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

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ABSTRACT: Objective: To investigate the cardioprotective effect of methanolic extract of Gardenia gummifera root (MEGG) on Isoproterenol (ISO) induced myocardial infarction (MI) in rats. Methods: LCMS analysis of MEGG was done for the identification of cardioprotective constituents. Myocardial infarction was induced by the subcutaneous injection of ISO (6mg/100g body weight) at an interval of 24h for 2 days. MEGG (125 and 250 mg/kg, p.o) was given to rats once daily for 45 days prior to the ISO challenge. The myocardial damage was assessed by quantifying the serum levels of cardiac marker enzymes (LDH, AST, ALT, CK-MB), serum iron and iron binding capacity, uric acid and ceruloplasmin. Antioxidants such as catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST) and reduced glutathione (GSH) were altered in MI rats. The level of lipid peroxidation was measured as malondialdehyde (MDA), triphenyl tetrazolium chloride (TTC) test and quantification of histopathological changes also supported the dose dependent protective effects of MEGG. Results: MEGG significantly (p ≤ 0.05) protected the above mentioned parameters to fall from the normal levels. LCMS analysis of MEGG revealed the presence of cardioprotective constituents such as erythrodiol, lupeol, epicatechin, β-sitosterol, asiatic acid, myricetin, oleanolic aldehyde, vernolic acid, chlorogenic acid and dicaffeoylquinic acid. Conclusion: Our results suggest that MEGG affords a dose dependant cardio protection against isoproterenol-induced myocardial infarction.

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. 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.
Isoproterenol (L - β - (3, 4 - dihydroxyphenyl) – 2 – Isopropyl amino ethanol hydrochloride), a synthetic β-adrenergic receptor agonist, causes severe stress to the myocardium resulting in an infarct like necrosis of heart muscle 4. The rat model of isoproterenol (ISO) induced myocardial necrosis serves as a well accepted standardized model to evaluate several cardiac dysfunctions and to study the efficiency of various natural and synthetic cardioprotective agents 5, 6. 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 7. ISO induced necrosis is a multi factorial condition involving relative hypoxia, coronary insufficiency, alternations in metabolism, decreased level of high energy phosphate stores, intracellular Ca\(^{2+}\) overload, changes in electrolyte content and oxidative stress.

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 8.

**Gardenia gummifera** Linn. f. belonging to the family Rubiaceae is a large medicinal shrub with resinous buds. The resin is acrid, bitter, thermogenic, cardiotonic, carminative, antispasmodic, stimulant, diaphoretic, anthelmintic, antiseptic and expectorant. It is traditionally used in conditions of cardiac debility, obesity, lipolytic disorders, bronchitis, neuropathy and splenomegaly and is given to children in nervous disorders and diarrhoea due to dentition 9. The gum yielded flavones, including gardenin, de-Me-tangeretin and neyadenisn, wogonins, isoscutellarein, apigenin 10, 11. Oleanoic aldehyde, sitosterol, D-mannitol, erythrodioil and 19α-hydroxyerythrodioil were isolated and characterized from *G. gummifera* stem bark 12. Previously we have reported the antioxidant and antihepatotoxic activity of MEGG against thioacetamide induced oxidative stress in rats 13. So the present investigation was undertaken to evaluate the cardioprotective efficacy of MEGG on ISO induced MI in experimental rats.

**MATERIALS AND METHODS:**

**Chemicals**

Isoproterenol hydrochloride was purchased from Sigma chemicals (St. Louis, MO, USA). Assay kits for serum lactate dehydrogenase (LDH), transaminases (ALT, AST), creatine kinase isoenzyme (CK-MB), triglycerides, cholesterol, creatinine, glucose, iron and iron binding capacity, 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.

**Collection of plant material and preparation of plant extracts**

*Gardenia gummifera* Linn. f. were collected from its natural habitat (Idukki (Dist), Kerala, India) and identified. A voucher specimen (SBSBRL.05) is maintained in School of Biosciences, M.G University, Kottayam. Roots were cleaned, chopped, shade-dried and powdered. A 50 g of dried powder was soxhlet extracted with 400mL of methanol for 48 h. The extracts were concentrated under reduced pressure using a rotary evaporator and were kept under refrigeration. The yield of methanolic extract was 10.3 % (w/w). The concentrate was suspended in 5% Tween 80 for in vivo studies.

**Animals and diets**

Adult male wistar rats weighing 150 – 200 g 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 Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (Reg.No. B2442009/3) and conducted humanely.

**Liquid chromatography-mass spectrometry (LC-MS) Analysis of MEGG**

The MEGG was analyzed using *LC-MS 2010A* instrument (Shimadzu, Kyoto, Japan). 10µl of the filtered sample was injected to the manual injector using a Microsyringe (1-20µl, Shimadzu). The mobile phase used was acetonitrile: 0.1% OPA in methanol (80:20) in an isocratic mode. The column and pump used were Reverse Phase C-18 (25 cm X2.5mm) (phenomenex) and SPD 10 AVP-RD respectively. The separated compounds were then
ionized using Atmospheric pressure chemical ionisation method (APCI). The flow rate was maintained to 2 ml/min with a temperature of 25°C and spectral data were collected at 315 nm. Mass analysis was performed in the range 50-800 m/z, under both positive and negative ion mode. The class VP integration software was used for the data analysis. The constituents of the extract were identified by referring the LCMS library, Metwin 2009 (version 2.1).

**Toxicity study**

For acute toxicity studies, rats were divided into two groups of six animals in each group. Group I was treated as normal control; Group II received a single dose (5g/kg) of MEGG and kept under observation for 14 days. Initial and final body weights, water and food intake, state of faecal matter and body temperatures were monitored. Animals were sacrificed on 15th day. The serum levels of AST, ALT, LDH, glucose, urea, triglycerides, cholesterol and creatinine were estimated.

**Dosage fixation**

The effective doses of MEGG was fixed based on the activities of serum aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH) and levels of antioxidant enzymes in the preliminary study, preventive and curative effect of methanolic extract of *Gardenia gummiifera* Linn. f. on thioacetamide induced oxidative stress in rats.

**Experimental Protocol**

The animals were divided in to 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, the animals were sacrificed and the blood and tissues were collected.

**Biochemical analysis**

**Serum analysis**

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), uric acid, ceruloplasmin, 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).

**Tissue analysis**

Heart was excised, washed thoroughly in ice-cold saline to remove the blood. 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), lipid peroxidation product (Thiobarbituric Acid Reactive Substances – TBARS) and total protein.

Tissue CAT (EC 1.11.1.9) activity was determined from the rate of decomposition of H$_2$O$_2$. GPx (EC 1.11.1.9) activity was determined by measuring the decrease in GSH content after incubating the sample in the presence of H$_2$O$_2$ and Na$_3$BO$_3$. GR (EC 1.6.4.2) activity was assayed at 37°C and 340 nm by following the oxidation of NADPH by GSSG. GST (EC 2.5.1.18) activity was determined from the rate of increase in conjugate formation between reduced glutathione and CDNB. GSH was determined based on the formation of a yellow colored complex with DTNB.

The level of lipid peroxidation was measured as malondialdehyde (MDA), a thiobarbituric acid reacting substance (TBARS), using 1'1'3’3’-tetramethoxypropane as standard. Protein content in the tissue was determined using bovine serum albumin (BSA) as the standard.

**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. 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 0.1 cm 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 1 cm 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.

**Histopathological studies**

Small pieces of heart fixed in 10% buffered formalin were processed for embedding in paraffin.

**TABLE 1: LIST OF MAJOR ANTIOXIDANT/CARDIOPROTECTIVE COMPOUNDS IDENTIFIED IN MEGG BY LC-MS ANALYSIS**

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Name of the compounds</th>
<th>Library sequence No.</th>
<th>Molecular Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Erythrodiol</td>
<td>MTW/UM/2.1.1/0089/11</td>
<td>442.72</td>
</tr>
<tr>
<td>2</td>
<td>Lupeol</td>
<td>MTW/UM/2.1.1/0322/11</td>
<td>426.73</td>
</tr>
<tr>
<td>3</td>
<td>Epicatechin</td>
<td>MTW/UM/2.1.1/1166/11</td>
<td>578.54</td>
</tr>
<tr>
<td>4</td>
<td>β-sitosterol</td>
<td>MTW/UM/2.1.1/0009/11</td>
<td>414.71</td>
</tr>
<tr>
<td>5</td>
<td>Asiatic acid</td>
<td>MTW/UM/2.1.1/0008/11</td>
<td>488.71</td>
</tr>
<tr>
<td>6</td>
<td>Myricetin</td>
<td>MTW/UM/2.1.1/7474/11</td>
<td>318.25</td>
</tr>
<tr>
<td>7</td>
<td>Oleanolic aldehyde</td>
<td>MTW/UM/2.1.1/1655/11</td>
<td>440.70</td>
</tr>
<tr>
<td>8</td>
<td>Vernolic acid</td>
<td>MTW/UM/2.1.1/1578/11</td>
<td>296.45</td>
</tr>
<tr>
<td>9</td>
<td>Chlorogenic acid</td>
<td>MTW/UM/2.1.1/7816/11</td>
<td>354.32</td>
</tr>
<tr>
<td>10</td>
<td>Dicaffeoylquinic acid</td>
<td>MTW/UM/2.1.1/1199/11</td>
<td>516.49</td>
</tr>
</tbody>
</table>
Toxicity study:
The extract administration up to a high dose of 5 g/kg did not result in mortality or any change in behavior of the animals. There was no significant alteration in the biochemical parameters like AST, ALT, LDH, creatinine, triglycerides, cholesterol, glucose and urea (Table 2). MEGG showed no lethal effect at least up to a dose of 5 g/kg body weight indicating that LD50 if any should be higher than this dose.

TABLE 2: BIOCHEMICAL PARAMETERS OF RATS TREATED WITH MEGG IN ACUTE TOXICITY STUDIES

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
<th>LDH (IU/L)</th>
<th>Glucose (mg/dl)</th>
<th>Urea (mg/dl)</th>
<th>Triglyceride (mg/dl)</th>
<th>Cholesterol (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>155.71±5.42</td>
<td>56.34±3.62</td>
<td>294.28±5.13</td>
<td>125.30±2.82</td>
<td>35.16±1.56</td>
<td>55.69±3.72</td>
<td>75.67±3.76</td>
<td>3.49±0.24</td>
</tr>
<tr>
<td>MEGG (5g/kg)</td>
<td>157.21±4.73</td>
<td>57.26±3.62</td>
<td>295.60±4.56</td>
<td>124.64±2.36</td>
<td>35.25±1.47</td>
<td>54.83±5.39</td>
<td>76.41±3.10</td>
<td>3.70±0.27</td>
</tr>
</tbody>
</table>

Values are mean ± S.D., n = 6. p ≤ 0.05 versus normal control

Biochemical analysis
Fig: 2 showed 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).

ISO showed a significant (P≤0.05) increase in serum uric acid (Table. 3) and a significant (P≤0.05) decrease in ceruloplasmin levels (Table. 3) when compared to control rats (Group I). In ISO myocardial infarcted rats, 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 3). Upon MEGG pre treatment, the free iron concentration was decreased with a significant increase in serum iron binding capacity (P≤0.05) when compared to myocardial infarcted rats (Group: II). MEGG alone treated rats (Group: III) showed no significant changes in these parameters when compared to control rats.

MEGG at a dose of 125 and 250 mg/kg showed a protection of 82.3% and 91.1% in uric acid, 42.2% and 65.5% in ceruloplasmin, 46.5% and 74.6% in serum iron binding capacity. The protective effect of the extract in decreasing and increasing the serum enzymes and other parameters are in a dose dependent manner.
FIG 2: EFFECTS OF MEGG ON CHANGES IN SERUM ENZYME LEVELS OF RATS TREATED WITH ISO
(A) Aspartate aminotransferase, (B) Alanine aminotransferase, (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.

FIG 3: EFFECT OF MEGG ON SERUM IRON AND IRON BINDING CAPACITY
I - 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

TABLE 3: 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>Group I</td>
<td>3.4 ± 0.2</td>
<td>14.2 ± 2.2</td>
</tr>
<tr>
<td>Group II</td>
<td>6.9 ± 0.6a</td>
<td>3.3± 1.2a</td>
</tr>
<tr>
<td>Group III</td>
<td>3.5 ± 0.3</td>
<td>14.8 ± 2.1</td>
</tr>
<tr>
<td>Group IV</td>
<td>4.1 ± 0.4b</td>
<td>8.2 ± 1.2b</td>
</tr>
<tr>
<td>Group V</td>
<td>3.8 ± 0.3b</td>
<td>10.9± 1.4b</td>
</tr>
</tbody>
</table>

Values are mean ± S.D., n = 6.
*a p ≤ 0.05 versus normal control.
*b p ≤ 0.05 versus ISO control.

Tissue analysis
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 4). 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 and 250 mg/kg showed a protection of 54.3% and 63.5% in GST, 51.4% and 70.2% in GPx, 59.5 and 91.4% in GR, 77.1% and 86.7% in CAT and 59.6% and 72.5% in GSH levels. The significant increase (P≤0.05) in tissue MDA levels was observed in ISO alone treated rats (Table.4). However ISO induced elevation of MDA concentration was lowered significantly (P≤0.05) by 64.1 and 88.1% 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 4: PROTECTIVE EFFECTS OF MEGG AGAINST ISO INDUCED CHANGES IN THE CARDIAC ANTIOXIDANT STATUS**

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>GSH (nmol/mg protein)</th>
<th>GST (µmol CDNB-GSH conjugate formed/min/mg protein)</th>
<th>GR (nmol of GSSG utilized/min/mg protein)</th>
<th>GPx (nmol of GSH oxidized/min/mg protein)</th>
<th>CAT (U/mg protein)</th>
<th>MDA (nmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>9.3 ± 0.9</td>
<td>26.6 ± 1.3</td>
<td>6.2 ± 0.4</td>
<td>15.1 ± 0.8</td>
<td>12.4±1.3</td>
<td>1.5±0.4</td>
</tr>
<tr>
<td>ISO (6mg/100g ;S.C)</td>
<td>3.1± 0.5a</td>
<td>9.3 ± 0.8a</td>
<td>1.5 ± 0.1a</td>
<td>5.0 ± 0.6a</td>
<td>4.1 ±0.8a</td>
<td>8.2±3.1a</td>
</tr>
<tr>
<td>MEGG (250 mg/kg)</td>
<td>9.7± 0.9</td>
<td>27.2 ± 1.1</td>
<td>6.1 ± 0.3</td>
<td>15.3 ± 0.9</td>
<td>12.2±1.4</td>
<td>1.5±1.3</td>
</tr>
<tr>
<td>MEGG (125mg/kg) +ISO</td>
<td>6.8± 0.6b</td>
<td>18.7 ± 0.9b</td>
<td>4.3 ± 0.2b</td>
<td>10.2 ± 0.6b</td>
<td>10.5±0.9b</td>
<td>3.9±1.0b</td>
</tr>
<tr>
<td>MEGG (250mg/kg) +ISO</td>
<td>7.6± 0.7b</td>
<td>20.3 ± 1.2b</td>
<td>5.8 ± 0.3b</td>
<td>12.1 ± 0.8b</td>
<td>11.3±1.1b</td>
<td>2.3±0.5b</td>
</tr>
</tbody>
</table>

Values are mean ± S.D 

a$p ≤ 0.05$ versus normal control. 
b$p ≤ 0.05$ versus ISO control

**Effect of MEGG treatment on histopathology 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 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 4B).

Rats received only MEGG (250mg/kg) did not showed any adverse effect on myocardial histology (Fig 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 4D).

MEGG pretreated at 250mg/kg showed almost the absence of myonecrosis, edema and inflammation (Fig 4E).
FIG 4. HISTOPATHOLOGICAL CHANGES OCCURRED IN GROUPS TREATED WITH ISO AND PREVENTION BY THE TREATMENT WITH MEGG (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.

Effect of MEGG on macroscopic enzyme assay (TTC)

TTC macroscopic enzyme mapping assay of sections of heart from control and experimental (Figure. 5) is direct evidence of myocardial necrosis. Fig. 5 A showed a section of heart from control rat with viable myocardial tissue. Fig. 5B 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 (Fig.5 C) had no effect on heart tissue when compared to control group. MEGG pretreated rat (125mg/kg) administered ISO showed a moderately low infarct size (Fig.5 D) and 250mg/kg MEGG pretreated rats administered ISO showed results almost similar to that of normal rats (Fig.5 E), indicating that the prior oral administration of methanolic extract may have prevented membrane damage by ISO, there by retaining near normal.

FIG 5. MACROSCOPIC ENZYME MAPPING ASSAY (TTC TESTS) OF HEART TISSUE IN CONTROL AND EXPERIMENTALRATS

(A): Normal control, (B): ISO control (6 mg/100g s.c), (C): Methanolic extract alone (250mg/kg), (D): Methanolic extract (125mg/kg) pretreated+ ISO, (E) Methanolic extract (250mg/kg) pretreated+ ISO.

DISCUSSION: The results of the present study showed that there was significant increase in oxidative stress after myocardial ischemia 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. 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. Free radical- mediated lipid peroxidation and consequent changes in membrane permeability are the primary factors for cardiotoxicity induced by ISO 24. Oxidative stress increases cAMP levels by exhausting ATP and decreases sarcolemmal Ca\(^{2+}\) transport, resulting in intracellular calcium overload, which leads to ventricular dysfunction 3.

Erythrodiol, lupeol, epicatechin, β- sitosterol, asiatic acid, myricetin, oleanolic aldehyde, vernolic acid, chlorogenic acid and dicafeoylquinic acid are the major compounds identified in MEGG with the potential to improve the cardiac health. Erythrodiol is a triterpene, exhibits both antioxidant and antithrombotic properties 25. Sudhakar et al 26 demonstrated lupeol’s triterpene’s mode of action by a restoration of several transmembrane enzymes, total cholesterol, triglycerides and phospholipids to normal levels, preventing hypertrophic cardiac histology. Epicatechin reduces blood pressure and limits infarct size in animal models of myocardial ischemia reperfusion injury 27. β-sitosterol reduces the blood levels of cholesterol and was used in treating hypercholesterolemia 28. Asiatic acid was reported to possess a wide spectrum of biological activities including antioxidation, anti-inflammation, antitumor, antidepression, anti-Alzheimers disease, cardiovascular protection, and hepatoprotective.
effect. Myricetin could be of benefit in the prevention of atherosclerotic lesions because it was a potent inhibitor of oxidative modification of LDL by macrophages. In addition, oleanolic aldehyde, vernolic acid, dicaffeoylquinic acid and chlorogenic acid are well-known for its antioxidant activities. Oral administration of MEGG at a dose of 5 g/kg body weight did not produce significant changes in biochemical, behavior, breathing, cutaneous effects, and gastrointestinal effects in rats. There are no adverse effects observed during the administration of MEGG, indicating that the median lethal dose (LD50) is higher than 5 g/kg in rats. 5g/kg dose used in the acute toxicity study were 10 to 20 times higher than those used in our previous in vivo pharmacological study, such as antioxidant and antihepatotoxic activity of MEGG against thioacetamide induced oxidative stress in rats.

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. 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. 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. CK-MB was 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.

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. 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 transaminasases were the diagnostic indicators of MI. This hypothesis is supported by the results of our study, as we have observed a significant increase in serum activity of cardiotoxicity enzymatic indices (AST, ALT, LDH, CK-MB) after ISO treatment. We observed that pretreatment with MEGG significantly prevented the depletion of these myocardial marker enzymes following ISO administration.

Heme iron is directly related and total iron-binding capacity which is inversely related to the risk of myocardial infarction. In ISO-induced myocardial necrosis, free iron was released from heme dependent proteins like hemoglobin and myoglobin and decreases the ironbinding capacity and thus increases prostaglandin metabolism and in vivo lipid peroxidation. 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 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. 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. 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, in the present study, significantly reduced serum uric acid level compared to ISO administered rats.

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 was due to release of oxygen derived free radicals, which causes cardiac dysfunction, increased lipid peroxidation and depletion of endogenous antioxidants\(^4^2\). 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\(^4^3\).

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\(^4^4\) 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.

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\(^4^5\). The significantly\( (p \leq 0.05)\) elevated level of GR activity in the cardiac tissues shows the role of MEGG to maintain the GSH level in these tissues. GPx catalyzes the GSH dependant reduction of \(H_2O_2\) and other peroxides and protects the organism from oxidative damage\(^4^6\). The significant\( (p \leq 0.05)\) restoration of GPx activity after pre-treatment with MEGG is also due to the antioxidant activity by detoxifying the endogenous metabolic peroxides generated after ISO injury in cardiac tissues.

Catalase is responsible for breakdown of \(H_2O_2\), an important ROS, formed during the reaction catalyzed by SOD\(^4^7\). Reduced activity of catalase after exposure to ISO in the present finding could be correlated to increased generation of \(H_2O_2\). 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\(^4^8\). MDA is a major lipid peroxidation end product, increased levels resulting in irreversible damage to hearts of animals subjected to ISO-induced damage. The results from the present study indicate that treatment with MEGG inhibits ISO-induced MDA accumulation in heart. Histopathological studies and TTC assay also provide supportive evidence for biochemical analysis. Therapy of MEGG significantly improved cellular morphology in a dose dependent manner.

**CONCLUSION:** 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 erthrodial, lupeol, epicatechin, β-sitosterol, asiatic acid, myricetin, oleanolic aldehyde, vernolic acid, chlorogenic acid and dicaffeoylquinic acid may exert the antioxidant and cardioprotective activity in ISO treated groups.

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