



Research Article

PROSPECTIVE FUNCTION AND CARDIOPREVENTIVE EFFECT OF *d*-LIMONENE AGAINST ADRIAMYCIN-INDUCED MYOCARDIAL DYSFUNCTION IN ALBINO RATS

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ABSTRACT

Adriamycin (ADR) an effective cytotoxic anthracycline antibiotic used for treating many solid tumours shows limiting significant in cardiac dysfunction. Partial side effect of adriamycin was due to oxidative stress and produce free radicals generate cardiotoxicity. *d*-Limonene a powerful terpenoid found naturally in all citrus fruits which avert oxidative damage and protects from cardiac damage induced by ADR. The object of the present study is to evaluate preventive role of *d*-limonene against ADR-mediated myocardial dysfunction. *d*-Limonene (100mg/kg.b.w) was given orally for 2 weeks prior to ADR (15mg/kg.b.w). Serum enzymes, such as SGOT, SGPT, CPK, CK-MB, troponin and C-reactive protein, were evaluated as markers for myocardial damage. Cardiac markers, GSH and GSSH, were also determined. Significant amount of *d*-limonene administration down regulates the activities of marker enzymes gradually. Histopathological PAS staining were complete to assess glycoconjugates assertion during cardiac damage. Hence, the results suggest *d*-limonene protects ADR-induced oxidative damages and boost up anti-oxidant to fight against cardiotoxicity.

Keywords: ADR, *d*-Limonene, Oxidative damage, Glycoconjugates, Cardiotoxicity

INTRODUCTION

Adriamycin, an important anti-neoplastic anthracycline chemotherapeutic drug, used in various human tumours like breast cancer, ovarian cancers and hematological malignancies¹. Among various anticancer drugs, adriamycin hampered by cumulative and irreversible dose-dependent toxicities like acute and chronic cardio toxicity², with most effective cardiac failure. Exact pathogenesis of myocardial damages has not understood properly. According to previous evidence, adriamycin induced oxidative stress by generating free radicals such as ROS and nitric oxide. However, free radicals accumulation induces lipid peroxidation³⁻⁵, calcium overloading, abnormalities in iron metabolism, DNA damage, dysfunction in mitochondria and finally cell apoptosis⁶⁻⁸. Thus, adriamycin promotes several abnormalities due to cumulative fixation and induces cardiotoxicity. *d*-Limonene vibrant monocyclic monoterpene found in many citrus fruits, vegetables, beverages, used as essential flavoring agents in various factories and household products⁹⁻¹². Moreover, *d*-limonene inhibits several physical stress and psychological stress¹¹.

Previous reports suggest, *d*-limonene emerging lipid peroxidation prevent free radical damages¹³ and well execute anti-oxidant levels¹⁴. *d*-Limonene, identified to be very specific monoterpene against free radicals because it contain high potential anti-oxidant which reduces oxidative stress. Thus, the present study is to focus cardiopreventive action of *d*-limonene against adriamycin-induced cardiotoxicity by enhancing anti-oxidant potential with nutritional event.



Figure 1: (a) Citrus fruits and (b) *d*-limonene

MATERIALS AND METHODS

Drugs

Doxorubicin (Dox) or adriamycin (ADR), *d*-limonene and chemicals for biochemical assays were purchased from Sigma-Aldrich, Mumbai, India. Sterile distilled water was used for biochemical assay. SGOT, SGPT, CPK, CK-MB, troponin and C-reactive protein assay kit were obtained from Teco diagnostics, USA and Aspen laborites Pvt. Ltd, India. All reagents and chemicals used were highly analytical grade.

Animal's Ethic Statement

Animals used for the experimental procedure were approved according to ethical norms and guidelines set by animal ethical committee of our institute with approval number of IAEC NO.35/03/2014

Animals

Adult albino Wistar strain of about (140-160g) was obtained from Tamilnadu Veterinary and Animal Science University, Chennai, India. All weighted animals were randomly placed according to groups in polypropylene cages with husk bedding. Animals were housed under standard temperature of (25±2°C) and humidity of about 30-70%. 12 hrs light and dark cycles was followed with standard chow feed and water ad libitum

Experimental Protocol

The animals of 4 groups with six rats were randomly divided according to the following experimental groups.

Group I: Control group (0.9% NaCl) by intra gastric gavage.

Group II: (2.5mg/kg body weight) of adriamycin for period of two weeks for cumulative dose of 15mg/kg body weight¹⁵.

Group III: Drug treatment (100mg/kg body weight) of *d*-limonene was administrated orally for four weeks.

Group IV: *d*-limonene of (100mg/kg body weight) was administrated for period of four weeks, two weeks prior and two weeks along with adriamycin administrated.

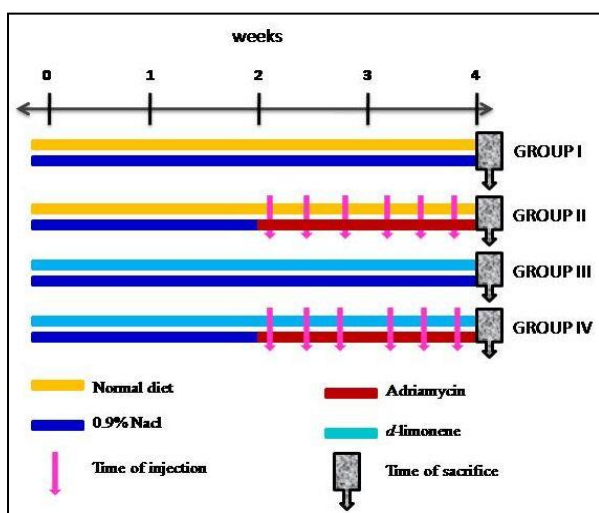


Figure 2: Chart demonstration of experimental design

Preparation of Serum Sample

At the end of experimental period all animals were anesthetized by using ketamine (90mg/kg) and xylazine (10mg/kg), sacrificed by cervical decapitation. Blood was collected by cardiac puncture method and centrifuged at 4000 rpm for 15 min. Serum was separated for analysis of various biochemical parameters like serum CPK, SGOT, SGPT, CK-MB, troponin and C-reactive protein by using diagnosing kit according to the manufactures instructions.

Preparation of Tissue Sample

Animal's heart were separated by cervical decapitation and washed with phosphate buffer saline (0.05M, pH 7.4). Heart were minced and chopped into small pieces and homogenized by using Tris 0.1M HCl buffer (pH 7.4). Homogenate was centrifuged by using Remi cooling centrifuge for 30 min at 8,000 rpm. The supernatant was separated and used for the estimation of various enzymes like GSH and GSSH.

Histological Examination

Heart tissue was fixed with 10% formalin and entrenched using paraffin. Sectioned for 5µm thickness using microtome and stained with PAS (Periodic acid Schiff-base) for routine histopathological evaluation of glycoconjugates in stained heart tissues. Sectioned heart tissues were immersed in 0.1% periodic acid for 15 min and again immerse the slides in Schiff's reagents for 40 min. The stained slides were engrossed in running water for 10 min and allow the slides for counter stain with hematoxylin. Dehydrate the slides in graded ethanol and mounted in DPX medium.

Statistical Data

All the grouped data were evaluated with SPSS/17 software. One way ANOVA used as hypothesis testing followed by Tukey's post hoc test. P<0.05 were considered as statistical significance. All these results were expressed as mean±S.D for six animals in each group.

RESULTS AND DISCUSSION

Studies in Diagnostic Marker Enzymes

Biomarker is one of the substances widely used as an indicator of cardiac biologic state. Cardiac biomarker is important substance that released into the blood stream when heart is damaged. Due to myocardial damage, cardiac enzymes located in the cytosolic region of cardiomyocytes generally released into the blood stream, and finally elevated in serum levels. These elevated enzymes are released varying amount by dying cardiomyocytes and becomes one of the useful tool for the detection of myocardial injury leads to cardiac injury. To define cardiac markers, aid to diagnosis, prognosis and stratification of patients with initial cardiovascular diseases, some of the initial serum markers include CPK, SGOT and SGPT are well established markers for cardiac functions. Due to myocardial damages, the level of these enzymes are released from the cardiomyocytes and own elevated amount in blood stream, thus increasing in concentration of serum¹⁶.

The level of diagnosis marker enzymes in serum is noted in Table 1. These enzyme markers (CPK, SGOT and SGPT) in ADR-administrated rats show significant increase in serum enzymes accompanied by their concomitant reduction in heart homogenate confirms myocardial damage and it was reported earlier¹⁷.

ADR-mediated heart tissue injury was indicated by elevation in level of serum and heart tissue marker enzymes such as CPK, SGOT and SGPT. *d*-Limonene treated (Group IV) at a dose of 100 mg/kg body weight produced (P<0.05) significantly reduced in activity of cardiac markers in serum (CPK, SGOT, SGPT) when compared to that of ADR-induced cardiac damage. *d*-Limonene inhibit ADR when compared to serum of ADR treated rats. It was widely noted that, ADR generally induced free radicals generation and triggers peroxidation of membranes finally disturb cardiomyocytes, which partially increases the level of CPK in serum was also reported¹⁸. *d*-Limonene pre-co-treated, rates to control the CPK release which finally resulted in either complete reversal or considerable recovery of serum enzyme activities.

The other cardiac enzyme for the myocardial damage were found to be CK-MB. The enzyme creatinine kinase partially named as creatinine phosphokinase exists in 3 forms as CK-MM, CK-MB and CK-BB. Assay of activity of CK-MB in ADR-induced cardiomyopathy in serum was executed in (Figure

3A) these forms of isoenzyme are found in cytosolic region of cardiomyocytes which facilitates high energy membrane phosphatases in and out of the mitochondria. These enzymes are distributed in many tissues, but the percentage of fraction was found to be higher in heart compared to other tissue. A sequential change in CK-MB includes elevation above the normal level within 4 hours, and peak reduction was about in 16-24 h¹⁹.

CK and CK-MB are well known establish diagnostic marker for myocardial function²⁰. During myocytic injury, these enzymes were leaked into the serum, and it was easily detected in blood samples. The results also shows, the administration of ADR in rats significantly increase ($P < 0.05$) in activity of CK-MB in serum when compared with control group of rats, reports also supported in previous studies^{21,22}. The mechanism for the elevation of CK-MB in serum was due to the formation of free radicals might causes sequential cardiac dys-functions, and also causes ventricular remodeling, myocyte degeneration etc. The cardiac injury performs to determine the effect of *d*-limonene on ADR-induced cardiotoxicity. Administration of *d*-limonene to ADR-treated rats shows significant decreases ($P < 0.05$) in level of CK-MB to confirm the protective effect in Group IV rats when compared to (Group II) animals, and there was no significant changes observed in level of cardiac marker on drug alone (Group III) when compared to that of control.

Troponins are regulatory proteins, highly found in skeletal and cardiac muscles, these proteins control the calcium mediating interaction between actin and myosin. Cardiac troponin (CTn) exists in 3 isoforms as troponin C, troponin I and troponin T. Among these proteins, CTnT was specific for heart muscle. Troponin T anchors troponin complex to tropomyosin. In serum concentrate cardiac troponin T was found to be highly sensitive marker for myocardial damages. Hence, troponin T is one of the sensitive marker for ADR²³⁻²⁶.

Our results shows, the level of Troponin-T indicated in (Figure 3B) was significantly elevated in ADR-induced rats when compared to control rats. The significance was about ($P < 0.05$) level. Troponin-T attaches troponin-tropomyosin complex to form thin filament of actin that form free pool in the cytosolic region of cardiomyocytes²⁷. ADR induces transient leakage in the cytosolic pool and result in loss of cell membrane integrity leads to prolonged leakages due to degeneration of myofilaments in ADR-induced irreversible cell injury. *d*-Limonene treated rats show, the level of troponin-T were significantly reduced when compared to ADR administrated groups. This was due to prevention of *d*-limonene in cardiac muscle from balancing the troponin marker in cytosolic pool and regulates loss of cell membrane integrity.

Among the list of cardiac diagnostic marker, C-reactive protein (CRP) is a non-specific inflammatory response marker during myocardial damages. It exhibits an exquisitely sensitive marker for cardiac death.

(Figure 3C) presents the level of CRP is experimental group of rats. ADR administrated rats found to be increase in CRP level when compared to control (Group I). Several studies demonstrated that elevated level of CRP associated with

systemic blunted endothelial vasodilator function shows systemic inflammatory response²⁸. ADR administrated rats show inflammatory cells accumulate in cardiac cells leads to elevation in CRP level in serum²⁹. *d*-Limonene treated (Group IV) rats shows markedly release of CRP levels and this diminished effect correlates the evidence for the prevention of cardiac cell by *d*-limonene as anti-inflammatory property.

ENZYMATIC ANTIOXIDANT PROFILE

Figure 4 demonstrated the levels of enzymic thiols in cardiac tissue of control and experimental groups. (Group II) shows remarkable decline about ($P < 0.05$) in the level of GSH in ADR administrated myocardial damage when compared to normal control (Group I) rats. Glutathione redox cycle is one of the important intracellular antioxidant complex. Generally, GSH is present in higher concentration in all animal cells. The key function of GSH is to serve as a reductant of toxic peroxides, and also keep the enzymes in active state by preventing the conversion of -SH (sulfydaryl) group. GSH is one of the essential compounds for maintaining cell integrity and its depletion causes damage to membrane lipids or damage to macromolecules. ADR administrated rats showed depletion of cardiac GSH and it may result in the formation of oxygen free radicals. The GSSG/GSH status is an important parameter that indicates the formation of reaction oxygen species leading to oxidative damages. In the present study significant reduction in GSH levels was highly noted in ADR administrated group. It has been reported that low cardiac glutathione level is a high risk of forming ADR-induced cardiomyopathy³⁰. Our present study also reported that treatment with *d*-limonene increased GSH levels which is generally regenerated from GSSG by enzyme glutathione reductase nearly depends on the NADPH availability and prevented cellular damage by scavenging reactive oxygen species.

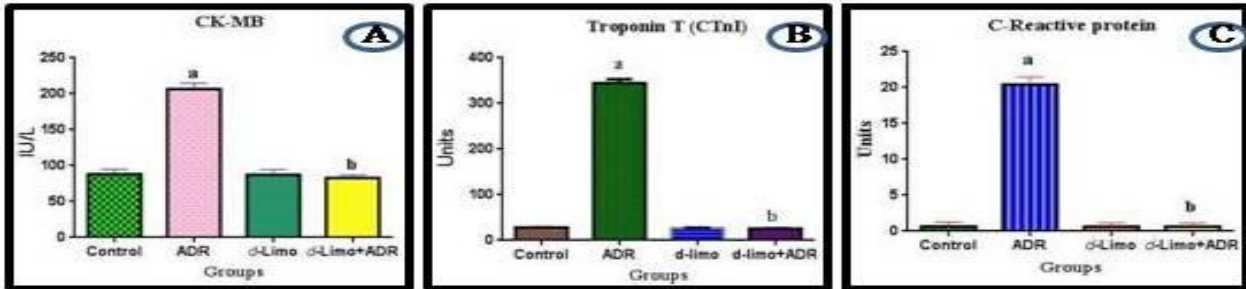
PAS STAINING FOR GLYCOCONJUGATES DEGRADATION

The Periodic acid-Schiff (PAS) stain is to identify glycoconjugates expression in cardiac tissue. Glycoconjugates are specific markers for identifying oxidative damage caused by reactive oxygen species resulting in lipid peroxidation and cardiac damage (Figure 5) shows PAS staining of control and experimental group of rats. The cardiac extracellular matrix consists of hetero-polysaccharides (glycosamino glycans), glycoproteins (heparin sulphate and chondroitin sulphate proteoglycans), microfibrillar proteins (fibrillin and fibulin) and elastin. The extracellular matrix is involved in the maintenance of structural integrity³¹. Control animals show the normal level of glycoconjugates whereas, ADR-induced (Group II) rats showed loss of glycoconjugates due to lipid peroxidation. Formation of reactive oxygen species results in overproduction of lipid peroxidation causing damage of the extracellular components disturbing the membrane integrity. Pre-co-treatment with *d*-limonene (Group IV) shows lesser amount of extracellular damage and maintains the membrane bounded glycoconjugates. Hence, *d*-limonene stabilizing effect on membrane bounded glycoconjugates may increase cardiac membrane stability and protect myocytes from damage.

Table 1: Effect of *d*-limonene on the activity of marker enzymes in serum of control and experimental groups of rats

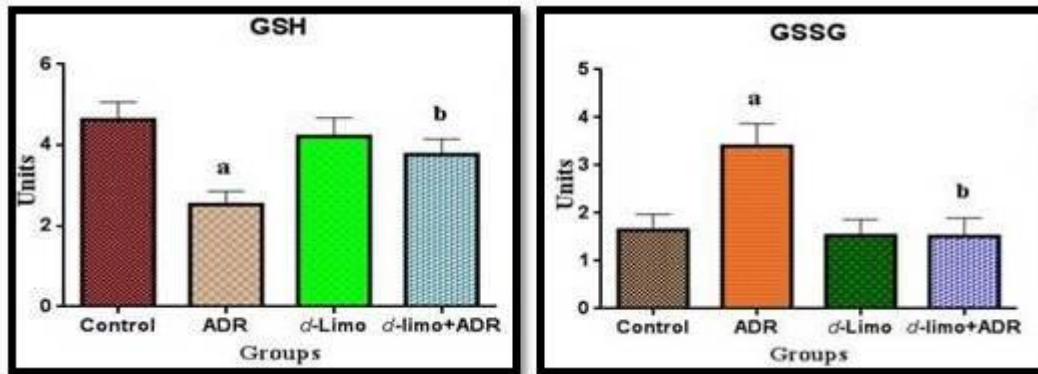
S.no	Groups	SGOT	SGPT	CPK
1.	Control	76.52±1.85	20.07±1.43	79.69±3.56
2.	ADR	150.61±1.87 ^a	40.30±2.86 ^a	186.60±2.36 ^a
3.	<i>d</i> -limo	75.27±3.84	21.21±2.13	79.27±2.33
4.	<i>d</i> -limo+ADR	82.48±3.07 ^b	25.61±2.19 ^b	88.02±2.86 ^b

Results are expressed as mean ± SD (n=6). One way ANOVA followed by Tukey's Post hoc test P<0.05. Compared with (a) Group I (Control) and (b) Group II (ADR)



Results are expressed as mean ± SD (n=6). One way ANOVA followed by Tukey's post hoc test P<0.05. Compared with (a) Group I (Control) and (b) Group II (ADR).

Figure 3: Effect of *d*-limonene on the activity of serum marker CK-MB, Troponin and C-reactive protein of control and experimental group of rats



Results are expressed as mean ± SD (n=6). One way ANOVA followed by Tukey's post hoc test P<0.05. Compared with (a) Group I (Control) and (b) Group II (ADR).

Figure 4: Effect of *d*-limonene on the level of cellular thiols in heart of control and experimental group of rats

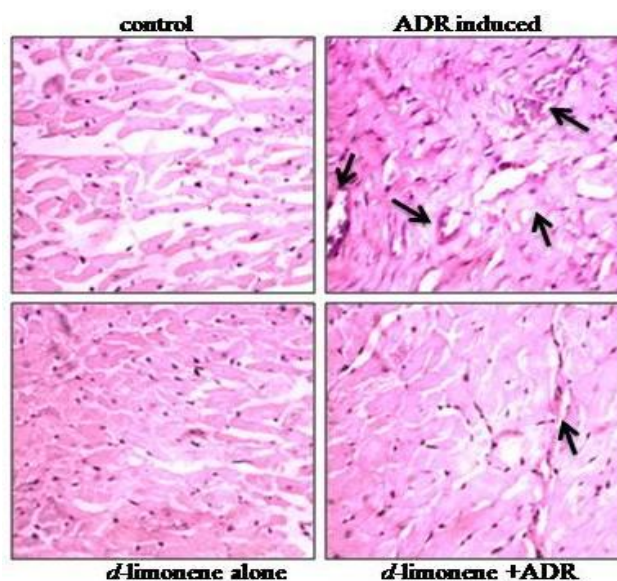


Figure 5: Evaluation of glycoconjugates in heart of control and experimental animals (40X)

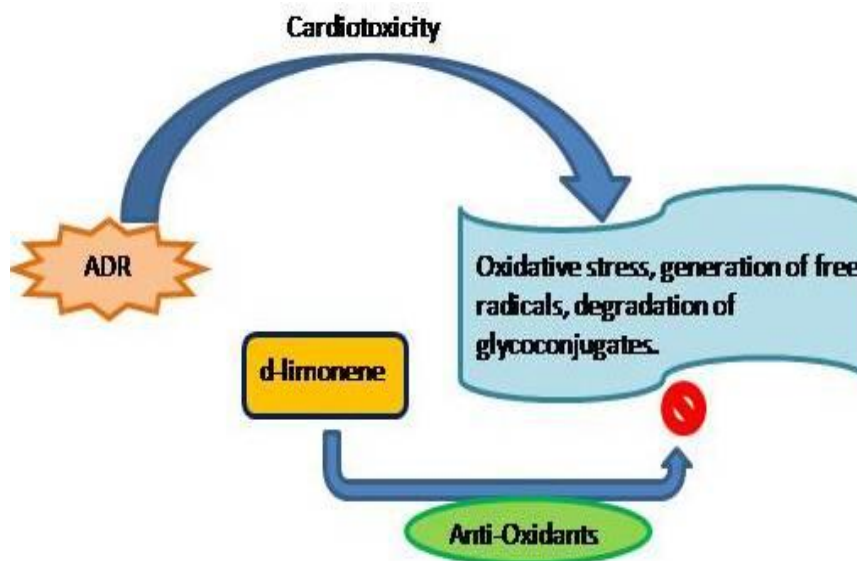


Figure 6: Overall schematic representation

CONCLUSION

Overall result of this study shows *d*-limonene an enriched anti-oxidant probably scavenges free radicals by protecting lethal cardiac side effects caused by adriamycin in clinical chemotherapy. Whereas, the literal mechanism for adriamycin-induced myocardial damage remain to be unclarified. Though, *d*-limonene could be an efficient drug to reduce ADR-mediated cardiotoxicity in future perception.

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