FERONIA ELEPHANTUM MODULATES ISOPROTERENOL INDUCED MYOCARDIAL INFARCTION BY ANTIOXIDANT PATHWAY IN EXPERIMENTAL RATS

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(Received 7 November 2016) (Accepted 6 February 2017)

Abstract
The present study evaluates Feronia elephantum alcoholic fruit extract (FEAFE) against isoproterenol induced myocardial infarction (MI), since, no studies have been reported to ascertain its cardio protective activity. Rats (n=6) were divided into five groups, namely, Group I: Normal control, Group II: ISO (200mg/kg s.c). Group III, IV, & V: FE (400 mg/kg orally) and Vit E (50 IU/kg orally) respectively, administered for 21 day, by end of treatment isoproterenol was administered for two days except Group III. ECG was recorded after 21 days. Blood samples and histopathological studies of isolated heart were performed by biochemical estimations, respectively. Isoproterenol induced rats show increased ST segment, QRS complex and QT interval, besides significant increase in CK-MB, LDH, AST, ALT and Troponin-I levels. Further, they showed significant increased MDA level, decreased SOD, GSH, total protein level. Pre-treatment with FEAFE significantly restored above parameters, supported by histopathological data. Pretreatment with FEAFE modulates isoproterenol induced MI by antioxidant pathway in rats.

Keywords: Feronia elephantum, isoproterenol, myocardial infarction, ECG.

Introduction
Cardiovascular diseases (CVDs) are the major health problem of developed as well as developing countries in the world1. Myocardial infarction (MI), commonly known as heart attack, is a disease that occurs when the blood supply to a part of the heart is interrupted, causing death of heart tissue. Oxidative stress resulting from increased production of free radicals plays a major role in CVD such as myocardial infarction, ischemic heart disease, atherosclerosis, congestive heart failure, cardiomyopathy and arrhythmias. Damage to the myocardial cells arises due to the generation of free radicals and reactive oxygen species (ROS)2.

Oxidative stress in myocardium causes infarct like necrosis of cardiac muscles, which can be induced by a well known β-adrenergic agonist, isoproterenol3. The proposed mechanisms to explain the damage to cardiac myocytes induced by isoproterenol (ISO) include hypoxia due to myocardial hyperactivity, coronary hypotension, calcium overload, depletion of energy reserve and excessive production of free radicals resulting from oxidative metabolism of catecholamine. Free radical mediated peroxidation of membrane phospholipids and consequent changes in membrane permeability are the primary reasons for cardiotoxicity induced by isoproterenol4.

There is an increasing recognition of herbs which can influence the course of heart diseases and its treatment by providing an integrated structure of nutritional substances which aid in restoring and maintaining the balanced body systems5. Traditional systems of medicine like Ayurveda, Unani and Chinese system prescribe numerous herbal drugs for cardiovascular disorders; however, a number of herbal drugs are still to be evaluated pharmacologically. Therefore, it is rational to use natural resources for identifying, selecting inexpensive and safer approaches for the management of cardiovascular diseases along with the current therapy. Various plant extracts such as Withania somnifera, Ocimum sanctum and Mangiferin have been proven protective against isoproterenol induced MI by virtue of their antioxidant properties6-8. Feronia elephantum (FE) (family Rutaceae), commonly known as wood apple9, is an indigenous plant of India; reported to possess multiple therapeutic properties like antulcer10, antimicrobial11, hepatoprotective12, antidiabetic13 and antitumor14 activities. The fruits possess Vitamin C and flavonoids which may be responsible for its antioxidant activity15.

However, the effect of FE fruit on isoproterenol induced myocardial infarction has not been reported. Hence taking
into consideration the reported activities and the various active chemical constituents, it is proposed in the present study that FE fruit may have beneficial effect against isoproterenol induced myocardial infarction.

**MATERIALS AND METHODS**

**Preparations of Feronia elephantum alcoholic fruit extract (FEAFE)**

A specimen sample of fresh fruits of FE was identified, authenticated and preserved in the herbarium section with the voucher No.RLSI/BOT/AUG-2 for future reference.

The fruits of FE were dried under the shade and powdered. The powder was subjected to extraction in Soxhlet extractor and was defatted with petroleum ether (40-60), later extracted with methanol (40-60) and extraction was continued for 12 cycles until the solvent in the thimble was clear. The solvent was evaporated by using rotary evaporator and extract obtained was stored under refrigerating condition. The extract was suspended in 1% Tween - 80 and used for oral administration.

**Animals**

Healthy male Wistar rats (150-200 g) were used for this study and housed in standard laboratory condition at room temperature along with 12 h light/dark cycle. The animals were provided with standard pelleted diet and water ad libitum. After seven days of acclimatization period, they were randomly selected for different experimental groups. The study was reviewed and approved by the Institutional Animal Ethics Committee for conducting this study (IAEC Reg. No.: 627/02/a/CPCSEA). Isoproterenol and Ellman’s reagent were purchased from Sigma-Aldrich whereas thiobarbituric acid and trichloroacetic acid were purchased from Himedia Ltd.

**Acute oral toxicity test**

The acute oral toxicity was carried out as per the guidelines set by OECD Guidelines 423. Rats weighing 150-200 gm (8-12 weeks) were used for toxicity study. FE fruit extract of 3000 µg/kg (p.o.) was selected as a initial dose and 3 animals used each at step. LD₅₀ of FEAFE was carried out and 400 mg/kg dose were selected as an effective dose.

At the end of the treatment period i.e. on 21st day, for induction of myocardial infarction isoproterenol (200 mg/kg s.c) was dissolved in normal saline and administered in two consecutive doses at an interval of 24 hours. The animals were anaesthetized using pentobarbital sodium 30-40 mg/kg i.p. The lead II ECGs of all animals were recorded using Biopac Student Lab PRO 3.7 software (Model No. MP35)

**Estimation of marker enzymes in serum**

The serum was used for determination of myocardial infarction biomarker such as alanine transaminase (ALT) and aspartate transaminase (AST) by using commercially available diagnostic kit (ERBA Diagnostics, Germany), lactate dehydrogenase (LDH) and creatinine kinase-MB fraction (Crest Biosystem, Goa, India) and troponin I (Biomed Industries India).

**Collection of Heart tissue sample**

Animals were dissected, heart was isolated and washed immediately with ice-cold saline. Later, the homogenate was prepared in 0.1N tris HCl buffer (pH 7.4); later, the homogenate was centrifuged and supernatant was collected which was used for the assay of malondialdehyde (MDA), reduced glutathione (GSH), super oxide dismutase (SOD) and total protein.

**Estimation of Malonaldehyde (MDA)**

MDA level was estimated by the method of Ohkawa M et al. Briefly 100 µL of sample was pipetted out into test tubes and 200 µL of 8.1 % SDS solution was added. To this, 1.5 mL of 20 % acetic acid and 1.5 mL of 0.8 % aqueous solution of TBA was added to each tube. Final volume was made up to 4mL with biological grade

**Experimental study design**

Rats were divided into five groups having six members in each. The alcoholic extract of FE fruit was dissolved in distilled water with 1% Tween 80. Rats of all groups were orally treated except isoproterenol induced group. Group I (normal control) received normal saline, Group II (Disease control) received normal saline and isoproterenol (200mg/kg) s.c. for last two consecutive days. Group III (Drug control) received alcoholic extract of FE fruit (400 mg/kg). Group IV (Drug treated) received alcoholic extract of FE fruit (400 mg/kg) and isoproterenol (200mg/kg) s.c. for last two consecutive days. Group V- Standard treated: - received Vit.E (50 IU/kg) and isoproterenol (200 mg/kg) s.c. for last two consecutive days. At the end of treatment, 24 hours after second dose of isoproterenol the ECGs of rats were recorded and animals were sacrificed. Blood was collected and the serum was separated immediately by cold centrifugation (at 3000RPM for 10 min) for biochemical estimation. The hearts were dissected out for enzyme estimation and histological examination.

The animals were anaesthetized using pentobarbital sodium 30-40 mg/kg i.p. The lead II ECGs of all animals were recorded using Biopac Student Lab PRO 3.7 software (Model No. MP35)
water. The tubes were heated at 95 - 100°C in boiling water bath for one hour. Further, they were centrifuged at 4000 rpm for 10 min and supernatant was collected and optical density was read at 532 nm using UV-Visible spectrophotometer17.

**Estimation of glutathione reduced (GSH)**

GSH level was measured by the method of Ellaman GL18. GSH level was expressed in umoles/mg of tissue. Briefly, to the 2.5 mL of reaction buffer and 50 µL of Ellamn’s reagent, 250 µL of brain homogenate was added. Mixed well and incubated, absorbance was read at 412 nm within 15 min using UV-visible spectrophotometer.

**Estimation of superoxide dismutase (SOD)**

SOD level was measured by the method of Anuradha N et al.19. Briefly to the 5 µL of heart homogenate was added 2.9 mL of Tris buffer (0.05 m) and 0.1 mL of Pyrogallol. Later, the absorbance was measured at 420 nm in UV-visible spectrophotometer.

**Estimation of total protein**

Total protein was estimated by the method of Lowry OH et al.20. Different dilutions of BSA solutions are prepared by mixing stock BSA solution (1 mg/ mL) and water in the test tube. The final volume in each of the test tubes is 5 mL. The BSA range is 0.05 to 1 mg/mL. From these different dilutions, 0.2 mL protein solution was pipette out to different test tubes and 2 mL of alkaline copper sulphate reagent was added and mixed well; later incubated at room temperature for 10 min. Further, 0.2 mL of reagent Folini Ciocalteau solution was added to each tube and incubated for 30 min. By using colorimeter, the optical density was measured at 740 nm. Absorbance was plotted against protein concentration to get a standard calibration curve. Concentration of sample was determined by using standard curve.

**Histopathological studies**

A portion of the heart was fixed in formalin (10%) and subjected to histopathology studies. The section of the heart was processed and embedded in paraffin wax. Sections of about 4-6 µm were made and stained with hematoxylin and eosin and observed under 10X magnification.

**Statistical analysis**

Values were expressed as Mean ± SEM. The data were analyzed by one-way ANOVA followed by Dunnet’s Multiple Comparison Test. P<0.05 was considered significant.

**RESULTS**

Acute toxicity studies showed no mortality at a dose of 2000 mg/kg, during period of 14 days. This helps to predict that it does not contain any type of toxicity and it is safe.

**Effect of FEAFE on isoproterenol induced changes in the ECG parameters.**

The administration of ISO causes ST segment elevation and significant (P<.001) increase in time for QRS complex and QT interval. Pretreatment with FE fruit extract significantly (P<0.05) reduced the time of QRS complex and QT interval when compared with ISO treated group. Similar results were shown by pretreatment with vitamin E. The ECG data of the experimental animals, including the QRS peak, QT interval are presented in Table I.

**Table I: Effect of Feronia elephantum fruit extract on isoproterenol induced changes in the ECG parameters.**

<table>
<thead>
<tr>
<th>Group</th>
<th>QRS (Time in sec.)</th>
<th>QT Interval (Time in sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>0.04167±0.0010</td>
<td>0.05167±0.0010</td>
</tr>
<tr>
<td>ISO treated</td>
<td>0.05417±0.00083 a*</td>
<td>0.0650±0.0012 a*</td>
</tr>
<tr>
<td>FE</td>
<td>0.0425±0.0011</td>
<td>0.0525±0.0011</td>
</tr>
<tr>
<td>FE + ISO</td>
<td>0.0475±0.0021 b^</td>
<td>0.05917±0.0015 b^</td>
</tr>
<tr>
<td>Vitamin E + ISO</td>
<td>0.0450±0.0018 b*</td>
<td>0.05417±0.0015 b*</td>
</tr>
</tbody>
</table>

All values are expresses as mean±SEM for six animals in each. a comparison made with Group I. b comparison made with Group II. * statistically significant at P< 0.001 **. # statistically significant at P<0.01 **. ^ statistically significant at P<0.05**.

**Effect of FEFE on serum markers in isoproterenol induced myocardial infarction.**

The ISO induced group rats showed significant elevation in serum biomarkers such as AST, ALT, LDH and CK-MB as compared to control group. FE fruit extract pretreatment followed by ISO significantly (P<0.001) reduced the levels of these biomarker when compared to isoproterenol induced MI in rats. Vitamin E pretreatment followed by ISO showed significant decrease in levels of serum biomarkers as compared to ISO treated group. (Table II)

**Effect of FEFE on Troponin I in isoproterenol induced myocardial infarction.**

The ISO induced MI group showed the presence of Troponin I in serum as compared to normal group. The
pre-treatment of FEAFE and Vit. E treated group showed the absence of Troponin I in serum as compared to ISO induced group. (Table II)

**Effect of FEFE on MDA, GSH, SOD and Total protein in isoproterenol induced myocardial infarction.**

The ISO treated group showed significant increase in MDA level and decreased GSH, SOD and total protein levels as compared to normal group. Pre-treatment of FEFE and VitE followed by ISO treated in both the groups showed significant decrease in MDA level and increased level of GSH, SOD and total protein as compared to ISO induced MI group (Table III).

**Histopathological study**

The cardiac sections of the ISO control group revealed degenerative changes in the muscle fiber, showing a coagulative necrosis characterized by more homogenous eosinophilic cytoplasm. The nuclei of myofibril revealed pyknotic nucleus. Interstitial edema was present in the connective tissue spaces. Pretreatment with FEAFE showed a protective effect with normal myofibrillar structures with striations. Similarly pretreatment with vitamin E showed protective effect. (Fig. 1)

**DISCUSSION**

Myocardial ischemic injury, damage of cardiomyocytes, is due to inadequate coronary blood supply and

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**Table II: Effect of *Feronia elephantum* fruit extract on serum markers in isoproterenol induced myocardial infarction.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum marker (Mean ± SEM)</th>
<th>Serum Troponin-I</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALT</td>
<td>AST</td>
</tr>
<tr>
<td>Group I (normal)</td>
<td>94.67±5.823</td>
<td>353.5±9.49</td>
</tr>
<tr>
<td>Group II (iso)</td>
<td>153.3±5.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>475.8±8.45&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group III (FE)</td>
<td>93.33±4.31</td>
<td>354.3±5.69</td>
</tr>
<tr>
<td>Group IV (iso + FE)</td>
<td>110.0±4.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>376.0±6.43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group V (iso + vit. E)</td>
<td>102.0±6.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>363.3±5.25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SEM for six animals in each group.

<sup>a</sup> comparison made with Group I
<sup>b</sup> comparison made with Group II
<sup>*</sup> Statistically significant at P<0.001
<sup>**</sup> Statistically significant at P<0.01
<sup>***</sup> Statistically significant at P<0.05
<sup>‡</sup> Statistically significant at P<0.05

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**Table III: Effect of *Feronia elephantum* fruit extract on MDA, GSH, SOD and Total protein in isoproterenol induced myocardial infarction.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Tissue Parameters (Mean ± SEM)</th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>MDA (µg/g wet tissue)</td>
<td>GSH (µg/g wet tissue)</td>
</tr>
<tr>
<td>Group I (normal)</td>
<td>25.52±0.3219</td>
<td>973.7±3.65</td>
</tr>
<tr>
<td>Group II (iso)</td>
<td>35.67±0.3748&lt;sup&gt;b&lt;/sup&gt;</td>
<td>551.0±5.11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group III (FE)</td>
<td>26.00±0.3983</td>
<td>981.8±2.98</td>
</tr>
<tr>
<td>Group IV (iso+FE)</td>
<td>30.88±0.3728&lt;sup&gt;b&lt;/sup&gt;</td>
<td>893.7±4.25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group V (iso+vit.E)</td>
<td>28.10±0.2966&lt;sup&gt;b&lt;/sup&gt;</td>
<td>939.7±3.62&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SEM for six animals in each group.

<sup>a</sup> comparison made with Group I
<sup>b</sup> comparison made with Group II
<sup>‡</sup> Statistically significant at P<0.05
Fig. 1: Effect of *Feronia elephantum* fruit extract on isoproterenol induced changes in the histopathology of rat heart.

Iso-proterenol, a sympathomimetic β-adrenergic agonist, causes severe stress results in infarct called necrosis of cardiac smooth muscles. High doses of ISO leads to cardio toxic effects acts by mechanisms such as functional hypoxia, ischemia, coronary insufficiency, metabolic anomalies, deprivation of high energy phosphate store, accumulation of intracellular Ca$^{2+}$, changes in electrolyte content as well as oxidative stress$^{22}$. In the animal experiment model, ISO induced abnormal changes in ECG, alterations represents damage to integrity of myocardial cells and function of heart by elevation of ST segment, prolongation of QT segment and attenuation of PR, QRS and RR segments$^{23}$. Hence, in this study ISO was used as standard model to evaluate cardiac dysfunction.

In the present study, administration of isoproterenol (200 mg/kg s.c.) in male Wistar rats showed significant increase in MDA levels, which indicates generation of cytotoxic free radicals and rise in lipid peroxidation.

Significant decrease in level of endogenous antioxidant viz, SOD, GSH as compared to normal saline treated group is observed. The isoproterenol induced myocardial damage rats showed a marked increased in level of serum biomarkers such as ALT, AST, LDH, CK-MB and Troponin-I. Further electrocardiographic changes and ultra structural changes in isoproterenol treated rat confirmed the injured state of the myocardium.

Pretreatment with methanolic extract of FE fruit at a dose of 400 mg/kg in isoproterenol induced myocardial damage animals restores the ultra structural and electrocardiographic changes in rats. Further, the levels of serum biomarkers viz, MDA (marker of lipid peroxidation) were significantly decreased in treatment group whereas the levels of SOD, GSH (endogenous antioxidant) and total protein were significantly increased when compared to isoproterenol induced myocardial infarcted rats (group II) indicating its antioxidant, anti-lipid peroxidation and cardio protective potential activity.

Vitamin E standard antioxidant treated animal shows similar result as that of obtained from extract treated animals in isoproterenol induced myocardial damage.

The results obtained from the present study shows that therapeutic efficacy of extract may be due to its antioxidant, anti-lipid peroxidation and free radical scavenging property. Effect of reactive oxygen species can be abolished by the antioxidant drugs. FE has been already reported to have hepatoprotective$^{24}$ properties by virtue of its antioxidant constituents, so the present effect seems to be due to the presence of vitamin C and flavonoids as active principles.

In conclusion, the cardio protective effect of FE is probably due to its antioxidant, anti-lipid peroxidation and free radical scavenging activity. Hence, FE modulates isoproterenol induced myocardial infarction by antioxidant pathway in rats. Further investigation is needed to identify the lead molecules responsible for the observed cardio-protective activity.
ACKNOWLEDGEMENTS

Authors are thankful to Dr. A. D. Taranalli Principal and Dr. V. P. Rasal, Head Department of Pharmacology, KLEU College of Pharmacy, Belagavi for supporting and providing all facilities to conduct this study. Authors are also thankful to Prof. R. S. Goudar for verifying and authenticating specimen of Feronia elephantum fruits and Sri Venkateswara Enterprises, Bengaluru, for providing rats to conduct this study.

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