Chapter 7

EFFECT OF METHANOLIC EXTRACT OF GARDENIA GUMMIFERA LINN. F. ON ISOPROTERENOL INDUCED MYOCARDIAL INFARCTION IN RATS
7.1. Introduction

Ischemic heart diseases (IHD) remain the principal cause of death in both developed and developing countries, accounting for about 20% of death per year worldwide. Myocardial infarction (MI) is the major form of IHD and is characterized by an imbalance of coronary blood supply and myocardial demand which results in ischemia and myocardial death. Experimental and clinical studies have shown that during ischemic injury, oxidative stress produced by the generation of reactive oxygen species (ROS) plays a key role in the development of MI (Dhalla et al., 2000). In ischemic tissues, the free radicals and ROS have been implicated in oxidative chain reactions which damage the cell membrane and subsequently, structural and metabolic alterations, leading to cardiac dysfunction and ultimately cell death (Tappia et al., 2001).

Isoproterenol (L-β-(3,4-dihydroxyphenyl)-2-Isopropylamino ethanol hydrochloride), a synthetic β- adrenergic receptor agonist, causes severe stress to the myocardium resulting in an infarct like necrosis of heart muscle (Prabhu et al., 2006; Arya et al., 2006). The rat model of isoproterenol (ISO) induced myocardial necrosis serves as a well accepted standardized model to evaluate several cardiac dysfunctions (Ithayarasi and Devi, 1997) and to study the efficiency of various natural and synthetic cardioprotective agents (Rathore et al., 1998). MI induced by ISO has been reported to show many metabolic and morphological aberrations in the heart tissue of the experimental animals similar to those observed in human MI (Noronha-Dutra et al., 1985). ISO induced necrosis is a multifactorial condition involving relative hypoxia, coronary insufficiency, alternations in metabolism, decreased level of high energy phosphate stores, intracellular Ca²⁺ overload, changes in electrolyte content and oxidative
stress. ISO-induced myocardial necrosis involves membrane permeability alterations that bring about loss of function and integrity of myocardial membranes (Todd et al., 1980). Administration of ISO depletes the energy reserve of cardiac muscle cells and complex biochemical and structural changes causing cell damage, which is prelude to necrosis (Rona, 1985).

Remiao et al., 2001 reported that oxidative metabolism of catacholamines produce excessive free radicals. These free radicals have been culpably involved in oxidative ischemic injury and are the central component of cellular damage that severely affects the myocardium. As a consequence, a great deal of research is focused on the prevention of diseases in which free radicals contribute significantly in pathophysiology (atherosclerosis, ischemic-reperfusion injury, hypertension, diabetic cardiomyopathy, hypertrophy and congestive heart failure) and their modulation by antioxidants (Bahorun et al., 2006). Biochemical alterations in ISO induced cardiomyopathy represent a complex pattern of changes in cardiac marker enzymes, Lipid profile, lipid metabolizing enzymes, enzymatic and non enzymatic antioxidants levels etc. The necrosis is maximal in the subendocardial region of the left ventricle and in the interaventricular septum (Peer et al., 2008).

Our previous studies revealed the efficacy of the methanolic extract of Gardenia gummifera (MEGG) against thioacetamide induced oxidative stress and high fat diet induced atherosclerosis. In addition, the phytochemical constituents identified by the LC-MS analysis of MEGG such as erythrodiol, lupeol, epicatechin, β-sitosterol, asiatic acid, myricetin, oleanolic aldehyde, vernolic acid, dicaffeoylquinic acid and chlorogenic acid are known for its antioxidant, hypolipidemic and/or antiatherogenic effects. With this back ground the present
study was designed to test the efficacy of MEGG against isoproterenol induced myocardial infarction.

7.2. Materials and Methods

7.2.1. Chemicals

Isoproterenol hydrochloride was purchased from Sigma chemicals (St. Louis, MO, USA). Assay kits for serum Lactate dehydrogenase (LDH), Serum transaminases (ALT, AST), Creatine kinase isoenzyme (CK-MB), Triglycerides, cholesterol, uric acid and ceruloplasmin were purchased from Agappe Diagnostic Ltd., India. 2,3,5 – Triphenyl Tetrazolium Chloride (TTC) was purchased from Sisco Research Laboratories (SRL). All other chemicals were of analytical grade.

7.2.2. Preparation of plant extracts

The shade-dried roots of *G. gummifera* were powdered and subjected to Soxhlet extraction with methanol (50 g in 400 mL) and concentrated under reduced pressure using a rotary evaporator. The yield of methanolic extract was 10.3% (w/w). Extract was suspended in 5% Tween 80.

7.2.3. Animals and diets

Adult male wistar rats weighing 150.7 ± 10.3g (Mean ± S.D) were used in this study. The rats were fed with standard laboratory chow (Hindustan Lever Foods, Bangalore, India) and provided with water ad libitum. The Animals were maintained at a controlled condition of temperature of 26 – 28°C with a 12 h light: 12 h dark cycle. Animal studies were followed according to Institute Animal Ethics Committee (IAEC) regulations approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (Reg. No. B2442009/3) and conducted humanely.
7.2.4. Experimental Protocol

Male Wistar rats weighing 150 ± 5.5 gm (Mean ± S.D, n = 30) were used in this study. The animals were divided into 5 groups (Six rats/group).

- **Group I**: Vehicle control: (5% Tween 80 and normal saline instead of MEGG and ISO respectively).
- **Group II**: Toxic control: Animals were given subcutaneous injection of ISO (6mg/100g) dissolved in 0.1ml normal saline at an interval of 24hrs for 2 days.
- **Group III**: Drug control: Animals were given MEGG orally for 45 days (250mg/kg body weight).
- **Group IV**: Animals were given MEGG orally for 45 days (125mg/kg body weight) and ISO was administered s.c. twice at an interval of 24 h as the dose mentioned in Group 2.
- **Group V**: Rats pretreated with MEGG orally for 45 days (250 mg/kg body weight) and then ISO was administered s.c. twice at an interval of 24 h as the dose mentioned in Group 2.

At the end of the experimental period (24 hours after the second ISO injection) the animals were sacrificed, the blood and tissues were collected.

7.2.5. Serum analysis

The blood collected from each animal was allowed to clot for 45 min at room temperature and the serum was separated by centrifugation at 2000 rpm at 4°C for 15 min. Cardiotoxicity was assessed by quantifying the serum levels of AST (EC 2.6.1.1), ALT (EC 2.6.1.2), LDH (EC 1.1.1.27), CK- MB (EC 2.7.3.2), total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C),
phospholipids (PL), uric acid, ceruloplasmin and serum iron and iron binding capacity by using the kit of Agappe Diagnostic Ltd., India. Activities of these serum enzymes, uric acid and ceruloplasmin were measured using semi autoanalyzer (RMS, India). Low density lipoprotein cholesterol (LDL-C) and very low density lipoprotein cholesterol (VLDL-C) were calculated as per the standard methods. (Friedwald et al., 1972).

7.2.6. Tissue analysis

At the end of the experimental period, the animals were anesthetized with pentothal sodium followed by neck decapitation. Liver, heart and kidney were immediately excised and washed with ice cold saline. The tissues were then cut into fragments and ten percent of homogenate was prepared in 0.1M Tris HCl buffer (pH - 7.4). The homogenate was centrifuged at 3000 rpm for 20 min at 4 °C and the supernatant was used for the estimation of catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), reduced glutathione (GSH) and lipid peroxidation (Thiobarbituric Acid Reactive Substances - TBARS).

GSH levels in tissues were determined based on the formation of a yellow colored complex with DTNB (Ellman, 1959). GST (EC 2.5.1.18) activity was determined from the rate of increase in conjugate formation between reduced glutathione and CDNB (Habig et al., 1974). GR (EC 1.6.4.2) activity was assayed at 37 °C and 340 nm by following the oxidation of NADPH by GSSG (Carlberg and Mannervik, 1985). GPx (EC 1.11.1.9) activity was determined by measuring the decrease in GSH content after incubating the sample in the presence of H₂O₂ and NaN₃ (Rotruck et al., 1973). Tissue CAT (EC 1.11.1.6) activity was determined from
the rate of decomposition of H$_2$O$_2$ (Beers and Sizer, 1952). Malondialdehyde (MDA), a product of lipid peroxidation was determined by thiobarbituric acid reaction as described by Niehuis and Samuelsson (1968). Protein contents of the tissues were determined using bovine serum albumin (BSA) as the standard (Lowry et al., 1951).

(Detailed protocols were given under chapter 2, section 2.2.10. Procedures for in vivo antioxidant assays)

The tissue lipids were extracted (Folch et al., 1957) and estimated for total cholesterol (Zak, 1957), phospholipids (Connerty et al., 1961) and triglycerides (van Handel and Zilversmith, 1957). Detailed protocols were given under chapter 2, sections 2.2.12 - 2.2.15

7.2.6.1. Macroscopic enzyme mapping of ischemic myocardium.

The triphenyl tetrazolium chloride (TTC) test, used for the macroscopic enzyme mapping assay of the ischemic myocardium was done according to the method of Lie et al. (1975). The heart was washed rapidly in cold water to remove excess blood, taking care not to macerate the tissue. The excess epicardial fat was lightly trimmed off and the left ventricle was separated. The heart was transversely cut across the left ventricle to obtain slices no more than 0.1cm in thickness. The heart slices were placed in the covered, darkened glass dish containing pre-warmed (1%) TTC solution in phosphate buffer and the dish was put in an incubator heated between 37 and 40°C for 30-45 minutes. The heart slices were turned over once or twice to make certain that it remains immersed and covered by 1cm of the TTC solution. At the end of the incubation period, the heart slice was placed in fixing solution. When not only fixes the tissue but also enhances the color contrast
developed. The expected reaction of the TTC test was as follows: normal myocardium (succinate dehydrogenase (SDH) or lactate dehydrogenase (LDH) enzyme active) turned bright red ischemic myocardium. SDH or LDH enzyme deficient turned to pale grey or grayish yellow or uncolored area, fibrous scars turned to white.

7.2.6.2. Histopathological studies

Small pieces of liver, heart and kidney were fixed in 10% buffered formalin were processed for embedding in paraffin. Sections of tissues (5-6 µm) were cut and stained with haematoxylin and eosin and examined for histopathological changes under the microscope (Motic AE 21, Germany). The microphotographs were taken using Moticam 1000 camera at original magnification of 100x.

7.2.7. Statistical Analysis

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

7.3. Results

7.3.1. Activities of AST, ALT, LDH and CK-MB in serum.

Fig.7.1 Shows the activities of serum marker enzymes such as AST, ALT, CK-MB and LDH in serum of control and experimental groups of rats. The treatment with MEGG at doses of 125 and 250 mg/kg showed a significant decrease (P≤0.05) of AST, ALT, LDH and CK-MB. Treatment with 125 mg/kg and 250 mg/kg MEGG exhibited a protection of 67.5% and 86.2% in AST levels, 59.4% and 87.9% in ALT levels, 53.4% and 73.1% in LDH levels and 46.5% and 72.2% in CK-
MB levels. MEGG alone treated rats (Group III) showed no significant change when compared to control rats (Group I).

Fig. 7.1. Effects of MEGG on changes in serum enzyme levels of rats treated with ISO. (A) Aspartate aminotransferase, (B) Alanine aminotransferase

I - Normal control, II - ISO control (6 mg/100g s.c), III – Drug control (MEGG – 250 mg/kg), IV - MEGG – 125 mg/kg, V - MEGG – 250 mg/kg. Values are mean ± S.D, error bar indicating the standard deviation, n = 6 animals. † p ≤ 0.05 vs. normal control. *p ≤ 0.05 vs. ISO control.
Fig. 7.1. (Cont.) Effects of MEGG on changes in serum enzyme levels of rats treated with ISO. (C) Lactate dehydrogenase (D) Creatine kinase-MB.

I - Normal control, II - ISO control (6 mg/100g s.c), III – Drug control (MEGG – 250 mg/kg), IV - MEGG – 125 mg/kg, V - MEGG – 250 mg/kg. Values are mean ± S.D, error bar indicating the standard deviation, n = 6 animals. †p ≤ 0.05 vs. normal control. *p ≤ 0.05 vs. ISO control.
7.3.2. Concentration of uric acid and ceruloplasmin in serum

ISO showed a significant (P ≤ 0.05) increase in serum uric acid (Table 7.1) and a significant (P ≤ 0.05) decrease in ceruloplasmin levels (Table 7.1) when compared to control rats (Group I). MEGG at a dose of 125mg/kg showed a decrease in serum uric acid levels. Treatment with MEGG 250 mg/kg further decreased the uric acid levels. The treatment with MEGG 125 mg/kg brought about an increase in ceruloplasmin level. The ceruloplasmin level was further increased by MEGG 250 mg/kg treatment. MEGG alone treated rats showed no significant changes when compared to normal groups.

Table 7.1. Effect of MEGG on serum uric acid and ceruloplasmin level in rats treated with isoproterenol

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Uricacid (mg/dl)</th>
<th>Ceruloplasmin (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Normal control</td>
<td>3.4 ± 0.2</td>
<td>14.2 ± 2.2</td>
</tr>
<tr>
<td>II ISO(6mg/100g ;s.c)</td>
<td>6.9 ± 0.6 a</td>
<td>3.3 ± 1.2 a</td>
</tr>
<tr>
<td>III MEGG(250mg/kg)</td>
<td>3.5 ± 0.3</td>
<td>14.8 ± 2.1</td>
</tr>
<tr>
<td>IV MEGG(125mg/kg) +ISO</td>
<td>4.1 ± 0.4 b</td>
<td>8.2 ± 1.2 b</td>
</tr>
<tr>
<td>V MEGG(250mg/kg) +ISO</td>
<td>3.8 ± 0.3 b</td>
<td>10.9 ± 1.4 b</td>
</tr>
</tbody>
</table>

Values are mean ± S.D., n = 6. Statistical significance: a p ≤ 0.05 – ISO group differs significantly from normal control group b p ≤ 0.05 - 125mg/kg MEGG+ ISO and 250 mg/kg MEGG+ ISO groups differs significantly from ISO control group.

7.3.3. Effect of MEGG on serum iron and iron binding capacity

In ISO induced myocardial infarction the serum iron binding capacity was observed to be decreased significantly (P ≤ 0.05) with a concomitant increase in free iron concentration (P ≤ 0.05) when compared to control rats (Fig. 7.2). Upon MEGG 125 mg/kg pretreatment, the free iron concentration was decreased with a significant increase in serum iron binding capacity (P ≤ 0.05) when compared to myocardial
infracted rats (Group: II). MEGG at a dose of 250mg/kg showed greater significance in decreased free iron concentration and increased iron binding capacity. MEGG alone treated rats (Group: III) showed no significant changes in these parameters when compared to control rats.

![Fig. 7.2. Effect of MEGG on serum iron and iron binding capacity of ISO treated rats](image)

1 - Normal control, II - ISO control, III – Drug control, IV - MEGG – 125 mg/kg, V - MEGG – 250 mg/kg. Values are mean ± S.D, error bar indicating the standard deviation, n = 6 animals. † p ≤ 0.05 vs. normal control. *p ≤ 0.05 vs. ISO control

7.3.4. Concentration of Tissue Antioxidants

The antioxidant enzyme activities such as GST, GPx, GR and CAT were found to be decreased significantly (P≤0.05) in ISO administered rats when compared to control rats (Table 7.2, 7.3, 7.4 and 7.5) in all the tissues. The reduced glutathione (GSH) level was decreased significantly (P≤0.05) in ISO administered rats (Group: II). In the present study MEGG at a dose of 125 mg/kg showed a
significant increase in GST, GPx, GR, CAT and GSH levels. MEGG 250mg/kg treated rats produced further increase in GST, GPx, GR, CAT and GSH levels.

Table 7.2. Protective effect of MEGG against ISO induced changes in the liver antioxidant status

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>ISO (6mg/100g; s.c)</th>
<th>MEGG (250 mg/kg)</th>
<th>MEGG (125mg/kg) +ISO</th>
<th>MEGG (250mg/kg) +ISO</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH(^1)</td>
<td>19.58 ± 0.73</td>
<td>6.91 ± 0.13(^a)</td>
<td>20.07 ± 0.64</td>
<td>14.82 ± 0.36(^b)</td>
<td>17.69 ± 0.78(^b)</td>
</tr>
<tr>
<td>GST(^2)</td>
<td>45.19 ± 1.60</td>
<td>19.37± 0.83(^a)</td>
<td>44.87 ± 1.94</td>
<td>33.67 ± 1.09(^b)</td>
<td>41.08 ± 1.30(^b)</td>
</tr>
<tr>
<td>GR(^3)</td>
<td>21.20 ± 0.42</td>
<td>7.5 ± 0.41(^a)</td>
<td>22.64 ± 0.51</td>
<td>13.26 ± 0.54(^b)</td>
<td>18.09 ± 0.65(^b)</td>
</tr>
<tr>
<td>GPx(^4)</td>
<td>14.19 ± 0.85</td>
<td>5.62 ± 0.54(^a)</td>
<td>15.04 ± 0.59</td>
<td>9.28 ± 0.42(^b)</td>
<td>11.05 ± 0.71(^b)</td>
</tr>
<tr>
<td>CAT(^5)</td>
<td>24.89 ± 1.32</td>
<td>10.36 ± 0.24(^a)</td>
<td>25.27 ± 1.40</td>
<td>15.53 ± 0.93(^b)</td>
<td>20.38 ±1.18(^b)</td>
</tr>
</tbody>
</table>

Table 7.3. Protective effect of MEGG against ISO induced changes in the cardiac antioxidant status

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>ISO (6mg/100g; s.c)</th>
<th>MEGG (250 mg/kg)</th>
<th>MEGG (125mg/kg) +ISO</th>
<th>MEGG (250mg/kg) +ISO</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH(^1)</td>
<td>9.3 ± 0.92</td>
<td>3.1 ± 0.51(^a)</td>
<td>9.7 ± 0.91</td>
<td>6.8 ± 0.64(^b)</td>
<td>7.6 ± 0.72(^b)</td>
</tr>
<tr>
<td>GST(^2)</td>
<td>26.6 ± 1.35</td>
<td>9.3 ± 0.84(^a)</td>
<td>27.2 ± 1.10</td>
<td>18.7 ± 0.91(^b)</td>
<td>20.3 ± 1.22(^b)</td>
</tr>
<tr>
<td>GR(^3)</td>
<td>6.2 ± 0.48</td>
<td>1.5 ± 0.16(^a)</td>
<td>6.1 ± 0.32</td>
<td>4.3 ± 0.23(^b)</td>
<td>5.8 ± 0.34(^b)</td>
</tr>
<tr>
<td>GPx(^4)</td>
<td>9.71 ± 0.85</td>
<td>4.03 ± 0.67(^a)</td>
<td>10.30 ± 0.90</td>
<td>6.24 ± 0.68(^b)</td>
<td>8.10 ± 0.86(^b)</td>
</tr>
<tr>
<td>CAT(^5)</td>
<td>12.4±1.36</td>
<td>4.1 ± 0.80(^a)</td>
<td>12.2 ± 1.43</td>
<td>10.5±0.90(^b)</td>
<td>11.3 ± 1.17(^b)</td>
</tr>
</tbody>
</table>

\(^1\)(nmol/mg protein); \(^2\)(µmol CDNB-GSH conjugate formed/min/mg protein); \(^3\)(nmol of GSSG utilized/min/mg protein); \(^4\)(nmol of GSH oxidized/min/mg protein); \(^5\)(U/mg protein).

Values are the mean ± S.D from 6 rats in each group. Statistical significance: \(p \leq 0.05\). \(^a\) ISO group differs significantly from normal control group. \(^b\) MEGG–125 mg/kg + ISO and MEGG–250 mg/kg + ISO groups differ significantly from ISO alone treated group.
Table 7.4. Protective effect of MEGG against ISO induced changes in the kidney antioxidant status

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>ISO (6mg/100g; s.c)</th>
<th>MEGG (250 mg/kg)</th>
<th>MEGG (125mg/kg) +ISO</th>
<th>MEGG (250mg/kg) +ISO</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH¹</td>
<td>13.68 ± 0.63</td>
<td>4.31 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.03 ± 0.41</td>
<td>6.53 ± 0.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.49 ± 0.70&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GST²</td>
<td>30.14 ± 0.63</td>
<td>9.29 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.97 ± 0.74</td>
<td>22.60 ± 0.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.18 ± 0.58&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GR³</td>
<td>18.70 ± 0.82</td>
<td>5.03 ± 0.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.14 ± 0.79</td>
<td>10.46 ± 0.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.90 ± 0.75&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GPx⁴</td>
<td>10.16 ± 0.94</td>
<td>4.02 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.64 ± 0.89</td>
<td>6.48 ± 0.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.92 ± 0.76&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAT⁵</td>
<td>15.09 ± 0.62</td>
<td>3.26 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.78 ± 0.50</td>
<td>11.73±0.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.08 ± 0.52&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

¹(nmol/mg protein); ²(µmol CDNB-GSH conjugate formed/min/mg protein); ³(nmol of GSSG utilized/min/mg protein); ⁴(nmol of GSH oxidized/min/mg protein); ⁵(U/mg protein).

Values are the mean ± S.D from 6 rats in each group. Statistical significance: *p* ≤ 0.05. <sup>a</sup> ISO group differs significantly from normal control group. <sup>b</sup> MEGG–125 mg/kg + ISO and MEGG–250 mg/kg + ISO groups differ significantly from ISO alone treated group.

7.3.5. Concentration of lipid peroxidation products in liver, heart and kidney.

The significant increase (P ≤ 0.05) in tissue MDA levels was observed in ISO alone treated rats. However ISO induced elevation of MDA concentration was lowered significantly (P ≤ 0.05) in cardiac tissue of rats treated with MEGG at a dose of 125 and 250mg/kg. MEGG alone treated (Group: III) rats showed non-significant changes in all these parameters as compared with control group.
Table 7.5. Concentration of lipid peroxidation products in liver, heart and kidney

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>MDA (nmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>I  Normal control</td>
<td>1.83 ±0.23</td>
</tr>
<tr>
<td>II ISO(6mg/100g ;S.C)</td>
<td>10.21 ±2.19&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>III MEGG (250 mg/kg)</td>
<td>1.72 ±0.54</td>
</tr>
<tr>
<td>IV MEGG (125mg/kg)+ISO</td>
<td>4.73 ±1.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>V  MEGG (250mg/kg) +ISO</td>
<td>3.81±0.43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± S.D., n = 6. Statistical significance: <sup>a</sup>p ≤ 0.05 – ISO group differs significantly from normal control group. <sup>b</sup>p ≤ 0.05 - 125mg/kg MEGG+ ISO and 250 mg/kg MEGG+ ISO groups differs significantly from ISO control group.

7.3.6. Effect of MEGG on macroscopic enzyme assay (TTC)

Results are given in Fig. 7.3. TTC macroscopic enzyme mapping assay of sections of heart from control and experimental (Fig. 7.3) rats showed direct evidence of myocardial necrosis. Group I (Fig. 7.3 A) showed a section of heart from control rat with viable myocardial tissue. Group II (Fig. 7.3 B) showed a section of heart from an ISO administered rat, necrotic tissues are visible as light gray patches. One of the characteristic features of ISO administration is the loss of LDH activity from myocardium, and may reflect the consequence of cellular injury. Administration of MEGG alone (Group III, Fig. 7.3 C) had no effect on heart tissue when compared to control group. Group IV (Fig. 7.3 D) MEGG pretreated rat (125mg/kg) administered ISO showed a moderately low infarct size and Group V (MEGG 250mg/kg pretreated rats administered ISO) showed results almost similar to that of normal rats, (Fig. 7.3 E) indicating that the prior oral administration of
methanolic extract may have prevented membrane damage by ISO, thereby retaining near normal.

Fig. 7.3. Macroscopic enzyme mapping assay (TTC tests) of heart tissue in control and ISO treated rats

(A): Normal control, Group I. (B): ISO control (6 mg/100g s.c), Group II (C): Methanolic extract alone (250mg/kg), Group III (D): Methanolic extract (125mg/kg) pretreated+ISO, Group IV (E) Methanolic extract (250mg/kg) pretreated+ISO, Group V.
7.3.7. Concentration of serum TC, TG, HDL-C, LDL-C, VLDL-C and phospholipids (PL)

Results were given in Table 7.6. The ISO control group (group II) produced significantly higher serum lipid components (TC, TG and PL) compared to the normal group (group I). Treatment with MEGG at a dose of 125mg/kg b.w. showed significant decrease in the serum lipid components when compared to ISO control group (group II). Pretreatment with MEGG at a dose of 250mg/kg b.w. lowered the level of TC, TG, LDL-C, VLDL-C and phospholipids. The HDL-C was found to be significantly increased in the case of rats treated with MEGG (125 and 250mg/kg). MEGG alone treated group showed no significant change when compared with normal group.
Table 7.6. Effect of MEGG on serum lipid profile - Total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), very low density lipoprotein cholesterol (VLDL-C) and phospholipids (PL) in control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>TC (mg/dl)</th>
<th>TG (mg/dl)</th>
<th>HDL-C (mg/dl)</th>
<th>LDL-C (mg/dl)</th>
<th>VLDL-C (mg/dl)</th>
<th>PL (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I: Normal control</td>
<td>59.97 ± 2.32</td>
<td>72.35 ± 2.27</td>
<td>47.28 ± 3.21</td>
<td>23.12 ± 1.95</td>
<td>10.20 ± 0.73</td>
<td>135.18 ± 5.91</td>
</tr>
<tr>
<td>Group II: ISO (6mg/100g; s.c)</td>
<td>106.68 ± 7.2\textsuperscript{a}</td>
<td>98.62 ± 5.21\textsuperscript{a}</td>
<td>30.42 ± 2.42\textsuperscript{a}</td>
<td>69.04 ± 3.87\textsuperscript{a}</td>
<td>18.35 ± 1.72\textsuperscript{a}</td>
<td>182.46 ± 8.52\textsuperscript{a}</td>
</tr>
<tr>
<td>Group III: MEGG (250mg/kg)</td>
<td>58.70 ± 2.31</td>
<td>71.98 ± 2.12</td>
<td>47.80 ± 3.06</td>
<td>22.91 ± 1.94</td>
<td>9.89 ± 0.59</td>
<td>136.05 ± 5.54</td>
</tr>
<tr>
<td>Group IV: MEGG (125mg/kg) +ISO</td>
<td>78.30 ± 3.53\textsuperscript{b}</td>
<td>86.43 ± 3.25\textsuperscript{b}</td>
<td>39.82 ± 3.31\textsuperscript{b}</td>
<td>36.41 ± 2.29\textsuperscript{b}</td>
<td>15.37 ± 1.32\textsuperscript{b}</td>
<td>167.23 ± 6.09\textsuperscript{b}</td>
</tr>
<tr>
<td>Group V: MEGG (250mg/kg) +ISO</td>
<td>62.57 ± 3.16\textsuperscript{b}</td>
<td>78.32 ± 2.11\textsuperscript{b}</td>
<td>43.65 ± 3.87\textsuperscript{b}</td>
<td>30.56 ± 2.17\textsuperscript{b}</td>
<td>12.15 ± 0.86\textsuperscript{b}</td>
<td>143.71 ± 5.76\textsuperscript{b}</td>
</tr>
</tbody>
</table>

Values are mean ± S.D., n = 6. Statistical significance: \textsuperscript{a}p ≤ 0.05 – ISO group differs significantly from normal control group \textsuperscript{b}p ≤ 0.05 - 125 mg/kg MEGG+ ISO and 250 mg/kg MEGG+ ISO groups differs significantly from ISO control group.
7.3.8. Concentration of total cholesterol (TC), triglycerides (TG) and phospholipids (PL) in the heart tissue

Results were given in Table 7.7. In ISO treated rats the conc. of TC and TG increased significantly when compared to control rats. In ISO control PL decreased significantly. Treatment with MEGG at two different doses significantly reduced the TC and TG while PL showed an increase. But no significant changes were observed in drug alone treated rats.

Table 7.7. Effect of MEGG on the concentration of total cholesterol (TC), triglycerides (TG) and phospholipids (PL) in heart tissue of control and experimental animals

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>TC (mg/dl)</th>
<th>TG (mg/dl)</th>
<th>PL (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I: Normal control</td>
<td>7.61 ± 0.53</td>
<td>12.52 ± 1.38</td>
<td>42.68 ± 2.88</td>
</tr>
<tr>
<td>Group II: ISO control 6mg/100g;s.c)</td>
<td>9.44 ± 1.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.28 ± 3.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.36 ± 2.18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group III: MEGG (250mg/kg)</td>
<td>7.57 ± 0.62</td>
<td>11.63 ± 1.85</td>
<td>42.16 ± 2.56</td>
</tr>
<tr>
<td>Group IV: MEGG (125mg/kg) +ISO</td>
<td>8.38 ± 0.70&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.15 ± 2.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36.27 ± 2.67&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group V: MEGG (250mg/kg)+ISO</td>
<td>7.92 ± 0.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.74 ± 2.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39.50 ± 2.79&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± S.D., n = 6. Statistical significance: <sup>a</sup><sub>p</sub> ≤ 0.05 – ISO group differs significantly from normal control group. <sup>b</sup><sub>p</sub> ≤ 0.05 - 125mg/kg MEGG+ ISO and 250 mg/kg MEGG+ ISO groups differs significantly from ISO control group.

7.3.9. Effect of MEGG treatment on histopathological changes of rat myocardium.

The microscopic observations of myocardial histoarchitecture were qualitatively graded on the basis of myonecrosis, inflammatory cells and edema. The myocardium of control group showed a normal histoarchitecture (Fig.7.4A). Myocardium of ISO control rats showed massive necrosis of myofibers with cell infiltration, edema and increased connective tissue among myocardial fibers along with extra vasation of red blood cells (Fig.7.4B). Rats received only MEGG
(250mg/kg) did not show any adverse effect on myocardial histology (Fig. 7.4C). On the other hand, pretreatment with MEGG (125mg/kg) in ISO-treated rats showed mild edema and inflammatory cells as compared to ISO control group (Fig.7.4D). MEGG at a dose of 250 mg/kg body weight showed almost the absence of myonecrosis, edema and inflammation (Fig. 7.4E).

Fig. 7.4. Effect of MEGG on ISO induced histopathological changes in heart tissue (hematoxylin and eosin, 100×).

(A) Normal control; (B) ISO control, (6 mg/100g s.c.); (C) MEGG alone treated groups (250 mg/kg); (D) MEGG (125 mg/kg) + ISO; (E) MEGG (250 mg/kg) + ISO.
7.3.10. Effect of MEGG treatment on histopathological changes of rat liver

Rats treated with ISO, the normal architecture of liver (Fig. 7.5B) was completely lost with the appearance of centrilobular necrosis and lymphocyte infiltration. Animals administered with MEGG at a dose of 250 mg/kg, b.w. showed a significant \( p \leq 0.05 \) protection from ISO induced histopathological changes in liver (Fig. 7.5 E). MEGG at a dose of 125mg/kg, b.w. showed hepatic architectural pattern with mild to moderate histopathological changes (Fig. 7.5 D). MEGG alone treated group exhibited no significant histopathological changes when compared with normal control (Fig. 7.5 C).
Fig. 7.5. Effect of MEGG on ISO induced histopathological changes in liver tissue (hematoxylin and eosin, 100×).

(A) Normal control; (B) ISO control, (6 mg/100g s.c.); (C) MEGG alone treated groups (250 mg/kg); (D) MEGG (125 mg/kg) + ISO; (E) MEGG (250 mg/kg) + ISO.
7.3.11. Effect of MEGG treatment on histopathological changes of rat kidney

Histological study of the kidney of the normal rats showed normal glomerulus surrounded by the Bowman’s capsule, proximal and distal convoluted tubules without any inflammatory changes. The kidneys of ISO control rats showed dilatation in glomerular capillaries, the lumens of the tubules and Bowman’s space were enlarged, mononuclear cell infiltration, degeneration in nephrons, including glomerulosclerosis and thickening in the basal membrane of glomeruli and tubules were seen (Fig. 7.6B). Treatment with MEGG at a dose 125mg/kg, b.w. showed mild histopathological changes such as mononuclear cell infiltration and edema (Fig. 7.6 D). MEGG at a dose of 250 mg/kg b.w. showed absence of mononuclear cell infiltration and edema (Fig. 7.6 E). MEGG alone treated groups exhibited no significant alterations in histopathological architecture (Fig. 7.6 C).
Fig. 7.6. Effect of MEGG on ISO induced histopathological changes in kidney (hematoxylin and eosin, 100×).

(A) Normal control; (B) ISO control, (6 mg/100g s.c.); (C) MEGG alone treated groups (250 mg/kg); (D) MEGG (125 mg/kg) + ISO; (E) MEGG (250 mg/kg) + ISO.
7.4. Discussion

The results of the present study showed that there was significant increase in oxidative stress after myocardial infarction in rats and MEGG showed a significant protective effect against this oxidative stress. ISO, a synthetic β-adrenergic agonist by its positive inotropic and chronotropic actions, increases the myocardial oxygen demand that leads to ischemic necrosis of myocardium in rats (Mohanty et al., 2008). A number of pathophysiological mechanisms have been proposed to explain the ISO induced myocardial damage, including altered permeability, increased turnover of norepinephrine, and generation of cytotoxic free radicals on auto-oxidation of catecholamine (Sharma et al., 2001). Free radical-mediated lipid peroxidation and consequent changes in membrane permeability are the primary factors for cardiotoxicity induced by ISO (Nirmala and Puvanakrishnan, 1996). Antioxidants suppress the formation of reactive oxygen species and shift the balance towards antioxidants from pro-oxidants, which accumulate to protect the myocytes (Haleagrahara et al., 2011). Oxidative stress increases cAMP levels by exhausting ATP and decreases sarcolemmal Ca\(^{2+}\) transport, resulting in intracellular calcium overload, which leads to ventricular dysfunction (Tappia et al., 2001).

The myocardial cells contain many cardiac enzymes like creatinine kinase, lactate dehydrogenase, aspartate transaminase etc. Upon administration of isoproterenol, the oxygen demand of the heart increases with increase in ionotropic effect in the heart, resulting in prolonged ischemia and glucose deprivation (Saravanan and Prakash, 2004). The cells were damaged with increased muscle contractility, which results in increasing the cell membranes permeability allowing cardiac enzymes to leak out into the blood stream (Wang et al., 2009). Creatine
kinase is an enzyme capable of reversibly transferring a phosphate group from the energy storage form of creatine phosphate, to a molecule of ADP, producing ATP (Rosalki et al., 2004). CK-MB is localized predominantly in the heart and this makes it a valuable diagnostic tool for MI since damage specific to the myocardium would result in elevation of CK-MB levels (Loh et al., 2007).

LDH has been used traditionally as a nonspecific diagnostic tool for myocardial infarction. A rise in the proportion of LDH in the serum can be diagnostic of myocardial infarction (Nigam, 2007). LDH usually rises within 6–12 hours of MI. Level of LDH peaks within 48 hours, remains at that peak for 4–14 days. Serum levels of CK-MB, LDH and transaminases are the diagnostic indicators of MI. In this study a decline in the activities of cardiac specific enzymes were observed in MEGG treated experimental groups. These results also unravel the cardioprotective efficacy of methanolic extract of G. gummifera.

Heme iron is directly related and total iron-binding capacity is inversely related to the risk of myocardial infarction (Prabhu et al., 2006). In ISO-induced myocardial necrosis, free iron was released from heme dependent proteins like hemoglobin and myoglobin and decreases the iron binding capacity and thus increases prostaglandin metabolism and in vivo lipid peroxidation (Halliwell and Gutteridge, 1989). Sasikumar and Devi, 2000 reported that polyphenol treatment reduces lipid peroxidation and serum iron concentration due to its free radical scavenging and antioxidant activity. MEGG treatment reduces lipid peroxidation and serum iron concentration due to its free radical scavenging and antioxidant activity. Thus, the increased plasma iron binding could have prevented hemolysis and iron catalyzed lipid peroxidation. This could be the reason for the decreased
level of iron and increased level of plasma iron binding capacity in MEGG pretreated (Group 4 and 5) rats.

Ceruloplasmin is an extracellular antioxidant that can scavenge superoxide radicals (Lekse et al., 2001) and inhibits ferritin dependant lipid peroxidation by catalyzing the oxidative reincorporation of released iron into ferritin. Since ceruloplasmin has both ferroxidase and copper binding capacity, it could have been used more to neutralize the excess amount of free radicals and hence ISO-induced MI rats showed a decreased level of ceruloplasmin. MEGG pretreatment maintains cellular oxidant–antioxidant balance, which could have prevented the loss of ceruloplasmin, thereby reducing the iron and copper mediated myocardial damage.

Serum uric acid is considered to be a risk factor in myocardial infarction (Weir et al., 2003). During hypoxic condition tissues are disturbed, the enzyme xanthine dehydrogenase is converted to xanthine oxidase by the oxidation of essential-SH-groups. Xanthine oxidase catalyses the conversion of hypoxanthine to xanthine, uric acid and superoxide (Upaganlawar et al., 2011). This could be one of the reason for the elevated levels of serum uric acid in the present study. The association of serum uric acid level with myocardial infarction, left ventricular dysfunction and elevated inflammatory markers must be interpreted as an association and not as a causal relation. Supplementation of MEGG (125 and 250mg/kg) in the present study, significantly reduced serum uric acid level compared to ISO administered rats. MEGG (250mg/kg) showed maximum decreased concentration of uric acid.

Oxidative stress is the state of imbalance between the level of antioxidant defence system and production of the oxygen derived species. Antioxidants have
been linked with the prevention of ROS. ISO-induced myocardial damage might have occurred due to release of oxygen derived free radicals, which causes cardiac dysfunction, increased lipid peroxidation and depletion of endogenous antioxidants (Ojha et al., 2012). The intracellular antioxidant system comprises different free radical scavenging antioxidant enzymes along with some non-enzyme antioxidants like GSH and other thiols. CAT, GST, GPx and GR constitute the first line of cellular antioxidant defense enzymes (Mudagal et al., 2011).

Treatment with MEGG significantly enhanced the cardiac antioxidant activity including the GSH level when compared to the ISO alone treated rats. Glutathione is the major endogenous antioxidant, which forms an important substrate for other enzymes which is involved in the free radical scavenging (Erukainure et al., 2011) and detoxifies toxic metabolites of drugs, regulates gene expression, apoptosis and transmembrane transport of organic solutes and it is essential to maintain the reduced status of the cell/tissue. Arya et al., 2006 reported that the cardioprotective activity of Ocimum sanctum has been attributed largely to the antioxidant properties associated with its flavonoid and phenolic constituents, which are known to augment GSH and antioxidant enzyme levels and scavenge lipid peroxides.

Pretreatment with MEGG prior to ISO intoxication significantly ($p \leq 0.05$) enhanced the GST activity, a phase II enzyme. This was attributed to the decreased bioactivation of ISO caused by the MEGG pre-treatment. GST offers protection against lipid peroxidation by promoting the conjugation of toxic electrophiles with GSH (Carlberg and Mannervik, 1985). The significantly ($p \leq 0.05$) elevated level of GR activity in the cardiac tissues showed the role of MEGG to maintain the GSH
level in these tissues. GPx catalyzes the GSH dependant reduction of H$_2$O$_2$ and other peroxides and protects the organism from oxidative damage (Ansil et al., 2011). The significant ($p \leq 0.05$) restoration of GPx activity after pre-treatment with MEGG is also a clear manifestation of the antioxidant effect of MEGG. This may be elicited by detoxification of the endogenous metabolic peroxide generated after ISO injury in cardiac tissue.

Catalase is responsible for breakdown of H$_2$O$_2$, an important ROS, formed during the reaction catalyzed by SOD (Ramanathan et al., 2002). Reduced activity of catalase after exposure to ISO in the present finding could be correlated to increased generation of H$_2$O$_2$. Presumably, a decrease in CAT activity could be attributed to cross-linking and inactivation of the enzyme protein in the lipid peroxides. The pre-treatment with MEGG significantly ($p \leq 0.05$) aided to maintain the CAT activity near to normal level in cardiac tissues. This evidently shows the antioxidant property of the extract against oxygen free radicals.

Lipid peroxidation a sensitive index of oxidative stress, but also it is an important pathogenic event in myocardial necrosis, and accumulation of lipid hydroperoxides reflects damage of cardiac constituents (Saravanan and Prakash, 2004; Zhou et al., 2012). MDA is a major lipid peroxidation end product, increased levels resulting in irreversible damage to hearts of animals subjected to ISO-induced stress. The results from the present study indicate that treatment with MEGG inhibits ISO-induced MDA accumulation in heart. 

Lipid metabolism plays an important role in myocardial necrosis produced by ischemia (Mathew et al., 1981). Isoproterenol-treated rats showing altered lipid profiles in the serum and heart agrees well with a previous report.
(Senthil et al., 2007). In the present study, the total cholesterol, triglycerides and phospholipids were increased significantly in the serum of ISO treated rats. Strong evidence suggested that hypercholesterolemia induces oxidative stress by causing a reduction in the enzymatic antioxidant defence potential of tissue and generation of oxygen free radicals like superoxide anions. As a result of these metabolic events, peroxidation reactions are accelerated leading to cellular injury (Anandan et al., 2007). An increase in serum LDL and VLDL fractions along with a decrease in HDL cholesterol was observed in ISO induced rats. Pretreatment with MEGG significantly restored the levels of cholesterol, triglycerides and lipoproteins (LDL, VLDL and HDL) as compared to ISO control rats.

The significant increase observed in the heart tissue lipid profiles except phospholipids in the rat treated with ISO alone could be due to enhanced lipid biosynthesis by tissue cAMP. Changes in membrane cholesterol content affect its fluidity, permeability to ions, activities of membrane-bound enzymes, and increased degradation of phospholipids (Yeagle, 1985). The elevated levels of lipids observed in ISO treatment were found to return to a near normal value in MEGG pretreated experimental rats. This could be due to the ability of MEGG to inhibit cAMP and thereby maintain the normal fluidity and less alteration in the property and function of the myocardial membrane (Wang and Polya, 1996).

Biological membranes are rich in phospholipids. A significant decrease in phospholipid content in ISO-treated rats might have been due to the breakdown of membrane phospholipids. Accelerated phospholipid degradation could produce membrane dysfunction, resulting in cell injury and ultimate cell death. Previous findings reported that ISO increases the activity of phospholipase, the observed
decreased content of PL in ISO induced myocardial infarction is due to accelerated membrane degradation of PL by phospholipases (Karthikeyan et al., 2007). MEGG pretreatment significantly prevented the degradation of phospholipids thereby increases phospholipids. From the above observation it can be inferred that MEGG pretreatment significantly prevent the degradation of membrane phospholipids.

Histopathological studies of the tissues of ISO treated rats showed myonecrosis, inflammatory cells and edema in myocardium, centrilobular necrosis and lymphocyte infiltration in liver and mononuclear cell infiltration, degeneration in nephrons, including glomerulosclerosis and thickening in the basal membrane of glomeruli in kidney. In the TTC test, normal myocardium succinate dehydrogenase (SDH) or lactate dehydrogenase (LDH) enzyme active turned bright red, ischemic myocardium, SDH or LDH enzyme deficient turned to pale grey or grayish yellow or uncolored area (Prabhu et al., 2006). MEGG (250mg/kg) pretreated rats significantly improved cellular morphology.

MEGG alone treated rats did not show any necrosis or scar formation or hypertrophy. This indicates that MEGG does not possess any adverse effects to myocardium under normal conditions.

The result of serum biochemical parameters, level of cardiac lipid peroxides, tissue antioxidants, TTC assay and histopathological studies together support the highly potent cardioprotective and antioxidant activity of MEGG. The identified class of components such as erythrodiol, epicatechin, lupeol, asiatic acid, β-sitosterol, oleanolic aldehyde, vernolic acid, chlorogenic acid, dicaffeoylquinic acid and myricetin may exert the antioxidant and cardioprotective activity in ISO treated groups.
Thus it can be concluded that the methanolic extract of the *G. gummifera* root evoke cardioprotective activity at doses of 125 and 250 mg/kg body weight respectively. Accordingly the use of this medicinal plant by the traditional healers is scientifically validated.