CHAPTER 4
MATERIALS AND METHODS

4.1 EXPERIMENTAL DESIGN

The entire study was designed into three phases to assess the effect of *Crataegus oxycantha* phytopharmacological potential in the treatment of myocardial infarction and endothelial dysfunction.

Phase–I

Acute and sub-acute toxicity of *Crataegus oxycantha* dry fruit Ethanol extract

Phase-II

Standardisation of Isoproterenol Dose – animal model of MI

Phase-III

Effect of *Crataegus oxycantha* on Isoproterenol induced myocardial infarction and endothelial dysfunction in Rats

Phase-I

**Table 4.1 Acute and Sub-acute Toxicity Studies**

<table>
<thead>
<tr>
<th>Group (N=6)</th>
<th><em>Crataegus oxycantha</em> (oral administration)</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>Control (Normal saline)</td>
</tr>
<tr>
<td>II</td>
<td>100 mg/kg B.W</td>
</tr>
<tr>
<td>III</td>
<td>200 mg/kg B.W</td>
</tr>
<tr>
<td>IV</td>
<td>500 mg/kg B.W</td>
</tr>
<tr>
<td>V</td>
<td>1000 mg/kg B.W</td>
</tr>
<tr>
<td>VI</td>
<td>2000 mg/kg B.W</td>
</tr>
</tbody>
</table>
Phase-II

Table 4.2 Isoproterenol Dose Fixation

<table>
<thead>
<tr>
<th>Group (N=1)</th>
<th>Isoproterenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5 mg/kg B.W s.c</td>
</tr>
<tr>
<td>II</td>
<td>85 mg/kg B.W s.c</td>
</tr>
<tr>
<td>III</td>
<td>150 mg/kg B.W s.c</td>
</tr>
</tbody>
</table>

Table 4.3 Effect of COC on Isoproterenol induced myocardial infarction and endothelial dysfunction in rats

<table>
<thead>
<tr>
<th>Group (N=6)</th>
<th>Treatment</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>Normal saline (Control)</td>
</tr>
<tr>
<td>II</td>
<td>Isoproterenol 85 mg/kg B.W (same dose from Group 3 to 9)</td>
</tr>
<tr>
<td>III</td>
<td>COC 50 mg/kg + ISO</td>
</tr>
<tr>
<td>IV</td>
<td>COC 100 mg/kg + ISO</td>
</tr>
<tr>
<td>V</td>
<td>COC 200 mg/kg + ISO</td>
</tr>
<tr>
<td>VI</td>
<td>Metoprolol 50 mg/kg + ISO (Positive control)</td>
</tr>
<tr>
<td>VII</td>
<td>COC 50 mg/kg + Metoprolol 50 mg/kg + ISO</td>
</tr>
<tr>
<td>VIII</td>
<td>COC 100 mg/kg + Metoprolol 50 mg/kg + ISO</td>
</tr>
<tr>
<td>IX</td>
<td>COC 200 mg/kg + Metoprolol 50 mg/kg + ISO</td>
</tr>
</tbody>
</table>

4.2 COLLECTION OF THE PLANT MATERIAL

Berries of the Crataegus oxycantha were collected from Chamba district, Himachal Pradesh, India. The plant and berries were identified and authenticated by Taxonomist Dr. K.G Bhat, Retired Professor, Department of Botany, Poorna Prajna College, Udupi, Karnataka, India.
4.3 PREPARATION OF ETHANOLIC PLANT EXTRACT

The 1 Kg dried fruit powder of *Crataegus oxycantha* was continuously extracted using soxhlet apparatus in 5:1 ratio of ethanol and dried fruit powder respectively. The subsequent crude extract was evaporated to dryness using a rotary evaporator at 70°C. The final yield obtained was 80 gm., which was stored at 4°C in the fridge and used in the entire study duration the residue was suspended in distilled water and administered through orally by using feeding gavage to all the experimental rats.

4.4 PHYTOCHEMICAL SCREENING OF *Crataegus oxycantha*

The qualitative phytochemical screening of plant ethanolic extract were carried out to identify the presence of various plant phytochemical constituents like Phenols, flavonoids, alkaloids, tannins and saponins.

4.4.1 Detection of Phenols

1. Ferric chloride test

To the 0.5 ml of plant extract 1ml of alcoholic ferric chloride solution was added. Appearance of bluish or black color indicates the presence of phenols.

2. Test for Tannins

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

4.4.2 Detection of Flavonoids

1. Shinoda test

To 0.5 ml of the plant extract, few magnesium turnings were added followed by few drops of concentrated hydrochloric acid. The entire mixture boiled in a boiling water bath for five minutes. Crimson red coloration indicates the presence of flavonoids
2. **Alkaline reagent test**

To 0.5 ml of plant extract, 1.0 ml of sodium hydroxide solution was added. Formation of intense yellow color, which becomes color less on addition of dilute acid, confirms the presence of flavonoids.

3. **Ferric Chloride test**

To 1.0 ml of plant extract, 1ml of 10% of ferric chloride was added. Formation of Intense greenish color denotes the presence of flavonoid content.

4.4.3 **Test for Saponins**

1. **Froth test**

About 1.0 ml of extract was diluted with 4.0 ml of distilled water and shaken continuously. Formation of layer of foam indicates the presence of saponins.

2. **Emulsion test**

To 1.0 ml of extract, 2 drops of olive oil was added. The solution was shaken vigorously. Formation of emulsion indicates presence of saponins.

4.4.4 **Detection of alkaloids**

1. **Mayer’s Test**

1.0 ml of extract were treated with Mayer’s reagent (potassium mercuric iodide). Formation of cream colored precipitate indicates the presence of alkaloids.

2. **Wagner’s Test**

1.0 ml of extract were treated with Wagner’s reagent (Iodine in potassium iodide). Formation of brown/reddish precipitate denotes the presence of alkaloids.
4.5 SELECTION OF ANIMALS

Male Wistar albino adult rats weighing between 150 to 200 g were procured from National Institute of Nutrition (NIN), Hyderabad, Telangana, India. Ethical Clearance was obtained from the Institutional Animal Ethics committee, SRM Medical College Hospital and Research Centre, SRM IST, India. The IAEC Approval No is 084/835/IAEC-2014.

4.6 PREPARATION OF EXPERIMENTAL RATS

The animals were kept in automated 12 hours light and dark cycles at 26°C ± 2°C in an air exchange room. All animals were acclimatized to laboratory environments for 15 days prior to the experiment. The animals were fed with standard pellet diet provided by Sai Enterprise, Chennai, Tamilnadu, India. All animal were provided with RO water ad libitum. Autoclaved clean paddy husk was used as bedding material was changed thrice a week. Animal experiments were performed according to the ethical guidelines suggested by the institutional animal ethics committee (IAEC).

4.7 ACUTE TOXICITY STUDIES OF ETHANOLIC EXTRACT OF Crataegus oxycantha

Acute toxicity studies were conducted according to the guidelines of OECD. Thirty six Wistar albino male rats weighing between 150-200g were used for the acute toxicity study. The extract was administered orally to the Treatment Groups in varying doses (100, 200, 500, 1000, 2000mg/ Kg B.W) for 2 days. Control Group animals were given distilled water. They were constantly observed for 48 hours to identify any changes in behaviour or autonomic responses, spontaneous movements, corneal reflex, irritability, defecation and urination, mobility, aggressiveness, pain and respiratory movements. The aim of the acute toxicity studies is to determine the LD50 values. The mortality rate was measured and expressed as LD50.
LD50 = the apparent least dose lethal to all in a Group – Σ (a×b)/N [60]

Σ = Sigma, a = dose difference  b= mean mortality N = number of animals in each Group

4.8 SUB-ACUTE TOXICITY STUDIES OF Crataegus oxycantha

To determine the effective dosage of Crataegus oxycantha, subacute toxicity studies were carried out by the method of Biswas. The Extract of COC was suspended in water and administered orally at varying doses (100, 200, 500, 1000, and 2000mg/kg B.W.) A constant volume of 1 ml/ rat with above mentioned doses were administered to different Groups of animals daily for 28 days. The sub-acute toxicity studies were conducted according the OECD, 407 guidelines [137].

4.8.1 Body weight

The body weights of all rats were measured using a sensitive balance. During the acclimatization period, prior to treatment, 4 times during treatment (once weekly) and on the day of sacrifice the body weight was measured. (Table 5.1)

4.8.2 Mortality and clinical signs

During experimental period of one month, all the rats were observed daily for clinical signs, morbidity and mortality patterns prior to the dosing, immediately after dosing and up to 4 hour after dosing. On 30th day, 1.5ml of blood was collected from all the rats and serum was separated, which was used for biochemical analysis. On 31st day, euthanasia was induced by thiopentone among the rats. The liver, kidney and hearts were excised. All the tissues were weighed and were preserved in 10% formalin solution for histopathological evaluation.

4.8.3 Relative organ weight

Different organs namely the heart, liver, and kidneys were carefully dissected out and weighed in grams using sensitive balance. The relative organ weight of each animal was then calculated as follows and tabulated (Table 5.2).
**Relative organ weight =** \[
\frac{\text{Absolute organ weight (g)}}{\text{Body weight of rat on sacrifice day (g)}} \times 100
\]

4.8.4 Haematological assessment

The Blood samples were estimated for haematological parameters like haemoglobin, HCT, RBC, WBC, MCV,MCH, MCHC, PLT, Clotting time, PT,APTT by using SYSMAX - XT1800I auto analyser (Table 5.3 & 5.4).

4.8.5 Estimation of Biochemical parameters

Biochemical parameters such as SGOT (Serum glutamate Oxaloacetate transaminase), SGPT (Serum glutamate pyruvate transaminase), ALP (Alkaline phosphatase), urea, uric acid, total protein, total bilirubin, Na⁺, K⁺, Cl⁻, Albumin, Globulin, Bicarbonates in serum were assayed by using BECMAN COULTER - AU 480 auto analyser by using standard kits (Table 5.5).

4.8.6 Histopathological Evaluation

The liver, kidney and heart were fixed in buffered 10% formalin. 5 micrometres thick paraffin sections were made and stained with haematoxylin and eosin for microscopic examination to establish the safe nontoxic dose in the present study (Fig. no.5.12, 5.13& 5.14).

4.9 DOSE STANDARDISATION OF ISOPROTERENOL FOR INDUCTION OF MYOCARDIAL INFARCTION

Isoproterenol was administered by S.C route in 3 different doses (5 mg/kg B.W, 85 mg/kg B.W, and 150 mg/kg B.W) to 3 animals. Positive induction of MI was confirmed by the quantitative determination of troponin-I and CK-MB(biomarkers of MI), histopathological findings and survival of the animal, which determined the final optimum dose of Isoproterenol for inducing MI. Isoproterenol 85 mg/kg B.W was used for experimental induction of MI in the present study (Table 5.6).[138-141]
4.10 EFFECTOF Crataegus oxycantha ON MYOCARDIAL INFARCTION

4.10.1 Induction of Experimental MI

After the 28 days of pre-treatment with Crataegus oxycantha L., and metoprolol based on the experimental design, Isoproterenol (85mg/kg B.W) was dissolved in normal saline and injected subcutaneously to all rats except the control Group at an interval of 24 hours for two consecutive days to create experimental MI [142]. Blood was collected and centrifuged to obtain the serum, which was used for biochemical analysis. The rats were sacrificed after an overnight fasting. Hearts were dissected for tissue homogenate to conduct biochemical analysis and piece of the tissue was preserved for histopathological studies and one heart from each Group was obtained to conduct the macroscopic enzyme mapping with TTC special stain.

4.10.2 Estimation of Biomarkers of Cardiac necrosis

The biomarker enzymes for cardiac damage, like Troponin-I, Creatine Kinase-MB (CK-MB), Lactate dehydrogenase (LDH), Aspartate transaminase (AST), Alanine transaminase (ALT) and Uric acid were estimated in the serum by using commercially purchased kits.

4.10.2.1 Estimation of Serum Troponin-I

The distinctive isoform and tissue specificity of cTnI are the basis for its use as a marker of cardiac muscle damage during necrosis. Serum troponin-I was measured by the method of Juneman EB, 2012 [143] and Keller T, 2009 [144] using Life diagnostics, Inc ELISA Kit. The activity of cTnI was expressed as ng/ml. Optical density of Trop-I was measured at 450 nm using ELISA reader.

Assay Principle

The high sensitivity cTnI ELISA recognizes an epitope on Rat cTnI that is resistant to proteolysis in Rat serum, thereby improving detection capability. The assay uses two different purified antibodies. One is used for solid phase immobilization (on the microtiter wells). The second is conjugated to horse radish
peroxidase (HRP). The serum sample is allowed to react concurrently with the two antibodies, resulting in cTnI being sandwiched between the solid phase and HRP-conjugated antibodies. After incubation for one hour at room temperature on a plate shaker the wells are washed to remove unbound HRP conjugated antibodies. A solution of tetramethylbenzidine (TMB), an HRP substrate, is then added and incubated for 20 minutes, resulting in the development of a blue color. The color development is stopped by addition of 1N HCl changing the color to yellow. The Concentration of cTnI is proportional to the absorbance at 450 nm measured using Spectrophotometer.

4.10.2.2 Estimation of Serum Creatinine Kinase-MB (CK-MB)

Principle of the Assay

This assay is based on the quantitative sandwich enzyme immunoassay technique. Specific antibody designed for CK-MB has been pre-coated onto a microplate. Standards and serum samples are pipetted into the wells and any CK-MB present is bound by the immobilized antibody. A biotin-conjugated antibody specific for CK-MB is added to the wells after removing any unbound substances. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash with wash buffer to remove any unbound avidin-enzyme reagent present in the wells, a substrate solution is added to the wells and color develops in proportion to the amount of CK-MB bound in the first step. The color development is stopped and the intensity of the color is measured.

According to Agappe Diagnostic Kit. 0.1 ml of working reagent was added to 40 µl of serum samples, mixed and incubated at 37° C for 5min. After incubation, change in the optical density was estimated for 3min at an interval of 1 min against blank at 340nm.

The activity of creatine kinase-MB was expressed as IU/L. Optical density of CK-MB was estimated at 340 nm using UV spectrophotometer. The method was described by Henry, 1979[145].
4.10.2.3 Estimation of Lactate dehydrogenase (LDH)

**Assay Principle**

The Assay is based on sandwich enzyme-linked immunosorbent assay technology. Anti-DHA antibody was pre-coated onto 96-well plates. And the biotin conjugated anti-DHA antibody was used as detection antibodies in the samples. The standards, test samples and biotin conjugated detection antibody were added to the wells consequently, and wash with wash buffer. HRP-Streptavidin was added and unbound conjugates were washed away with wash buffer. TMB substrates were used to visualize HRP enzymatic reaction in the samples. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the DHA amount of sample captured in plate. Read the O.D. absorbance at 450nm in a microplate reader, and then the concentration of Ldha can be calculated.

The activity of LDH was read at absorbance of 340 nm in a UV spectrophotometer by a method of Teitz, 1976[146] using Aspen diagnostic kit.

4.10.2.4 Estimation of AST and ALT

**Principle of the Assay**

This assay is based on the principle that AST and ALT catalyse the transfer of amino Group from L-aspartate/L-alanine to $\alpha$-ketoglutarate to yield oxaloacetate/pyruvate respectively. Oxaloacetate/pyruvate can in turn oxidise NADH to NAD$^+$ in the presence of malate dehydrogenase/lactate dehydrogenase. The reduction in absorbance at 340 nm in a spectrophotometer due to the oxidation of NADH is observed kinetically and is proportional to AST/ALT activity. To 100 µl of serum, 1000 µl of working reagent was added. The tubes were mixed well and the absorbance was measured after 60 sec and the change in absorbance was recorded for 2 min at 340nm in a spectrophotometer. AST/ALT activity is expressed as IU/L. The serum AST and ALT enzyme levels were estimated by the method of Bergermeyer H and Walefeld, M. et.al. 1978 [147] using spectrophotometric method.
4.10.2.5. Estimation of Uric Acid

Uricase is very important and specific enzyme acting on uric acid with end product being allantoin and peroxide. Peroxidase utilizes hydrogen peroxide (proportional to uric acid concentration) to translate chromogen to coloured complex. The intensity of color produced was proportional to uric acid concentration and it was measured photometrically at 520 nm. Absorbance of serum uric was measured by UV spectrophotometer at 520 nm using a method of fossati et al. 1980 [148]. The intensity of color produced is proportional to the uric acid concentration and is measured by spectrophotometer.

4.10.3 Preparation of Tissue Homogenate for Estimation of Cardiac antioxidant status

Cardiac tissue from control and treated Groups were weighed and homogenized (10% w/v) in chilled Tris buffer(10mM; pH 7.4) tissue homogenate in 0.15 M potassium chloride was prepared using Potter-Elvehjem homogenizer at 0° C and centrifuged in cold (0 to4°C) at 12,000 rpm for 45 minutes. The supernatant thus obtained was distributed into eppendorf tubes, labelled and stored at 20° C and all the antioxidant enzymes were assessed at the earliest.

4.10.3.1 Estimation of Lipid Peroxidation

Lipid peroxidative extent was measured by the formation of TBRAS by using the method of Buege and Aust,1978 [143]. Malondialdehyde has been identified as the product of lipid peroxidation that reacts with TBA to give a chromogen whose absorbance was read at 535 nm by spectrophotometer.

4.10.3.2 Estimation of Superoxide dismutase

SOD activity was measured by the ability of the enzyme to inhibit the auto oxidation of pyrogallol, which was described by Marklund and Marklund, 1974 [150].
4.10.3.3 Estimation of Catalase

Catalase catalyses the breakdown of $H_2O_2$ to $H_2O$ and $O_2$ and the rate of decomposition of $H_2O_2$ was measured spectrophotometrically at 240nm following the method of Beers and Sizer, 1952 [151]. The activity was measured as nmol of $H_2O_2$ decomposed/min/mg protein.

4.10.3.4 Estimation of reduced glutathione (GSH)

Total reduced glutathione content was measured by Ellman’s method, 1959[152]. This method was based on the development of yellow color, when 5, 51-dithio-2-nitro benzoic acid (DTNB) reacts with the compounds containing sulphydryl Groups with a maximum absorbance at 412 nm.

4.10.3.5 Estimation of Vitamin E

Vitamin E levels were measured by the method of Baker and Frank, 1968 [153]. The formation of a red coloured complex with 2, 2’ –dipyridyl and the absorbance of the chromophore was measured at 520 nm using UV spectrophotometer.

4.10.4 ESTIMATION OF CARDIAC MEMBRANE BOUND ENZYMES

4.10.4.1 Estimation of Na\(^+\)-K\(^+\) - ATPase

The incubation mixture was contained 1.0 ml of Tris-HCl buffer, 0.2 ml each of magnesium sulphate and potassium chloride, sodium chloride, EDTA, ATP and the cardiac tissue homogenate. The mixture was incubated at 37°C for 15 min. The reaction was stopped by the addition of 1.0 ml of 10 % TCA, mixed well and then centrifuged. The phosphorus content of the supernatant was calculated according to Fiske and Subborow method 1925 [154]. The activities of Na\(^+\)-K\(^+\) ATPase were assayed using slight modifications of the method of Bonting, 1970 [155]. The enzyme activity was expressed as µmol phoshateliberated/mg protein.
4.10.4.2 Estimation of Ca\(^{2+}\) - ATPase

The incubation mixture containing 0.1 ml each of Tris-HCl buffer, calcium chloride, ATP and cardiac tissue homogenate. The contents were incubated at 37°C for 15 min, the reaction was then arrested by the addition of 1.0 ml ice cold TCA. The quantity of phosphorus liberated was estimated according to the method of Fiske and Subbarow (1925) [153]. The enzyme activity was expressed as μmoles of phosphorus liberated/min/mg protein under incubation conditions. Ca\(^{2+}\)-ATPase activity in the tissue homogenate was measured by the method of Hjerten and Pan, 1983 [156]. The amount of Phosphorous liberated was estimated by the method of Fiske and subbarow, 1925 [154]

4.10.4.3 Estimation of Mg\(^{2+}\) - ATPase

The incubation mixture contains 0.1 ml each of Tris-HCl buffer, magnesium chloride, ATP and the cardiac tissue homogenate. The reaction mixture was incubated at 37°C for 15 min. The reaction was arrested by the addition of 1.0 mL of ice cold TCA. The liberated phosphorus was assessed according to the method of Fiske and Subbarow (1925) [154]. The enzyme activity was expressed as μmoles of phosphorus liberated/min/mg protein. Mg\(^{2+}\) - ATPase activity in the tissue homogenate was measured by the method of Ohinishi et al, 1982[157].

4.10.5 Estimation of Lipids

4.10.5.1 Estimation of Triglycerides

Glycerol released from hydrolysis of triglycerides by lipoprotein lipase is converted by glycerol kinase into glycerol -3-phosphate. Glycerol-3 phosphate is oxidised by glycerol phosphate oxidase to dihydroxyaceton phosphate and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide oxidizes phenolic chromogen to a red colored compound which is measured at 510 nm [158].
4.10.5.2 Estimation of total cholesterol

Cholesterol esterase hydrolyses cholesterol esters into free cholesterol and fatty acids. Cholesterol oxidase converts 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 color is proportional to the amount of total cholesterol [159].

4.10.5.3 Estimation of High Density Lipoprotein cholesterol (HDL-C)

Phosphotungstate/ mg$^{+2}$ precipitate chylomicrons and low density lipoprotein (LDL) fractions. HDL fraction remains unaffected in supernatant. Cholesterol content of HDL fraction is assayed using autozyme cholesterol diagnostic kit [160]

4.10.5.4 Estimation of Very Low density lipoprotein (VLDL) and Low Density Lipoprotein (LDL)

VLDL and LDL were calculated using the friedewald et al, 1972[161] formula as follows.

$$\text{VLDL}= \frac{\text{TG}}{5}$$

$$\text{LDL}= \text{Total Cholesterol} - \frac{\text{TG}}{5} - \text{HDL}$$

4.10.6 Estimation of biochemical markers of endothelial function

V-CAM, CRP and TNF-α activities were estimated in the serum samples by using ELISA method.

4.10.6.1 Estimation of V-CAM

Principle of the Assay

The serum of test and standard samples was separated by centrifugation at 3000 rpm for 10 min and stored at -20°C until used. This kit was designed based on the principle of sandwich enzyme-linked immune-sorbent assay technology. Anti-
VCAM-1 antibody was pre-coated onto 96-well plates. And the biotin conjugated anti-VCAM-1 antibody was used as detection antibodies. The standards, test serum samples and biotin conjugated detection antibody were added to the wells and washed with wash buffer. HRP-Streptavidin was added and unbound conjugates were washed away with wash buffer. TMB substrates were used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to develop a blue colour product that changed into yellow after adding acidic stop solution. The density of yellow colour is proportional to the VCAM-1 amount of sample captured in plate. Record the O.D. absorbance at 450nm in a microplate reader, and then the concentration of VCAM-1 can be estimated. The activities were measured by ELISA kit (Ray biotech, USA) and optical density was read at 450 nm.

4.10.6.2 Estimation of C-reactive protein

Principle of the Assay

The Assay was based on the principle of a solid phase sandwich ELISA method (Enzyme-Linked Immunosorbent Assay). It employs an antibody specific for rat CRP coated on a 96-well plate. Standards or tests of serum samples are added to the wells, and any rat CRP present binds to the immobilized antibody. After the appropriate incubation, wells are washed and a horseradish peroxidase conjugated anti-rat CRP is added to produce an antibody-antigen-antibody “sandwich”. Following another incubation period, the wells are washed and a substrate solution is added, which produces a blue color in direct proportion to the amount of CRP present in the initial sample (development of a blue color indicates a positive reaction while negative reactions appear colorless). The reaction is interrupted with the Stop Solution, which changes the color from blue to yellow (negative reactions remain colorless or faintly yellow) [163]. Estimation of CRP was done by using method of Banerjee M. et al, 2003[164]. The activity of CRP was measured by ELISA kit purchased from life diagnostics and absorbance was read at 450 nm.
4.10.6.3 Estimation of TNF-α

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for rat TNF-α has been pre-coated onto a microplate. Standards control, test serum samples are pipetted into the wells and any TNF-α present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for rat TNF-α is added to the wells. Subsequent wash is carried out to remove any unbound antibody-enzyme reagent, and substrate solution is added to the wells. The enzyme reaction yields a blue product that changes yellow when the Stop Solution is added. The intensity of the color estimated is in proportion to the amount of TNF-α bound in the initial step. The sample values are then read off the standard curve. The Serum TNF-α was estimated by ELISA immunoassay method of Croft M et al. 2012[165] absorbance was read at 450 nm in a ELISA reader using a ELISA kit purchased from Ray biotech, USA.[166]

4.10.7 Morphometric Analysis of the Heart

Rat hearts were dissected and the weights were measured using sensitive balance. Then the ventricles were separated surgically and weighed using sensitive balance and values were recorded. The ventricle wall thickness was measured by using digital vernier calipers and values were noted. The results were plotted to compare the differences between the Groups.

4.10.8 Macroscopic enzyme mapping with TTC

Rat Hearts were kept in container having 1% TTC (2, 3, 5-triphenytetrazolium chloride; TCI chemicals, Tokyo, Japan) solution and incubated at 37 °C for 30 min for differentiation of viable tissue from necrotic tissue by the method of Li et al., 2011[167]. Observed tissue changes were photographed for interpretation of the findings of control and drug treated rats.