Protective Effect of \( d \)-Limonene Experimentally Induced Cardiotoxicity in Wistar Albino Rats

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ABSTRACT

Cyclophosphamide (CP) is one of the most widely used alkylating antineoplastic agents that damages normal cells while killing cancerous cells. The consumption of CP in treating cancer patients is limited due to its severe toxicity induced mainly by cardiac toxicity. Toxicity profiles of diverse anti-neoplastic drugs are extensively documented in past decades and the protective role of natural compounds against such toxic drugs plays a vital role in the evolution of chemoprevention. Hence the present study was designed to investigate the protective role of \( d \)-Limonene against CP induced cardio toxicity in male Wistar rats. The dose fixation studies the experimental designs were segregated into four groups for the cardio protective analysis in CP induced cardiotoxicity for 10 days. After the experimental period the body weight, relative heart weight, the level of lipid peroxidation, the activities of antioxidant enzymes were assessed. The administration of CP to rats results in increased relative heart weight and serum marker enzymes, and significant increase in cardiac lipid level, and significant decrease in the final body weight, cardiac mitochondrial dysfunction, tissue damage and the pathological changes assessed by histology study. Supplementation of \( d \)-Limonene significantly normalized the level of serum marker enzymes, and cardiac lipid level, cardiac mitochondrial function and cardiac function.

Keywords: Cyclophosphamide, \( D \)-Limonene, Cardiotoxicity,
reducing the activities of Krebs cycle enzymes with the subsequent separation of mitochondrial-linked ATP synthesis [14, 15]. In 1972, cardiotoxicity was first influenced by CP as a difficulty to the transplantation of bone marrow [16]. In the procedures of peripheral blood stem cell transplantation (PBSCT) and bone marrow transplantation for used high-dose of chemotherapy agent of CP. After one week administration CP results in acute heart failure and cardiotoxicity was occurrence rate is about 20% and mortality about 8% after bone marrow transplantation [17-18]. High dose of CP treatment usually associated with severe cardiotoxicity during various therapeutic regimens [19-20]. Therefore, the requirement of novel therapeutic agents, which helps in the protection of normal tissue from drug, induced toxicity with absence of toxic properties. Essential oils and their constituents have been used commonly in traditional medicine for diverse pharmaceutical actions. Recent days, plant-derived products are great focus due to their multiple pharmacological activities [21-25]. Monoterpenes are plant derivatives which possess medicinal properties and these components are considered as a novel class of chemo preventive agents. 7-Limonene, a monocyclic monoterpenes, also known as 1-methyl-4-(1-methyl ethenyl) cyclohexene. 7-Limonene (orange oil/essence oil) is widely distributed as a natural non-nutritive constituent in a variety of foods particularly fruits (citrus fruits, especially lemon and orange) (26-27), vegetables (carrots) (28), coffee, beverages, meat, and spices (nutmeg) (29). 7-Limonene inhibit lipid peroxidation and apprehend the free radical-induced damage. In addition, 7-Limonene is potent a biological activities, such as antioxidant property (30, 31), chemotherapeutic properties against many types of cancers (32), anti-inflammatory properties, hepatoprotective activities (33), immuno-modulatory effects (34) and there is scarce information about the cardio protective effect of 7-Limonene. Hence this study was designed in such a way to elucidate the role of 7-Limonene on experimentally induced cardio toxicity.

![Figure 1. (a)Structure of d-Limonene and (b) structure of Cyclophosphamide](image)

**II. MATERIALS AND METHOD**

**A. Chemicals**

Cyclophosphamide was purchased from Hi Media laboratories, India. 7-Limonene was purchased from Sigma-Aldrich, Bangalore. All other chemicals used were of analytical grade obtained from SRL/TCI/HIMEDIA laboratories, India.

**B. Animal Model**

Male, Wistar strain albino rats weighing about 150–180 g were obtained from The King Institute, Chennai, India. The animals were housed in cages under proper environmental conditions and were fed with a commercial pelleted diet (M/s Hindustan Foods Ltd., Bangalore, India). The animals had free access to water. All the experiments were designed and strictly conducted according to the ethical norms approved by Institutional animal ethics committee guidelines regulated by the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

**C. Experimental Design**

The rats were divided in to four groups, each group consisting of six animals (n=6).

**Group I** Normal control rats provided with standard diet and drinking water.

**Group II** Rats received standard diet and drinking water throughout the experimental period and on the
first day of experiment, a single dose of CP (200 mg/kg body weight, intraperitoneally using saline) was given and maintained for 10 days.

**Group III** Rats were treated with *d*-Limonene (150 mg/kg body weight-based on effective dose fixation studies) on all the days of experimental period (10 days).

**Note:** Various doses of *d*-Limonene (100, 150, 200, 250 mg/kg body weight) were administered orally to the CP intoxicated animals to optimize the *d*-Limonene dose for its maximum efficacy in the minimum dose, determined by the levels of serum marker enzymes for tissue damage (data shown in fig-2). It was found that 150 mg/kg body weight of *d*-Limonene has the maximum protective efficacy in the minimum dose.

**Group IV** Rats were treated with *d*-Limonene (150 mg/kg body weight) to the CP induced group of rats from the day 1 till end of the experimental period.

![Fig-2](image)

After the experimental period, the animals were fasted overnight, anesthetized with sodium pentothal and blood collected from jugular vein for serum isolation and sacrificed by cervical decapitation. The heart tissue was excised immediately and a portion of the tissue was homogenized in 0.1 M Tris buffer, pH 7.4 and used for various biochemical assays.

**D. Biochemical Parameters:**

The collected serum was used to estimate antioxidant enzymes such as superoxide dismutase (SOD) [29], catalase (CAT) [30], glutathione peroxidase GPx [31], glutathione reductase (GR) [32], Glutathione-S-transferases (GST) [33] and non-enzymatic antioxidants reduced glutathione (GSH) [34], vitamin C [35], vitamin E [36]. The Heart tissue homogenate was used to measure aspartate transaminases [37], alanine transaminase [38], alkaline phosphatase [39], Lactate dehydrogenase (LDH), was measured by the method of [40]. Lipid peroxidation level was determined by measuring thiobarbituric acid reactive substances (TBARS) according to the method of [41].

**E. Estimation of Serum lipoprotein fractions**

Serum lipoproteins such as LDL, HDL and VLDL were estimated in the serum samples (48).

**F. Histological evaluation:**

Histological examination portion of the heart tissue was fixed in 10% neutral buffered Formalin and embedded in paraffin wax for histological evaluation. Sections with thickness 5 µm were stained with hematoxylin and eosin (H & E), examined under high power light microscope.

**Statistical analysis:**

The data were analyzed with SPSS/10 Software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. P values of <0.05 were considered to indicate the statistically significance. All the results were expressed as mean ± standard error (SE) for six animals in each group.

**III. Result**

**Fixation of optimum dosage schedule:**

Fig-3 shows the effect of oral administration of diverse doses of *d*-Limonene (100mg/kg, 150mg/kg, 200mg/kg and 250mg/kg body weight of rat) for 10 days on the levels of the serum marker enzymes, CK, AST and ALT for tissue damage. In 100 mg/kg body weight of *d*-Limonene treated group of animals, the elevation of AST and ALT was controlled lesser than 150mg/kg body weight group of animals. The treatment of *d*-Limonene was more efficient in 150mg/kg body weight treated group of animals but the 200mg/kg and 250mg/kg body weight groups showed efficiency, but it have no significant difference when compared to 150mg/kg body mass group. 150mg/kg *d*-Limonene treated group showed the maximum protection in a minimum dose of treatment. Hence in the present study, the optimum
dosage of \( d \)-Limonene was fixed as 150 mg/kg body weight of rat. \( d \)-Limonene alone treated group which is an indicative of nontoxic nature of \( d \)-Limonene

**Fig-4**

**Fig-4 (a)** Showing the morphology of macroscopic structure of heart of control and experimental group of rats

**Fig-4 (b)** Effect of \( d \)-Limonene on Initial body weight and final body weight of control and experimental groups of rats

**Fig-4 (c)** Effect of \( d \)-Limonene on heart weight and relative heart weight of control and experimental groups of rats

Results are expressed as mean ± S.D for six rats in each group. Statistical significance at \( p<0.05 \) compared with \( a\) Compared with control and \( b\) Compared with CP administered group.

\( d \)-Limonene decreased the levels of serum marker enzymes in CP-induced cardiotoxicity animals

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**Effect of \( d \)-Limonene on body weight, heart weight and relative heart weight**

The cardio protective effect of \( d \)-Limonene against CP-induced cardiac damage was elucidated in male Wistar albino rats. **Fig-4(a)** and shows initial body weight, final body weight, heart weight and relative heart weight of control and experimental group of animals. In CP-induced group 2 animals, there is a significant decrease in the absolute body weight and significant increase in heart weight when compared with group 1 control animals (**Fig4 (b)**). The \( d \)-Limonene treated groups 4 showed a significant increase in the absolute body weight when compared with group 2 CP-induced animals during the course of the experiment, all rats showed greater tolerance to treatment with \( d \)-Limonene.**Fig-4 (c)** In group 2 animals, the relative heart weight is significantly improved when compared with group 1 animals and there is a significant reduced in the heart weight in \( d \)-Limonene treated groups 4 animals when compared with group 2 CP-induced animals. No obvious changes were observed between the control and
Table -1 shows the effect of d-Limonene on the levels of markers enzymes CK, LDH, AST, ALT and ALP in the serum of control and experimental group of rats. The marker levels were significantly increased in CP-induced animals when compared with control animals. Whereas CP + d-Limonene treated animals significantly decreased the levels of marker enzymes when compared with CP-induced animals. No significant changes observed between control and d-Limonene alone treated animals.

Table-1

<table>
<thead>
<tr>
<th>Groups</th>
<th>CK (µmol of phosphorus/h/mg protein)</th>
<th>LDH</th>
<th>AST</th>
<th>ALT</th>
<th>ALP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>85.25 ± 6.40</td>
<td>100.5 ± 10.9</td>
<td>71.32 ± 5.55</td>
<td>22.20 ± 1.17</td>
<td>0.65 ± 0.18</td>
</tr>
<tr>
<td>CP</td>
<td>175.34 ± 15.32</td>
<td>154.2 ± 13.45</td>
<td>294.2 ± 22.04</td>
<td>77.23 ± 5.25</td>
<td>25.32 ± 3.09</td>
</tr>
<tr>
<td>d-Limonene</td>
<td>85.74 ± 8.74</td>
<td>108.6 ± 12.3</td>
<td>71.84 ± 4.88</td>
<td>21.98 ± 2.11</td>
<td>0.78 ± 0.96</td>
</tr>
<tr>
<td>CP+ d-Limonene</td>
<td>159.24 ± 11.25</td>
<td>132.2 ± 13.86</td>
<td>129.2 ± 21.33</td>
<td>44.16 ± 4.27</td>
<td>12.26 ± 1.79</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.D. for six rats in each group. Statistical significance at p<0.05 compared with a Compared with control and b Compared with CP.

Units — CK: µmol of phosphorus/h/mg protein; LDH, AST and ALT: µmol of pyruvate/h/mg protein; ALP: KA U/l.

Effect of d-Limonene on serum lipoprotein-cholesterol profile

Fig-5 shows the serum lipoprotein fractions such as LDL, HDL and VLDL of control and experimental groups of rats. The level of HDL was significantly (p<0.05) reduced in CP induced group when compared with control animals. The levels of VLDL and LDL were significantly (p<0.05) increased in CP induced group when compared with control animals. However, significantly decreased levels of VLDL and LDL and elevated levels of HDL were observed in d-Limonene treated animals when compared to CP induced animals.

Fig-5

Effect of d-Limonene on antioxidant activities during CP induced cardiotoxicity

Table 2 and 3 indicates the enzymatic and non enzymatic antioxidant activities in the heart of the control and experimental groups. CP induced animals showed significant decrease in the activities of enzymatic antioxidants such as SOD, CAT, GPx, GR and GST when compared with control animals. Whereas CP + d-Limonene supplemented animals showed significant increase in these enzymes when compared with CP induced animals. Non enzymatic antioxidants such as GSH, G6PD, VIT C, VIT E and VIT A also found significantly decreased activities during CP induced animals when compared with control animals. In CP + d-Limonene treated animals, there is significant increase in the activities of GSH, G6PD, VIT C, VIT E and VIT A when compared with CP induced animals. No significant change was observed in d-Limonene alone treated animals when compared with control animal.

Table-2 Effect of d-Limonene on the activities of enzymic antioxidants in the heart of control and experimental groups of rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD</th>
<th>CAT</th>
<th>GPx</th>
<th>GR</th>
<th>GST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.16 ± 0.17</td>
<td>74.06 ± 5.39</td>
<td>21.23 ± 1.41</td>
<td>1.37 ± 0.16</td>
<td>1.06 ± 0.12</td>
</tr>
<tr>
<td>CP</td>
<td>0.98 ± 0.07</td>
<td>34.84 ± 3.92</td>
<td>14.96 ± 1.26</td>
<td>0.63 ± 0.05</td>
<td>0.64 ± 0.06</td>
</tr>
<tr>
<td>d-Limonene</td>
<td>2.21 ± 0.16</td>
<td>75.87 ± 4.96</td>
<td>21.10 ± 1.17</td>
<td>1.28 ± 0.08</td>
<td>1.01 ± 0.14</td>
</tr>
<tr>
<td>CP+ d-Limonene</td>
<td>1.89 ± 0.09</td>
<td>68.76 ± 6.23</td>
<td>18.28 ± 1.76</td>
<td>1.03 ± 0.11</td>
<td>0.82 ± 0.07</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.D. for six rats in each group. Statistical significance at p<0.05 compared with a Compared with control and b Compared with CP.
Analysis indicated that high therapeutic doses of CP induced animals manifested significant oxidative stress and tissue damage. Histological examination revealed the normal architecture of the heart and myocardial fibers in normal control animals (fig.6 (A)) showing almost normal myocytes and the abnormal pathological findings are reduced. These restorations may be due to the protective effect of d-Limonene against oxidative stress and tissue damage induced by CP.

**Table-3** Effect of d-Limonene on the activities of non-enzymic antioxidants in the heart of control and experimental groups of rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>GSH</th>
<th>G6PD</th>
<th>VITAMIN C</th>
<th>VITAMIN E</th>
<th>VITAMIN A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.76 ± 0.57</td>
<td>1.26 ± 0.13</td>
<td>1.21 ± 0.13</td>
<td>1.83 ± 0.25</td>
<td>1.16 ± 0.10</td>
</tr>
<tr>
<td>CP</td>
<td>2.59 ± 0.37</td>
<td>0.79 ± 0.06</td>
<td>0.80 ± 0.09</td>
<td>1.36 ± 0.07</td>
<td>0.75 ± 0.02</td>
</tr>
<tr>
<td>d-Limonene</td>
<td>4.92 ± 0.50</td>
<td>1.27 ± 0.12</td>
<td>1.21 ± 0.11</td>
<td>1.81 ± 0.27</td>
<td>1.16 ± 0.09</td>
</tr>
<tr>
<td>CP + d-Limonene</td>
<td>3.88 ± 0.90</td>
<td>1.06 ± 0.10</td>
<td>1.05 ± 0.10</td>
<td>1.50 ± 0.16</td>
<td>1.04 ± 0.07</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.D. for six rats in each group. Statistical significance at p<0.05 compared with

*a Compared with control and **b Compared with CP

Units: Vit C in mg/g of wet tissue, Vit E in mg/g of wet tissue, Vit A in mg/g of wet tissue and GSH in µg/mg protein.

**Histology examination:**

Histopathological alterations of heart tissue sections, stained with hematoxylin and eosin (H&E) was assessed under a light microscope. Control animals (fig.6 (A)) revealed the normal architecture of the heart and d-Limonene alone supplemented animals (fig.6(C)) also showed the normal histological appearance as compared to normal control animals. The CP induced animals (fig.6 (B)) showed myocytes inflammation and hyalinization at focal, whereas CP + d-Limonene treated heart (fig6 (D),) showing almost normal myocytes and the abnormal pathological findings are reduced. These restorations may be due to the protective effect of d-Limonene against oxidative stress and tissue damage induced by CP.

**Fig-6** Photomicrographs (40x) obtained from the heart sections (A) Control group, (B) CP alone group, (C) d-Limonene alone, (D) CP + d-Limonene group. Arrow shows swollen myocytes and damaged myocardial fibers.

**IV. DISCUSSION**

Cyclophosphamide (CP), a frequently used oxazaphosphorine alkylating agent, has been comprehensive from neoplastic diseases to organ transplantation and various disorders and as an immunosuppressive agent. The major limitation of CP is the damage of normal tissue, leading to several organ toxicity. It is well known that high therapeutic doses of CP could origin a lethal cardiotoxicity that has a combination of symptoms and signs of myopericarditis leading to lethal complication such as congestive heart failure, arrhythmias, cardiac tamponade and myocardial depression. Cellular mechanisms of CP-induced cardiotoxicity are consideration to be mediated by an increase in free oxygen radicals and the decrease in the antioxidant defense mechanism [49]. This study has been initiated to investigate the possible mechanisms whereby d-Limonene could prevent the development of CP-induced cardiotoxicity.

In this present study, administration of CP severely reduced in the final body weight and significantly increased the heart weight. Similarly CP administration adversely affects the tissue by increase in the serum markers CK, LDH, AST, ALT, and ALP. d-Limonene challenged along with CP animals significantly increase the body weight and attenuated the tissue damage thereby preventing the membrane enzymes leakage.
into the serum, this shows the protective effect of d-Limonene in myocytes against cardiotoxicity(50).

Clinical investigations also suggest that increased oxidative stress associated with CP causes cardiomyopathy that leads to heart failure. CP and its metabolites reduced the oxidant level that leads to accumulate abnormal free radical generation in the heart tissue (10). In this study, the oxidative stress associated tissue injury caused by CP results significant decreased in the activities of SOD, CAT, GPx, GR, GST and d-Limonene along with CP treatment restored the enzymic antioxidant levels which clearly shows the beneficial effect against CP induced cardiotoxicity (51) Depletion of GSH impairs the facility of the cells to defend against the free radicals and results in enhanced LPO. Vitamin E is a lipid soluble antioxidant which is there in cellular membranes where it plays an important role in the suppression of free radical induced LPO data from this study revealed that CP significantly increased cholesterol and triglycerides in serum. Hypercholesterolemia, hypertriglyceridemia induce by CP, which are well-known risk factors in cardiovascular diseases, has been reported earlier. Interestingly, d-Limonene supplementation completely inverted the CP-induced enhance in cholesterol and triglycerides to the organize values. Biochemical data were further confirmed by histopathological studies of the cardiac tissues. As CP induction caused distinct myocardial disintegration in the form of myofibrillar loss, inflammatory group infiltration, cytoplasmic vacuole development, interstitial edema and hemorrhage. These histopathological changes have been previously reported in CP-induced cardiotoxicity (52) d-Limonene treated animals revealed the normal architecture of the heart and showed improved cardio protection as observed by the absence of adverse pathological changes in the heart of CP induced cardiac damage. Thus, d-Limonene may reduce oxidative stress through the direct antioxidant effect, leading to the prevention of CP-induced cardiotoxicity

V. CONCLUSION

The present findings clearly shows the protective effect of d-Limonene on CP induced cardiotoxicity through maintaining systemic antioxidant activities, cellular membrane integrity and reducing hyperlipidemia conditions. Further studies are in the underway to investigate the intricate mechanism

VI. ACKNOWLEDGEMENT

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VII. REFERENCES

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