CARDIOPROTECTIVE EFFECT OF NARINGIN AGAINST DOXORUBICIN INDUCED CARDIOMYOPATHY IN RATS

V. Madhava Reddy Papasani*, B. Hanumantharayappa, A. Annapurna

Department of Pharmacology, A.U College of Pharmaceutical Sciences, Andhra University, Visakhapatnam-530 003, Andhra Pradesh, India

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ABSTRACT

The objective of present study is to evaluate the cardioprotective activity of Naringin against doxorubicin induced cardiomyopathy in male albino rats. Naringin is a flavanone glycoside which has the ability to act against oxidative stress, and increase the levels of enzymes that counteract oxidative stress. Rats were divided into 4 groups of 6 animals in each and treated for 8 weeks with naringin against doxorubicin. Doxorubicin was administered i.p. for a cumulative dose of 24 mg/kg body weight over a period of eight weeks in eight consecutive doses. Naringin 100 & 200 mg/kg dose were started one week prior to Doxorubicin, and was continued with daily dose of 100 & 200 mg/kg body weight. Serum samples and heart homogenates were used to estimate Troponin I, Creatine Kinase, LDH, SGPT, SGOT, LDH, SOD, CAT, GSH, MDA, Iron and Calcium. The results were expressed as the mean ± S.E.M. The results obtained were analyzed using One way ANOVA followed by Tukeys post hoc test. Doxorubicin treated rats showed significant increase in serum and oxidative biomarkers indicating cardiomyopathy. Naringin treatment significantly decreased serum biomarkers levels Troponin I, Creatine Kinase, SGPT, SGOT, Iron and Calcium. It also decreased oxidative biomarkers LDH and MDA and whereas it increased SOD and CAT levels. In conclusion, the present study indicates that Naringin offered dose dependent significant cardiac protection and healing effect against doxorubicin induced Cardiomyopathy in rats. The possible mechanisms involved in the cardioprotection of Naringin might be antioxidant and free radical scavenging properties.

*Corresponding author

V. Madhava reddy papasani

Department of Pharmacology,
A.U College of Pharmaceutical Sciences,
Andhra University, Visakhapatnam-530 003,
Andhra Pradesh, India,
madhavjournals@gmail.com,
09591117880

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INTRODUCTION

Cardiomyopathies are heterogeneous disease conditions of the myocardium [1] that are often characterized by cardiac dysfunction, causing it to lose its cardiac efficiency in pumping blood to the body [2]. Cardiomyopathy can result from different stimuli. Cardiomyopathy encompasses three main types, namely hypertrophic, restrictive and dilated Cardiomyopathy [3]. In humans, cardiomyopathies represent a major cause of morbidity and mortality in both children and adults and are a frequent reason for cardiac transplantation [4]. Cardiomyopathies either confined to the heart or are part of generalized systemic disorders, frequently causing cardiovascular death or progressive heart failure related disability [5].

It has been predicted that by 2020, two major ailments like cardiac diseases and stroke will be the leading cause for both mortality and disability around the globe [6]. The current epidemiology studies survey reveals that the mortality rate would increase to 20 million a year and expected to rise to 24 million by the end of 2030 [7]. The sedentary life style on an average increases 6 times the chances of occurrence of MI, there is even still high chances of occurrence in women, diabetes patients, geriatrics, dementia patients and also patients with history of heart failure [8].

Doxorubcin (DOX) an cytotoxic agent has been implicated for chronic treatment for several human malignancies either in combination or alone. One of the hindrance for utilization of doxorubicin for prolonged treatment as a potential chemotherapeutic agent is its Cardiomyopathy [9]. Several mechanism have been proposed to understand its toxicity. One of the important proposed mechanism is toxicity by oxidative stress through generation of free radicals [10]. Free radicals cause damage to DNA, proteins, membrane lipids and carbohydrates [11].

Naringin [the 7-β-neohesperidoside of narigenin (4',5,7-trihydroxiflavananone)] is found in citrus plants and is most abundant in Citrus paradisi species. Naringin is extracted from grapefruit (Citrus paradisi) powder with methanol [12]. Naringin is a bioflavonoid (flavanone glycoside) which gives bitter taste to grape fruit juice. In the body it is metabolized by the liver to flavanone naringenin. Naringenin and one more aglycone hesperetin both are aglyconic portions of naringin and hesperidin respectively. Several studies have already been done for various therapeutic beneficial effects of naringenin (aglycone portion of naringin) like cardioprotective, [13] cholesterol lowering, nephroprotective, antiaging, anti-alzheimer's, anti-hyperglycemic, anti-osteoporotic and gastroprotective etc [14, 15].

The purpose of present research was to evaluate the cardioprotective activity of Naringin by estimation of both serum and oxidative biomarkers of cardiomyopathy which includes, Troponin I, Creatine Kinase, LDH, SGPT, SGOT, LDH, SOD, CAT, GSH, MDA, Iron and Calcium against doxorubicin induced cardiomyopathy in rats.

MATERIALS AND METHODS

Drugs and Chemicals

Estimation of biochemical markers were done by using commercial kits. The kits for estimation of serum levels of Creatine Kinase-Myocardial band (CK-MB), Creatine Kinase-n Acetylcysteine (CK-NAC), Lactate Dehydrogenase (LDH) and Cardiac Troponin-I (cTnI), were procured from Erba chemicals and Life Diagnostics. The kits for Serum Glutamic-Pyruvic Transaminase (SGOT), Serum Glutamic Oxaloacetic Transaminase (SGPT) and Calcium kit were procured from Excel Diagnostics Pvt. Ltd. Iron estimation kit was procured from CREST BIOSYSTEMS. Doxorubicin hydrochloride injection (ADRIM 50mg/25ml) was purchased from local hospital pharmacy. Remaining all other chemicals and reagents used were of analytical grade.

Animals

Male albino rats weighing 250-270g were used. Animals were maintained under standard laboratory conditions at 25 ± 2°C, relative humidity 50 ± 15% and normal photoperiod (12 h dark/ 12 h light). After seven days of acclimatization period, they were randomly selected into experimental groups. They were given free access to food and water ad libitum.

Groups

Rats receiving various injection regimens were divided into four groups, with six rats assigned per each group. Naringin was dissolved in water and administered to rats orally using an intragastric tube daily [16]. Dose of Naringin 100, 200 mg/kg body weight were selected. All the rats in normal control (Group I) group were given eight intraperitoneal injections for eight weeks with normal saline. All the rats in the diseased control (Group II) group were given eight intraperitoneal injections of Doxorubicin at a dose of 3 mg/kg body weights over a eight week period. In (Group III & IV) Naringin 100 & 200 mg/kg dose were started one week prior to Doxorubicin, and was continued with daily dose of 100 & 200 mg/kg body weight orally over a period of eight weeks with eight consecutive doses of Doxorubicin (3 mg/kg) respectively. Finally (Group V) eight intraperitoneal injections of Doxorubicin were given at a dose of 3 mg/kg body weights over a eight weeks period and then treated with Naringin 200 mg/kg for 8 weeks.

Acute Toxicity Studies

The experimental protocol has been approved by the Institutional Animal Ethics Committee (Regd. No. 516/01/A/CPCSEA). Acute toxicity studies were carried out as per OECD guidelines by employing the Up and Down method prior to evaluation of cardioprotective activity.

Dose Selection and experimental induction of myocardial toxicity or cardiac stress : Dose of Doxorubicin was chosen based on preliminary and previous studies [17,18]. In this study commercially available Doxorubicin (ADRIM) was used for the experimental induction of myocardial toxicity or cardiac stress. Doxorubicin each dose containing 3 mg/kg body weight, was administered intra peritoneally, [19] for a cumulative dose of 24 mg/kg body weight over a period of eight weeks in eight consecutive doses. About 40% of mortality was observed during the study.
Assessment of Cardioprotective activity

Serum Analysis

Thirty-six hours after the treatment, under light ether anesthesia, orbital blood samples were obtained using heparinized micro capillaries for the estimation of biomarkers. The collected blood was allowed to stand for 45 minutes. The clot that was formed was disturbed by using a glass rod and was then centrifuged at 4000 rpm for 15 min. The serum was separated and used for the analysis.

Isolation of Heart: The rats were sacrificed by decapitation using a guillotine. The thoracic cavity was opened by midline incision, and the hearts were excised. Hearts were immediately washed with ice cold 0.1 M sodium phosphate buffer (pH 7.4) and placed on blotting paper for a few seconds to absorb excess washing solution and were used for the estimation of oxidative parameters.

Biochemical Parameters

Serum samples and heart homogenates were used to estimate Troponin I, Creatine kinase, LDH, SGPT, SGOT, LDH, Superoxide dismutase (SOD), Catalase (CAT), Reduced Glutathione (GSH), Malondialdehyde MDA, Iron and Calcium.

Statistical Analysis

The results were expressed as the mean ± S.E.M. The results obtained were analyzed using One way ANOVA followed by Tukeys post hoc test. Data obtained was computed for statistical analysis using software Graph Pad Prism. Differences between the data were considered significant at p<0.001.

RESULTS

Results of serum biomarkers and cardiac tissue biomarkers in Doxorubicin-induced cardiomyopathy in rats were presented in Table 1 & 2.

Table 1 shows that the treatment with Naringin 100 mg/kg and 200 mg/kg significantly decreased serum biomarkers levels Troponin I, CK-MB, CK-NAC, SGPT, SGOT, LDH, Calcium and Iron, when compared to Doxorubicin treated (diseased control) rats. The cardioprotective action was dose dependent, in terms of reducing elevated levels of serum biomarkers. Naringin 200 mg/kg action was more significant as compared to Naringin 100 mg/kg.

Table 1: Cardioprotective effect of Naringin (100 mg/kg and 200 mg/kg) on Serum biomarkers in Doxorubicin-induced cardiomyopathy in rats.

<table>
<thead>
<tr>
<th>Serum Biomarkers</th>
<th>NORMAL CONTROL (Mean± SEM)</th>
<th>DISEASE CONTROL (DOX 3 mg/kg) (Mean± SEM)</th>
<th>NARINGIN 100 mg/kg (Mean± SEM)</th>
<th>NARINGIN 200 mg/kg (Mean± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Troponin-I (ng/ml)</td>
<td>0.1583±0.0040</td>
<td>1.017±0.086&lt;sup&gt;$$&lt;/sup&gt;**</td>
<td>0.352±0.103&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.165±0.01&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>Creatine Kinase-MB (CK-MB) (IU/L)</td>
<td>654.8±9.607</td>
<td>1042±25.85&lt;sup&gt;$$&lt;/sup&gt;**</td>
<td>898.3±3.54&lt;sup&gt;0.04&lt;/sup&gt;**</td>
<td>669.3±14.46&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>Creatine Kinase (IU/L)</td>
<td>328.2±22.58</td>
<td>666.5±28.47&lt;sup&gt;$$&lt;/sup&gt;**</td>
<td>432.3±23.41&lt;sup&gt;***&lt;/sup&gt;</td>
<td>329.5±17.59&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>SGPT (IU/L)</td>
<td>47.67±4.573</td>
<td>95.17±5.160&lt;sup&gt;$$&lt;/sup&gt;**</td>
<td>61.17±4.854&lt;sup&gt;***&lt;/sup&gt;</td>
<td>48.00±4.633&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>SGOT (IU/L)</td>
<td>120.7±19.89</td>
<td>267.2±17.04&lt;sup&gt;$$&lt;/sup&gt;**</td>
<td>139.3±8.421&lt;sup&gt;***&lt;/sup&gt;</td>
<td>128.7±7.517&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lactate Dehydrogenase (LDH) (IU/L)</td>
<td>961.8±33.12</td>
<td>1386±82.30&lt;sup&gt;$$&lt;/sup&gt;**</td>
<td>1133±17.99&lt;sup&gt;***&lt;/sup&gt;</td>
<td>1001±8.262&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum Calcium(Ca&lt;sup&gt;2+&lt;/sup&gt;) (mg%)</td>
<td>9.100±0.2145</td>
<td>18.97±1.594&lt;sup&gt;$$&lt;/sup&gt;**</td>
<td>11.42±0.3763&lt;sup&gt;***&lt;/sup&gt;</td>
<td>9.183±0.2971&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum Iron (Fe&lt;sup&gt;3+&lt;/sup&gt;) (µg/dl)</td>
<td>88.50±2.918</td>
<td>202.2±3.341&lt;sup&gt;$$&lt;/sup&gt;**</td>
<td>119.0±2.921&lt;sup&gt;***&lt;/sup&gt;</td>
<td>89.00±2.436&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 2 shows that the treatment with Naringin 100 & 200 mg/kg significantly elevated the levels of oxidative stress biomarkers SOD, CAT, and GSH, when compared to Doxorubicin treated (diseased control) rats. Whereas the levels of lipid peroxidation product MDA decreased significantly when compared to Doxorubicin treated (diseased control) rats. The cardioprotective action offered was dose dependent, as the Naringin 200 mg/kg produced superior results than 100 mg/kg.
DISCUSSION

The troponin complex is located on the thin filament of striated muscle, along with actin and tropomyosin. Cardiac cell

Doxorubicin administration induced a significant elevation of serum cardiac cell isoenzyme (CK-MB) level, which is considered as an important marker of early and late cardiac cell injury during Doxorubicin therapy [22], which may be due to the inhibition of nucleic acid and protein synthesis [23]. Oral pretreatment with Naringin decreased the levels of serum creatine kinase-MB; this effect might be due to its major metabolite naringenin, which is the absorbable form. The ability of Naringenin to scavenge free radicals is an important property in its protection against oxidative stress [24].

Creatine Kinase (CK) is predominantly found in the cytoplasm; within myocytes, the enzyme occurs in close association with the sarcoplasmatic reticulum, mitochondria and myofibrils. Doxorubicin-induced cardiomyopathy significantly increases serum CK levels in rats due to the excessive production of free radicals and lipid peroxides that might have caused leakage of cytosolic enzymes and cell membrane damage. The serum creatine kinase (CK) levels in Doxorubicin treated rats were significantly increased compared with normal rats.

The increase in LDH level in serum and extracellular fluid suggests an increased leakage of this enzyme from mitochondria as a result of toxicity induced by treatment with Doxorubicin. This index has been recently used in other studies to test for cardiomyopathy [27]. Results shown in the above Table-1 shows that Doxorubicin-induced cardiomyopathy significantly increased the level of serum lactate dehydrogenase (LDH) in the rats as compared with the normal control group. Naringin because of its antioxidant effect decreases elevated LDH and maintains the levels of non-enzymic antioxidants [28].

Doxorubicin-induced cardiomyopathy is accompanied by an increase in intracellular calcium levels. Dysregulation of intracellular calcium concentrations leads to irregular rhythm of heart beat [29]. Imbalance of calcium ion concentrations in myocardial tissue occurs as a result of myocardial damage by ROS generation. Animal and clinical studies of morphological changes during the early stages of Doxorubicin-induced cardiomyopathy have suggested that the calcium regulation may be the early target of Doxorubicin-induced cardiomyopathy [30]. Pretreatment with Naringin increased the activity of Na+/K+ ATPase and decreased the activities of Ca2+, Mg2+ and ATPases. This can be due to the ability of Naringin to protect the ‘SH’ groups from the oxidative damage through the inhibition of peroxidation of lipids in the membrane, and likely to cause stabilization of membrane [31].

Iron (Fe) is a crucial biogenenic element vital for all living cells, where it is essential for oxidation-reduction catalysis and bioenergetics. Intracellular iron, free or ferritin bound may promote ROS formation in Doxorubicin exposed cells which include the two major pathways 1) Fenton and Haber-Weiss reactions and 2) Formation of Doxorubicin-Fe complexes [32]. Pretreatment with Naringin orally, decreased the levels of serum iron and increased the plasma iron binding capacity. It can be attributed to the chelating ability of Naringin, which significantly reduced the oxidative threat leading to reduction of pathological changes and restoration of normal physiological functions. Moreover, the increased plasma iron binding could have prevented hemolysis and iron catalyzed lipid peroxidation. This effect might be due to the free radical scavenging and antioxidant property of Naringin [31].

Doxorubicin-induced oxidative stress results from an imbalance between reactive oxygen species production and endogenous antioxidant defense mechanisms [33]. Doxorubicin-induced cardiomyopathy cause a decrease in the level of antioxidant enzyme superoxide dismutase of the heart Naringin induced elevation in antioxidant enzyme SOD may be due to the positive regulation of

Table 2: Cardioprotective effect of Naringin (100 mg/kg and 200 mg/kg) on Cardiac tissue biomarkers in Doxorubicin-induced cardiomyopathy in rats.

<table>
<thead>
<tr>
<th>Cardiac tissue Biomarkers</th>
<th>NORMAL CONTROL (Mean± SEM)</th>
<th>DISEASE CONTROL (DOX 3 mg/kg) (Mean± SEM)</th>
<th>NARINGIN 100 mg/kg (Mean± SEM)</th>
<th>NARINGIN 200 mg/kg (Mean± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide Dismutase (SOD) (U/mg protein)</td>
<td>44.97±1.800</td>
<td>24.91±1.225</td>
<td>34.57±0.9803</td>
<td>41.26±1.036</td>
</tr>
<tr>
<td>Catalase µmoles of H2O2 decomposed/mg protein/min</td>
<td>56.70±1.533</td>
<td>34.73±1.217</td>
<td>44.58±0.8813</td>
<td>52.05±1.406</td>
</tr>
<tr>
<td>Reduced Glutathione (µg/g wet tissue)</td>
<td>105.6±1.433</td>
<td>29.02±0.3137</td>
<td>66.62±0.8946</td>
<td>91.12±0.6397</td>
</tr>
<tr>
<td>Malondialdehyde (MDA) (nmol/ gm tissue)</td>
<td>19.08 ±1.088</td>
<td>90.46 ±2.404</td>
<td>47.38±1.267</td>
<td>23.45±0.6787</td>
</tr>
</tbody>
</table>

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gene expression of SOD enzyme. Naringin elevated serum SOD levels constantly works in conjugation with other H$_2$O$_2$ neutralizing enzymes [34]. The antioxidant enzymes viz. SOD and CAT of heart upon Doxorubicin treatment has shown a significant decrease.

However, the extent of lipid peroxidation; malondialdehyde increases in diseased control, when compared with the normal group [35, 36]. Naringin has the ability to scavenge free radicals, chelate metals and antioxidant properties and also offers some protection against mutagenesis. The antioxidant effects of Naringin have been shown to be similar to that of GSH and furthermore, it is reported to inhibit the hydrogen peroxide induced lipid peroxidation. Naringin increased myocardial GSH levels and conversion of H$_2$O$_2$ to water thereby reducing the oxidative attack of O$_2$. and H$_2$O$_2$ in the cells [37].

Naringin at a dose of 200 mg/kg has offered more degree of cardioprotection when compared to Naringin at a dose of 100 mg/kg, which could be due to free radical scavenging and anti-lipid peroxidation activity of Naringin causing stabilization of cardiac cell membranes from the peroxidative damage thus preventing the injury to myocardial tissues against doxorubicin toxicity in a dose dependent manner.

CONCLUSION

In Conclusion, the present study indicates that Naringin treatment significantly reduced the free radical production, generation of lipid peroxides and leakage of cytosolic enzymes characterized by decreased levels of biomarkers such as creatine kinase (CK), creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH), troponin-I (Tnl), serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), serum calcium ($Ca^{2+}$) and serum iron ($Fe^{3+}$) activities in animal subjected to Doxorubicin-induced cardiomyopathy. These findings might be helpful to understand the beneficial effects of Naringin against myocardial injury although further study is needed to confirm its mechanism. Further studies on these flavanone can bring better drugs to the society for the treatment of cardiomyopathies.

REFERENCES


