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Protective Effect of d-Limonene against Adriamycin Induced Cardiac Injury in Rats – A Preliminary and Biochemical Study

Indumathi Selvanathan[1*], Sasivarnam Tamilarasi[2], Chengalvarayan Subramani Pramila[3] and Thiruvengadam Devaki[4]

Department of Biochemistry, University of Madras, Chennai, India.
* Corresponding Author E-mail: indu13medbio@gmail.com

Abstract

Adriamycin (ADR) is the conventional anthracycline antineoplastic drugs, treated against various solid tumors and malignancies. Its adverse effect was formation of oxidative stress and reactive oxygen species leads to cardiotoxicity due to cumulative dose of Adriamycin in chemotherapy. D-Limonene is an oil nutrient, occurs naturally in lemon and orange peels and it have various pharmaceutical properties like anti-oxidant, anti-inflammatory and anti-cancer etc. In this present study cardio protective effect of D-Limonene on cardiac marker enzymes, anti-oxidant enzymes was investigated in Adriamycin induced rats. Animals induced with Adriamycin with 2.5 mg/kg/body weight ip) leads to myocardial damage that was manifested by the increase in the level of cardiac marker enzymes like (LDH, CPK, GOT, GPT) enzymes and significant reduction in the level of anti-oxidant enzymes like (SOD, CAT, GP_X, GST). Administration of D-limonene (100mg/kg/body weight) orally for two weeks prior before a period of injection of Adriamycin shows significant reduction in marker enzymes to normal range and increases in anti-oxidant activity. This results indicates, pre-co-treatment with D-limonene have effectively protect against Adriamycin induced cardiotoxicity. Hence this present study was framed to detect cardioprotective role of d-limonene (d-Limo) against Adriamycin induced cardiac damage in albino male rats.

Keywords: Adriamycin, cardiotoxicity, D-Limonene, antioxidant system, cardio-protective.

Introduction

Adriamycin is an anthracycline antibiotic, well known an antineoplastic agents against several tumors such as breast cancer, lung cancer, acute leukemias etc (Blum *et al.,* 1974). (Fig 1a) Adriamycin mediates cardiotoxicity due to cumulative effect of chemotheraphy. Possible mechanism for adriamycin mediating cardioxicity was not yet understood where as formation of free radicals and oxidative stress was considered to be the reason behind cardiotoxicity. Generally, free radicals causes damages in membrane stability, lipid peroxidation and changes in extracellular matrix which leads to cardiac dysfunction and results in cardiotoxicity (Kumar *et al.,* 2007; Olson RD *et al.,* 1990; Pacher *et al.,* 2002; Z.Zhang *et al.,* 2014; P.L.Crowell & M.N.Gould 1994)

d-Limonene is a flavoring and fragrance monocyclic monoterpene in many citrus fruit oil like lemon and orange (T.Shimada *et al.,* 2002; JECFA 1993) (fig 1b). It is known as a hypocholestremic compound by dissolving cholesterol containing gall stones and in relief of gastro esophageal reflux (Igimi *et al.,* 1976; Sun 2007) (fig 1b). Free radical scavenging activity of many fruits, vegetables and medicinal plants has been found. Whereas d-limonene possess free radical scavenging property in certain cancer cell due to presence of natural monoterpen. Studies have shown that monoterpenes have various pharmacological properties including antifungal, antibacterial, antioxidant, anticancer, and anti-spasmodic (Garcia *et al.,* 2008; Kato *et al.,* 1990; Singh *et al.,* 2010; Karkabounas *et al.,* 2006; Magalhães *et al.,* 1998). Besides the activities described above, monoterpenes also produce significant effects on the cardiovascular system, promoting, among other actions, vasorelaxation, decreased heart rate, and hypotension (Peixoto-Neves *et al.,* 2010; Bastos *et al.,* 2010; Aydin *et al.,* 2007; Magalhães *et al.,* 2008). Thus, these monoterpenes can be useful as agents for prevention and or treatment of cardiovascular diseases (CVD). So, the objective of this present study is to identify the cardio protective effect of d-limonene against Adriamycin induced cardiotoxicity in rats.

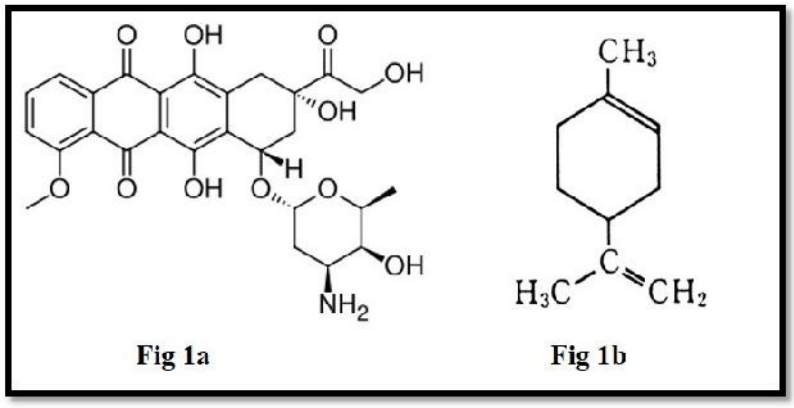

Fig 1: a) Doxorubicin b) d-Limonene

Materials and Methods

Experimental Animals

Adult male albino rats of Wistar strain weighing about 140-160 g were obtained from the Tamilnadu Veterinary and Animal Science University, Chennai, India. They were acclimatized to animal house conditions, fed commercial pelleted rat chow (Hindustan Lever Ltd., Bangalore, India) and had free access to water. This study was carried out as per the guidelines of the animal ethical committee of our institution. (IAEC NO.35/03/2014).

Chemicals

Adriamycin and D-limonene were purchased from Sigma chemicals Co. (St.Louis,MO,USA). All other chemicals used were of the highest quality and analytical grade.

Experimental protocol

The animals were divided into four groups of six rats. Each groups are as follows.

Group I: Animals were treated with distilled water orally by intragastric gavage.

Group II: Adriamycin was given intraperitonally (2.5mg/kg/body weight) in six equal injection over a period of two weeks for a cumulative dose of 15mg/kg/bw (Siveski-Iliskovic.N *et al*., 1994; Siveski-Iliskovic N, Hill M *et al*., 1995).

Group III: d-limonene was administrated orally (100mg/kg/body weight) for the period of two week

Group IV (Pre-co-treatment):d-limonene was administrated orally(100mg/kg/body weight) over a period of four weeks, two weeks prior to Adriamycin administrated and two weeks along with Adriamycin administrated.

At the end of the experimental protocol,the animals were sacrificed by cervical decapitation. The blood samples were collected and the serum was separated for the estimation of LDH, CPK, AST and ALT. The heart tissue was excised immediately, rinsed in ice-cold saline and fixed in 10% formalin for further histological studies. Other part of cardiac tissue were homogenized in Tris-HCL, pH 7.4 (0.1M) buffer, and used for the analysis of biochemical parameters.

Biochemical Parameters

Protein estimation

Protein was estimated by Lowry *et al.,*. Tissue homogenate or serum sample of 1.0ml was added with 0.9ml of water and 4.5ml of alkaline copper reagent and kept at room temperature for 10 minutes. 0.5ml of folins reagent was finally added and the colour developed was calculated at 640nm after 20 minutes .Proteins level was expressed as mg/g of tissue or mg/dl of serum.

Assay of Cardiac marker enzymes

Activities of cardiac marker enzymes like LDH (King 1965), (CPK) (Okinaka S, Kumogai H *et al.,* 1961), glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT) (Bergmeyer 1974).

Measurement of Total Reduced Glutathione (GSH)

The activity was followed by the method of Moron *et al*., (1979) (Moran I, Lescoat G *et al*., 1979). DTNB [5,5dithiobis (2-nitrobenzoic acid] used as reagent for this method. The range of standard glutathione concentration is (10-50µg) and the colour formation was read at 412 nm against blank reagent. The amount of glutathione expressed as nmoles/g heart tissue.

Measurement of Catalase (CAT)

Catalase activity was estimated by the method of Takahara *et al*., (1960) (Takahara, S, Hamilton *et al*., 1960). To 0.2ml of tissue homogenate add 50mM phosphate buffer, and the reaction was carried out by the addition of 30mM hydrogen peroxide solution of 1.0ml.Changes in the absorbance was measured at 240nm. The activity of enzymes was calculated as µmoles of H_2O_2 consumed/min/mg Protein.

Measurement of Superoxide dismutase (SOD)

The activity of SOD was assessed by the method of Misra and fridovich (1972) Homogenated cardiac tissue of 0.1ml was added with 0.75ml of ethanol + 0.15 ml of chloroform and centrifuged for 15 minutes at 2500 rpm. To .ml of supernatant, 0.5ml of EDTA and 0.1ml of 0.1M carbonate buffer were added. The reaction was initiated by the addition of 0.5ml of epinephrine. The absorbance was measured in shimadzu UV spectrophotometer at 480nm. SOD activity was expressed as 50% inhibition of epinephrine auto oxidation.

Measurement of Glutathione-s-transferase (GST)

The assay of GST was estimated by the method of Habig *et al.,* (1974). To 0.1ml of tissue homogenate add 0.1ml of 3mM phosphate buffer, followed by adding 0.1ml of water and 0.1ml of CDNB (1-chloro-2,4-dinitrobenzene) incubate at 37˚C for 15 minutes. The final volume was added with 0.1ml of GSH and optical density was read at 340nm. The activity of Glutathione-s-transferase was expressed as units/min/mg protein.

Measurement of GPx

This assay method was described by Rotruct *et al.,* (1973). In this method, reaction mixture contain 0.2ml of 0.8 m EDTA, 0.1ml of 10mM sodium azide. 0.1ml of H_2O_2, 0.2ml of reduced glutathione 0.4ml of 0.4m phosphate buffer and 0.2ml of tissue homogenate and kept for incubation for 10 minutes at 37˚C. To this solution add 10% TCA of 0.5ml to arrest the reaction and the tubes were centrifuged at 2000 rmp. For 10 minutes, two milliliter of supernatant solution was added with 3.0ml of disodium hydrogen phosphate and 0.04 % DTNB of 1.0ml. The absorbance of colour developed was read at 420nm immediately. GPx activity was expressed as µmoles of glutathione oxidized/min/mg protein.

Histological studies

Histological evaluation was performed on a portion of the heart tissue after fixation with 10% formalin, embedded in paraffin wax, sectioned to 3-5 µm thickness and was stained with hematoxylin and eosin to assess the pathological changes.

Statistical analysis

All the grouped data were evaluated with SPSS/17 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by LSD (least significant difference test. *P* values of less than 0.05 were considered to indicate statistical significance. All these results were expressed as mean ±S.D for six animals in each group.

Results

Cardioprotective effect of d-limo in cardiac marker enzymes in heart of control and experimental group of rats:

Table 1 represents the levels of LDH,CPK,AST and ALT in the heart of control and experimental groups of rats. LDH, CPK, AST and ALT levels were significantly increased ($p<0.05$) in Group II ADR induced animals when compared with Group I control animals. However, Group IV (D-limo + ADR) rats showed a significant reduction in myocardial marker enzymes when compared to Group II ADR induced animals. No significant changes were observed between GroupI control and Group III D-limo alone treated animals.

Table 1: Effect of d-limo on the activities of cardiac marker enzymes in the heart of control and experimental groups of rats.

Groups	LDH	CPK	AST	ALT
Control	12.33±0.08	26.17±0.40	7.52±0.11	13.27±0.07
ADR	13.09±0.40[a]	30.12±0.19[a]	12.11±0.02[a]	15.10±0.32[a]
d-limo alone	11.45±0.05	26.15±0.41	7.56±0.10	12.93±0.12
d-limo + ADR	12.14±0.07[b]	26.13±0.38[b]	7.58±0.09[b]	13.02±0.45[b]

Results are expressed as mean ± S.D for six rats in each group. Statistical significance at $p< 0.05$ compared with [a]group 1 and[b]group 2 based on LSD.

Units: CPK: µmol of phosphorus liberated/h/mg protein; LDH, AST and ALT: µmol of pyruvate liberated/h/mg protein.

Cardioprotective effect of d-limo on anti-oxidant enzymes in the heart of control and experimental group of rats:

Table 2 shows the level of SOD, CAT, GPX and GST in the cardiac tissue of control and experimental groups of rats. Activity of anti-oxidant enzymes were significantly ($p<0.05$) decreased in Group II ADR induced animals when compared with Group I control animals. However, treatment with d-limo (Group IV) showed significant increase ($p<0.05$) in the activities of antioxidant enzymes against Group II ADR induced animals. There is no significant difference between Group I and Group III animals.

Table 2: Effect of d-limonene on anti-oxidant enzymes in the heart of control and experimental group of rats.

Groups	SOD	CAT	GPx	GST
Control	8.60±0.34	86.14±0.95	1.38±0.54	0.27±0.03
Adriamycin	4.39 ±0.19[a]	60.97±0.34[a]	1.12±0.72[a]	0.12±0.32[a]
d-limo alone	9.24±0.20	88.74±0.77	1.47±0.09	0.23±0.36
d-limo + ADR	8.02±0.10[b]	85.29±0.29[b]	1.36±0.53[b]	0.28±0.39[b]

Results are expressed as mean± S.D for six rats in each group. Statistical significance at $p< 0.05$ compared with [a]group 1 and[b]group 2 based on LSD.

Units: SOD in units/mg protein, CAT in µmol of H_2O_2 decomposed/min/mg protein, GPx in µmol of GSH utilized/min/mg protein and GST in µmole of CDNB-GSH conjugate formed/min/mg protein.

Cardioprotective effect of pathological histology

The histology of the heart tissue (Figure-2) or the transverse section of control (Group 1) rats shows normal morphological myocardial muscle cells in the myocardial bundle (a). Adriamycin treated (Group II) longitudinal section (b and d) shows mononuclear infiltrations and congested blood vessels (in arrow). Also the transverse section (c) shows the increasing the number of microcytolysis (m), necrosis of muscle fiber (f) and congestion (e) shows in arrow. Treatment with d-limonene (h,i,j) (Group IV) revealed myocardium looks normally like control but with congested blood vessels (i) as well as modurate level of intestinal inflammatory cell infiltration (j) and edema, shows in arrow. (Group III) Reveals D-limonene alone treated group shows normal morphological features same as control group in (g). (Magnification 40X).

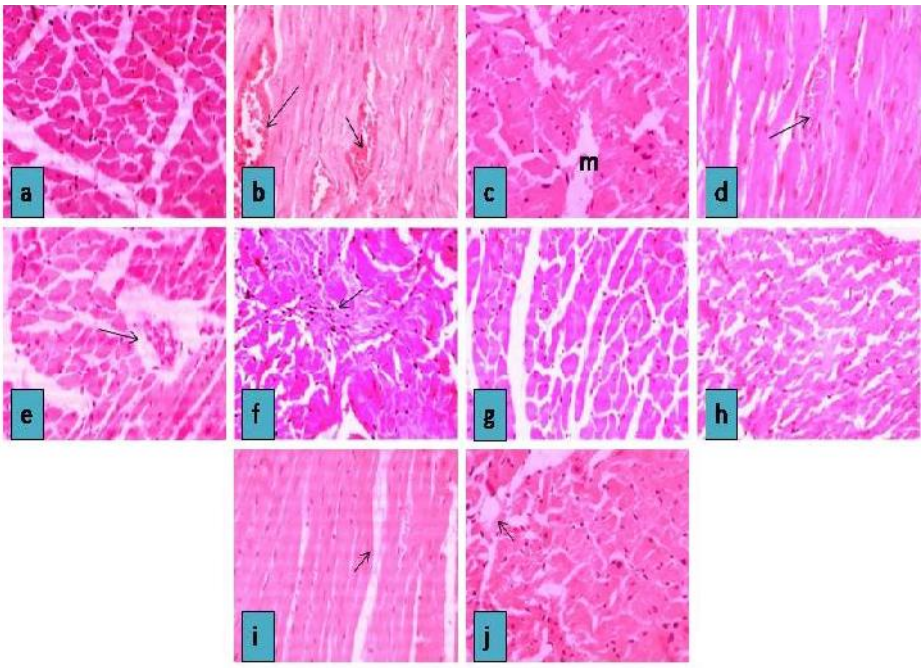

Figure 2: Histopathological changes of rat heart (a) Control, (b,c,d,e, and f) ADR (disease control), (g) d-Limo alone, (h,i,j) ADR+D-Limo. Original magnification: 40X. (m) microcytolysis.

Discussion

Adriamycin is an anthracycline, it is very effective and widely used as tumor drug for various cancers like acute leukemia, lymphoma and a number of solid human tumors. Unfortunately cumulative dose of Adriamycin is limited by cardiomyopathy and congestive heart failure as soon as after beginning of cancer therapy (Fadillioglu *et al.*, 2004; Patil and Balaraman 2004). Adriamycin induced cardiotoxicity is due to the over production of free radicals and it is mainly characterized by tachycardia, hypotension and arrhythmias (Garcia *et al.*, 2008; Kato *et al.*, 1990). In this study Adriamycin toxicity caused an elevation in the non-functional enzymes like LDH, CPK, AST, ALT and depletion in the level of anti-oxidant enzymes in cardiac tissue like SOD, CAT, GPX and GPT, it is already, in accordance with previous studies (Singh *et al.*, 2010).

Increased level of LDH, CPK, GOT and GPT is well established cardiac marker for myocardial function. Further studies reported that, these enzymes are released from the heart into blood vessels and leads to increases in the concentration. The result of rats pre-treated with d-limonene showed marked reduction in the level of biochemical marker enzymes towards normal by decreasing oxidatitve stress as evident by increasing levels of antioxidant (Karkabounas *et al.*, 2006). This is due to the presence of ingredient carvone or α-terpinol (antioxidant moiety) in d-limonene (Magalhães *et al.*, 1998) Thus could have anti-inflammatory and cardioprotective activity.

There are several mechanism postulated for Adriamycin cardiotoxicity (Peixoto-Neves *et al.*, 2010), among them disturbed mitochondrial metabolism formation of free radicals is the direct toxic effect for Adriamycin induced cardiotoxicity in muscles. Some of the reports shown that the reasons for Adriamycin induced cardiotoxicity is due to the formation of free radicals (Bastos *et al.*, 2010). Reactive oxygen species (ROS) generated in live cells are super oxide anions and its derivatives, which are highly reactive and damage hydroxyl radicals as per oxidation of cell membrane lipid layers (Aydin *et al.*, 2007). A cumulative dose of Adriamycin (15 mg/kgb.Wt) significantly increases the level of functional enzymes like LDH, CPK, AST, ALT and decrease in the level of anti-oxidant like SOD, CAT, GPX and GSH in cardiac tissue when compared to control groups. But d-limonene along with Adriamycin treated group shows marked elevation in the level of anti-oxidant compared to that of induced groups. As correlated in previous studies,

oxidative stress and production of lipid peroxidation by Adriamycin leads to cardiomyopathy. Studies on histopathology shows microcytolysis, dilated blood vessels, necrosis in muscle fiber with some congestion in Adriamycin induced rats. Previous studies have also been demonstrated similar obstruction on acute cardiotoxicity (Magalhães *et al.*, 2008).

Conclusion

The present study emphasizes the cardio protective property of d-Limo as a potential antioxidant compound in mitigating ADR induced oxidative stress by enhancing intracellular antioxidant defense in rats. d-Limo significantly reduced cardiac enzyme markers in pre-treated animals compared with the induced group. Hence this report suggests the use of this natural terpene as a potential antioxidant drug for clinical manifestations.

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