containing potassium or standard potassium salt.

Procedure: 1 ml of potassium reagent was taken into two test tubes labelled as standard and test. 0.05 ml of standard potassium was added to the first tube and 0.05 ml of serum was added to the second tube. The tubes were mixed well and incubated for 5 min at room temperature. The absorbance was measured at 620 nm.

23. Calcium (Ca^{2+})
The content of calcium was estimated by the method of Tietz (1995) by using Robonik Diagnostic Kit.

Principle: Calcium with Arsenazo, at neutral PH yields a blue colored complex, whose intensity is proportional to the calcium concentration. Interference by magnesium is eliminated by addition of 8-hydroxyquinoline-5-sulfonic acid.

Procedure: 1 ml of reagent was added to 10 μl of standard and 10 μl of test samples. After mixed well, the samples were incubated for 10 min and then the absorbance was read at 630 nm.

24. Lecithin cholesterol acyl transferase (LCAT)
LCAT activity was determined according to the self-substrate method explained by Ngasaki and Akanuma (1977).

Procedure: Lecithin suspension (0.25%) and 150mM sodium iodo-acetic acid solution was prepared using 50mM phosphate buffer (pH 7.4). The subject serum was prepared by adding 20 ml of 0.25% lecithin suspension and 20 ml of 50mM phosphate buffer (pH 7.4) to 40 ml of serum, meanwhile, the control serum was prepared by adding 20 ml of 0.25% lecithin suspension and 20 ml of 150mM sodium iodoacetic acid solution to 40 ml of the serum. After incubation of subject serum and control serum for 40 min at 37 °C, 20 ml of 150 mM sodium iodoacetic acid solution was added to the subject
serum and 20 ml of 50 mM phosphate buffer (pH 7.4) was added to the control serum, and the free cholesterol in both was determined using a commercial assay kit. LCAT activity was calculated as the decreased level (mg) of free cholesterol in 1 ml of serum per 1 h by subtraction of the free cholesterol level of subject serum from that of control serum.

25. Nitric Oxide (NO)
The levels of NO were measured by the method of Kauser et al. (1998).

**Procedure:** The level of NO was assessed indirectly by measuring the nitrite/nitrate concentration in serum using the Griess reagent (1% sulfanilamide in 5% H3O4 and 0.1% napthylethlenediamine dihydrochloride, in a ratio of 1 : 1). Blood was centrifuged to separate serum samples and stored overnight in a freezer. The day of the experiment, an aliquot of 750 ml of serum was mixed with 750 ml of the Griess reagent, protected from light, and maintained at room temperature for 15 min. The concentration of nitrite/nitrate in the samples was determined spectrophotometrically at 540 nm. For every NO assay, a standard curve was performed, using sodium nitrite (NaNO2) as a NO source.

25. Paraoxonase (PON)
The activity of PON was measured by the method of Gan et al. (1991).

**Procedure:** PON activity towards phenyl acetate was measured spectrophotometrically at 270 nm in an UV visible spectrophotometer. Buffer substrate was prepared by adding 50 mM Tris-HCl buffer pH 8.0 containing 2 mM Calcium. Phenyl acetate in Isopropyl alcohol was added such that the Phenyl acetate concentration was 2 mM and the isopropyl alcohol concentration was less than one percent. To 2.99 ml of buffered substrate 10 μl of the test sample was added and the optical density was measured at 270 nm continuously at intervals of 30 sec for 3 min. PON activity was measured by calculating the change in OD per min. One unit of activity was defined as that amount of enzyme which produced 1 micromole of phenol per min. The molar extinction coefficient of phenol was 1310 M⁻¹ cm⁻¹.
Heart tissue homogenate biochemical analysis

27. Extraction of tissue lipids

Total lipid was extracted from the different tissues according to the method of Folch et al., (1951).

Procedure: A known volume of suspension was mixed with 10 ml of chloroform-methanol mixture (2:1 v/v) and shake vigorously. Then it was filtered through Whatmann filter paper (No.42) into a separating funnel. The filtrate was mixed with 0.2 ml of physiological saline and the mixture was kept overnight undisturbed. The lower phase containing the lipid was drained off into pre weighed beakers. The upper phase was re-extracted with more of chloroform-methanol mixture and the extracts were pooled and evaporated to dryness.

The lipids were redissolved in 1 ml of chloroform-methanol mixture and aliquots were used for the estimation of lipid components namely cholesterol and phospholipids. The aliquots were dried at room temperature to evaporate the solvent before use.

28. Total Cholesterol (TC)

The level of heart tissue cholesterol was estimated by using Zlatkis et al. (1953) method. To 0.1 ml of lipid extract 4.9 ml of ferric chloride-acetic acid reagent (0.05 % of ferric chloride acetic acid was added and followed by 3 ml of concentrated sulphuric acid mixed. The contents were cooled and absorbance was read against reagent blank at 560 nm. Standard graph was made by using cholesterol standards ranging from 40-200 mg. Results were expressed as mg cholesterol/mg protein.

29. Triglycerides (TG)

Triglycerides (TG) were estimated by the method of Rice (1970). To 0.5 ml of lipid extract, 3.5 ml of isopropanol was added followed by 50 mg of activated alumina. It was mixed well and left for 15 min. It was then centrifuged and 2 ml of the
supernatant was taken for analysis. About 0.6ml of alkaline potassium hydroxide was added to all the tubes. The tubes were incubated at 60° C for 10 min. The tubes were cooled and 1ml of sodium metaperiodate reagent was added to the tubes followed by the addition of 0.5ml of acetyl acetone reagent. The tubes were cooled and the color developed was read at 405nm using blue filter.

30. Preparation of tissue extracts for glutathione and lipid peroxidation
   Immediately after separation of heart, 10% tissue homogenate was prepared in 0.1M Tris-HCl using Potter-Elvehjem homogenizer at 0 °C. The whole homogenate was used for estimation of glutathione and lipid peroxidation.

31. Sample preparation for assay of antioxidant enzymes
   10% tissue homogenate in 0.1M Tris-HCl was prepared using Potter-Elvehjem homogenizer at 0°C and centrifuged in cold (0-4°C) at 12,000 rpm for 45minutes. The supernatant thus obtained was distributed into eppendorf tubes, labeled and stored at -20°C and all the antioxidant enzymes were assayed at the earliest.

Oxidative stress & Antioxidant studies in Heart.

32. Xanthine oxidase
   The activity of the enzyme was measured according to the method described by Roussos (1967).
   **Procedure:** The assay mixture, in final volume of 3.0 ml, contained 0.30 ml of CuSO4, 0.05 ml of xanthine, 0.1 ml of enzyme source was added and the volume was make upto 3.0 ml with distilled water to make up the volume. Change in absorbance was recorded at 290 nm at 15 seconds interval for one minute. Suitable control was run simultaneously. One unit of activity has been defined as change in absorbance at 290 nm in 1minute by 1 ml enzyme preparation.
33. Reduced Glutathione

**Principle:** Total reduced glutathione content was measured by following method Ellman’s method (1959). This method was based on the development of a yellow colour, when 5,5'-dithio-2-nitrobenzoic acid (DTNB) reacts with the compounds containing sulphydryl groups with a maximum absorbance at 412nm.

**Procedure:** 0.5ml of heart tissue homogenate was deproteinized with 3.5ml of 5% TCA and centrifuged. To 0.5ml of supernatant, 3.0ml phosphate buffer and 0.5ml of Ellman’s reagent were added and the yellow colour developed was read at 412nm. A series of standards (4-20pg) were treated in a similar manner along with a blank. Values are expressed as pg GSH/mg protein.

34. Glutathione peroxidase

**Principle:** A known amount of the enzyme preparation was allowed to react with H$_2$O$_2$ in the presence of GSH for a specified time period according to the method of Rotsruck (1973) and remaining GSH was measured by Ellman’s method (1959).

**Procedure:** To 0.5ml buffer, 0.2ml enzyme source, 0.2ml GSH, 0.1ml H$_2$O$_2$ were added and incubated at room temperature for 10min along with a control tube containing all reagents except enzyme source. The reaction was arrested by adding 0.5ml of 10% TCA, centrifuged at 4000rpm for 5min. and GSH content in 0.5ml of supernatant was estimated. The activity was expressed as µg of GSH consumed/min/mg protein.

35. Glutathione-S-transferase

**Principle:** Glutathione-S-transferase activity was measured by monitoring the increase in the absorbance at 340nm using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate according to the method of Habig et al (1974).

**Procedure:** The assay system contained 1.7 ml of buffer, 0.2ml GSH and 0.04ml enzyme source (40 µg protein). The reaction was initiated by 0.06ml CDNB. The change in absorbance was recorded at 1 minute intervals at 340nm for 5min and the
activity was calculated using an extinction coefficient of CDNB-GSH conjugate as 9.6 mM$^{-1}$ and expressed as mmoles of CDNB-GSH conjugate formed/min/mg protein.

36. Catalase

**Principle:** Catalase catalyses the break down of H$_2$O$_2$ to H$_2$O and O$_2$ and the rate of decomposition of H$_2$O$_2$ was measured spectrophotometrically at 240nm following the method of Beers and Sizer (1952).

**Procedure:** The assay system contained 1.9ml buffer, and 1.0ml H$_2$O$_2$. The reaction was initiated by addition of 0.1ml enzyme source (45µg protein). The decrease in absorbance was monitored at 1 min interval for 5 min at 240nm and activity was calculated using a molar absorbance index of H$_2$O$_2$ 43.6. The activity was expressed as mM of H$_2$O$_2$ decomposed/min/mg protein.

37. Superoxide dismutase

**Principle:** SOD activity was measured based on the ability of the enzyme to inhibit the autoxidation of pyrogallol. A modified procedure described by Marklund and Marklund (1974) was adopted as followed by Soon and Tan (2002).

**Procedure:** The assay system contained 2.1ml of buffer, 0.02ml of enzyme source (35µg protein) and 0.86ml of distilled water. The reaction was initiated with 0.02ml of pyrogallol and change in absorbance was monitored at 420nm. The per cent inhibition was calculated on the basis of comparison with a blank assay system. One unit of SOD was defined as that amount of enzyme required to inhibit the auto-oxidation of pyrogallol by 50% in standard assay system of 3 ml. The specific activity was expressed as units/min/mg protein read in spectrophotometer.

38. Adenosine triphosphatases (ATPases)

**Principle:** ATPases transport electrolytes against concentration gradient at the cost of ATP molecule liberating inorganic phosphate (Pi). The liberated Pi is estimated by Fiske and Subbarow method.
39. Na⁺/K⁺ ATPase

The activity of Na⁺/K⁺ ATPase was estimated according to the method of Bonting (1970).

Procedure: The reaction mixture consisted of 100 µL tissue homogenate incubated in a medium containing Tris-HCl (75 mM, pH -7.5), NaCl (600 mM), KCl (50mM), MgCl₂ (5 mM), EDTA (1 mM) and ATP (3 mM) for 30 min at 30°C. The reaction was arrested by the addition of TCA. The results were expressed as µM of phosphorous liberated/min/mg of protein.

40. Ca²⁺ ATPase

The activity of Ca²⁺ ATPase was estimated according to the method of Hjerten and Pan (1983).

Procedure: Ca²⁺ ATPase was measured by determining spectrophotometrically inorganic phosphate released from ATP in a total reaction volume of 500 µL. The final concentrations of CaCl₂ and ATP were 10 mM and 2mM, respectively, in 25 mM Tris-HCl buffer, pH 8. The samples were incubated at 37°C for 30 min. The reaction was stopped by the addition of 250 µL of cold 20% TCA. The results were expressed in µM Pi liberated/min/mg of protein.

41. Mg²⁺ ATPase

The activity of Mg²⁺ ATPase was estimated according to the method of Ohnishi (1982).

Procedure: The reaction mixture consisted of 75 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, and 3 mM Na₂-ATP in a total of 0.5 ml. The reaction was terminated after 10 or 30 min by the addition of 0.15 ml of 30% TCA. The results were expressed as µM Pi liberated/min/mg of protein.

42. HMG CoA-reductase

The activity of HMG CoA-reductase was estimated according to the method of Venugopal rao and Ramakrishnan (1975).
**Principle:** HMG-CoA reductase catalyzes the sole rate limiting reaction of hepatic cholesterol Synthesis in animals. The ratio (absorbance of HMG-CoA/ absorbance of mevalonate) is taken as an index of the activity of HMG-CoA reductase required to convert HMGCoA to mevalonate in the presence of NADPH. If cholesterol biosynthesis is decreased in a clinical condition, the ratio by this method will increase, and vice versa.

**Procedure:** It is an indirect method of assessing HMGCoA reductase activity. Equal volumes of fresh 10% tissue homogenate and diluted perchloric acid were mixed and allowed to stand for 5 mm then centrifuged (2000 rpm, 10 mm). 1.0 ml of filtrate was treated with 0.5 ml of freshly prepared hydroxylamine reagent (alkaline hydroxylamine reagent in the case of HMG-CoA), mixed, and after 5 mm 1.5 ml of ferric chloride reagent was added to the same tube and mixed well. Readings were taken after 10 mm at 540 nm against similarly treated saline/arsenate blank.

43. Lipoprotein lipase (LPL)

The activity of lipoprotein lipase was estimated by the method of Shirai and Jackson (1982).

**Principle:** The enzyme LPL catalyzes the hydrolysis of substrate p-Nitrophenyl butyrate (PNPB) into p-Nitrophenol and Butyric acid. The increase in the absorbance is the measurement of LPL activity.

**Procedure:** The assay system containing 0.9 ml buffer (100 mM Sodium Phosphate Buffer with 150 mM Sodium Chloride and 0.5% (v/v) Triton X-100, pH 7.2 at 37°C), 0.10 ml of enzyme source was mixed well and 0.010 ml of PNPB substrate was added. The increase in absorbance was read at 400 nm immediately for 5 min at an interval of 1 min. The activity of LPL was expressed as U/mg of protein.

44. Inducible Nitric Oxide Synthase (iNOS)

**Western Blotting**
Protein samples (50 µg, iNOS) were subjected on 12% SDS-PAGE and then analyzed by western blotting. The protein was transferred onto the nitrocellulose membrane at 25 mA overnight. The membrane was blocked with 3% BSA in TTBS buffer for 1 h, then washed with TTBS (4x10ml) and incubated with primary anti-iNOS antibodies for 4 h. After washing with TTBS (4x10ml), the blot was incubated with goat anti-rat ALP antibodies (1:2500) for 1 h. After washing with TTBS (4x10ml) the blot was then developed using and was visualized by chemiluminescence using Kodak gel imager (Kodak Molecular Imaging System, USA).

45. MTT assay
The study of cell proliferation and cell viability requires the accurate quantification of the number of viable cells in a cell culture. MTT is indicative of metabolism and thus is a viable assay (can develop relationship between absorbance and concentration). MTT is a quantitative and reliable assay for determining the number of viable cells in a given culture. This homogeneous colorimetric assay is based on the conversion of a tetrazolium salt MTT, a pale yellow substrate, to formazan, a purple dye. This cellular reduction reaction involves the pyridine nucleotide cofactors NADH/NADPH and is only catalyzed by living cells. Dissolving the resulting formazan with a solubilization buffer permits the convenient quantification of product formation. The intensity of the product color, measured at 550 - 620 nm, is directly proportional to the number of living cells in the culture. For performing MTT assay, SK-N-MC cells were plated at a density of 500-10,000 cells in 96 well plate and incubated at 37°C, 5% CO₂ O/N to allow the cells to attach to wells. L-ODAP was added at different concentrations and incubated for 4 h and 24 h. MTT was added to a final concentration of 0.5 mg/ml and incubated at 37°C, 5% CO₂ for 3 h to allow MTT to be metabolized. Media was removed and 200 µl of DMSO was added to resuspend formazan (MTT metabolic product) and kept on a shaking table for 5 min. Absorbance was read at 560nm and background at 670 nm was subtracted. Absorbance is related with cell quantity. Assay was done in triplicates.
46. Docking studies

Docking studies of Gallic acid (GA), Ellagic acid (EA), Maslinic acid (MA) and Oleanolic acid (OA) were performed. The structures of these compounds were constructed and optimized using chemsketch software. To prepare the HMG CoA structure, the crystal structure was taken from the Protein Data Bank (PDB_ID:1HW9). Statins were removed from the binding site and the chain A was selected for docking studies. Hydrogen atoms were added to the enzyme. The molecular docking method was performed using the Gold version 3.0.1 program to study the binding orientation of compounds into the HMG CoA structure. The docking experiments were performed using the binding site of HMG coA reductase. The binding site identification was carried out using CastP server. A new program, CAST, for automatically locating and measuring protein pockets and cavities, is based on precise computational geometry methods, including alpha shape and discrete flow theory. CAST identifies and measures pockets and pocket mouth openings, as well as cavities. The program specifies the atoms lining pockets, pocket openings, and buried cavities; the volume and area of pockets and cavities; and the area and circumference of mouth openings.

Method

Docking was carried out using GOLD (Genetic Optimization of Ligand Docking) software which is based on genetic algorithm (GA). This method allows as partial flexibility of protein and full flexibility of ligand. The compounds are docked to the active site of the HMG coA reductase. The interaction of these compounds with the active site residues are thoroughly studied using molecular mechanics calculations. The parameters used for GA were population size (100), selection pressure (1.1), number of operations (10,000), number of island (1) and niche size (2). Operator parameters for crossover, mutation and migration were set to 100, 100 and 10 respectively. Default cutoff values of 3.0 Å (dH-X) for hydrogen bonds and 6.0 Å for vanderwaals were employed. During docking, the default algorithm speed was
selected and the ligand binding site in the alpha glucosidase was defined within a 10 Å radius with the centroid as CE atom of ALA410. The number of poses for each inhibitor was set 100, and early termination was allowed if the top three bound conformations of a ligand were within 1.5 Å RMSD. After docking, the individual binding poses of each ligand were observed and their interactions with the protein were studied. The best and most energetically favorable conformation of each ligand was selected.

**Gold Score fitness function:**

Gold Score performs a force field based scoring function and is made up of four components: 1. Protein-ligand hydrogen bond energy (external H-bond); 2. Protein-ligand vander Waals energy (external vdw); 3. Ligand internal vander Waals energy (internal vdw); 4. Ligand intramolecular hydrogen bond energy (internal- H- bond). The external vdw score is multiplied by a factor of 1.375 when the total fitness score is computed. This is an empirical correction to encourage protein-ligand hydrophobic contact. The fitness function has been optimized for the prediction of ligand binding positions.

\[
\text{GoldScore} = S(\text{hb\_ext}) + S(\text{vdw\_ext}) + S(\text{hb\_int}) + S(\text{vdw\_int})
\]

Where \( S(\text{hb\_ext}) \) is the protein-ligand hydrogen bond score, \( S(\text{vdw\_ext}) \) is the protein-ligand van der Waals score, \( S(\text{hb\_int}) \) is the score from intramolecular hydrogen bond in the ligand and \( S(\text{vdw\_int}) \) is the score from intramolecular strain in the ligand.

### 47. Histopathological studies

After sacrificing the rats, hearts were removed and preserved in buffered formalin for histomorphological examination.

**Fixation**

Tissue blocks with 3 mm thickness were cut from small pieces of heart tissue and were placed in a fixative solution (pH 7.0), prepared by adding 100ml of 37-40%
formaldehyde, 900ml of distilled water, in which 4g of Na$_2$HPO$_4$ and 6.5g of NaH$_2$PO$_4$ were dissolved.

**Tissue processing**

The tissue block was processed through a series of solvents: alcohol 80%-1hr, alcohol 90%-1hr, alcohol 95%-2 changes-1hr each, isopropyl alcohol-1hr, acetone-2 changes-1hr each, chloroform-3 changes-1hr each and paraffin-2 changes-1hr each as per scheduled for dehydration, clearing and paraffin infiltration. This block was then ready for embedding. During the process of embedding, the tissue blocks were oriented so that sections were cut in the desired plane of the tissue. Two L-shaped metal moulds were laid on metal plate so as to enclose a rectangular or square space. This is then partly filled with melted paraffin and the tissue was placed in it in the desired position. The container was then filled with melted paraffin and allowed to cool until reasonably firm so that the set block of paraffin with the tissue can be removed from the moulds. The block was trimmed to a suitable size and fixed on a metal objects holder. The block was further trimmed so that paraffin overlaying the piece of tissue is excluded and an adequate area of the tissue facing the knife is exposed. The block was then kept for cooling at 0°C.

**Section Cutting**

The section were cut at 5μm thickness and floated in a water bath between 38-49°C. The sections from the water were mounted on clean glass slides, which have been smeared with a drop of Mayer’s egg albumin. They were then dried on a hotplate at about 50°C for 30 min and the sections on the slides were then subjected to staining.

**Staining**

The slide containing the section was processed serially as follows-xylol 1-3 min, xylol II-3 min, acetone-3 min, 95% alcohol-3 min, running water-3 min, hematoxylin stain-20 min, wash in running tap water-20 min, eosin working solution-2 min-15 sec, 95% alcohol-2 to 3 dips, 95% alcohol-2 changes 1 to 2 min each, acetone-2 changes-3 min each , xylol-2 changes-3 min each and mounted in D.P.X. and
viewed under microscope. The nuclei stained with blue and cytoplasm in various shades of pink (Raghu ramulu et al., 1983).

48. Statistical analysis

Data were expressed as mean ± SD of eight replicates and subjected to one way analysis of variance (ANOVA). Means were separated by the Duncan multiple range (DMR) test to determine significant differences in all parameters. Values were considered statistically significant at p<0.05.