Effect of Enzymatic Changes in Vitamin D Combination with LIV-52 on Carbon Tetrachloride Induced Liver Disease in Wistar Rats

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Authors’ contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

ABSTRACT

Aim: The present study was conducted to investigate the hepatoprotective effect of enzymatic changes in vitamin D combination with Liv-52 on CCl4 induced liver toxicity in Wistar rats.

Background: The central organ of liver plays an essential vital role in the metabolism, and the liver is called as the metabolic “engine-room of the body”. Therefore, to maintain a healthy liver is a crucial factor for overall health and well being. The liver is the central organ for pharmaceutical drug or chemicals and xenobiotic detoxification metabolism, which regulates most of medication and
xenobiotic-related toxic activity. High metabolic and synthetic activity in this organ is an important place for the generation of free radicals.

**Materials and Methods:** Adult male Albino Wistar rats weighing 150-250 g were used in this study. The rats were split into six groups, each consisted of six rats. CCl₄ were admitted two days in a week for five weeks. Liver disease associated animals were treated with Vitamin D and Liv-52 for 5 weeks.

**Results:** The levels of AST, ALT, ALP, γ-GT, and AFP were significantly reduced in vitamin D, and Liv-52 treated animals when compared with CCl₄ induced animals. Moreover, the levels of Vitamin D and Liv-52, a good indicator of restoring the liver architecture, were also reversed in the damage after treatment. **Conclusion:** The results of the present study indicate that the combination drugs were more hepatoprotective effect when compared with the individual.

**Keywords:** Liver enzymes; vitamin D; liv-52 and carbon tetrachloride.

### 1. INTRODUCTION

Carbon tetrachloride (CCl₄) is a manufacture colorless chemical, and it is not available naturally in the environment, it is widely used for industrial purpose. Mainly used as heat carry liquid in refrigerating equipment and as aerosol propellants [1]. And also it is an important ingredient in numerous industrial fluids, it was an effective metal degreaser and an element in fire extinguishers [2]. Solid compounds of CCl₄ have a half-life of 6–12 months in soil or water and 30–100 years in the atmosphere. Humans can be exposed to CCl₄ in drinking water, air, foodstuffs, plastics, paints and industrial waste water since it has been used as a dry cleaning agent, grain fumigant, and solvent. Those workers are involved in the manufacture industry, or use CCl₄ are more likely to have significantly higher exposure to CCl₄ than are other persons. It is absorbed during ingestion, by inhalation, and, more slowly, from direct contact with the skin. After absorption, it may spread to organs with a high fat concentration and accumulate there. Depending on the dose, CCl₄ may be carcinogenic in humans, while acute exposure to high concentrations through ingestion or inhalation damages the liver [3].

CCl₄ is activated by drug-metabolizing enzymes in the endoplasmic reticulum and is metabolized by cytochrome P450 isoforms CYP2E1 and CYP3A4 produce trichloromethyl radicals, which are then oxidized to form the more reactive trichloromethyl peroxyl radicals [4]. This metabolic reaction results in covalent binding to macromolecule, causing lipid peroxidation. Prolonged exposure to CCl₄ may induce liver, kidney, and central nervous system injury. The liver is the most sensitive organ due to acute exposure to CCl₄ induces a hepatocellular injury, with the formation of lipid peroxidation and elevated levels of aspartate transaminase and alkaline transaminase and mainly in the centrilobular (zone 3) damage. This will happen because CYP2E1 enzymes are primarily occurs in the perivenous (zone 3) region of the hepatic acinus, and CCl₃ are produced in the highest concentrations of the region first, finally, necrosis can occur [5].

The liver plays an important vital role in the metabolism and it called the metabolic “engine-room of the body”. Therefore, maintaining a healthy liver is more important for overall health and well being [7]. The liver is the central organ for pharmaceutical chemicals and xenobiotic detoxification metabolism, which regulates most of the medication and xenobiotic-related toxic activity. High metabolic and synthetic activity in this organ is an important place for the generation of free radicals.

Early-stage of acute and chronic hepatic disease can be prevented by maintaining a healthy diet, lifestyle and avoiding drug and alcohol. It can also prevented by increasing natural substances of antioxidant levels and neutralizing the reactive oxygen species. Recent studies suggest that some vitamins have antioxidant properties in lowering the risk of suppressing the state of oxidative stress. When vitamin D level is adequate, the intracellular oxidative stress is positively correlated with the degree of CCl₄-induced hepatotoxicity [3,6].

The levels of AST, ALT, ALP, γ-GT, and AFP were significantly reduced in vitamin D, and Liv-52 treated animals when compared with CCl₄ induced animals. Moreover, the levels of Vitamin D and Liv-52, a good indicator of restoring the liver architecture, were also reversed in the damage after treatment. **Conclusion:** The results of the present study indicate that the combination drugs were more hepatoprotective effect when compared with the individual.

**Keywords:** Liver enzymes; vitamin D; liv-52 and carbon tetrachloride.
nucleus of target cells and either selectively activates gene expression or particularly represses gene transcription. Vitamin D is not only regulating calcium-phosphate level but also it is involved in modulating immune system in a response to infection [8]. It has been participating in cell division, proliferation and differentiation mechanisms. It is proved that vitamin D status is directly related to both innate and adaptive immune system additionally, the multifunctional role of vitamin D is under extensive study.

Vitamin D plays an important role in the immune system, and it decreases inflammation and fibrosis [9]. Proinflammatory signals in liver macrophages and monocytes may regulate the local metabolic synthesis of calciferol, auto-inducing the expression of CYP27B1 and the local production of 25(OH)D, and thus controlling the excessive inflammatory response [10]. Almost 90% of macrophages are present in the liver hepatocyte [11], which indicates that the hepatic production of 25(OH)D is reduced during the inflammatory diseases of the liver. Furthermore, high levels of VDR are present in both macrophages and biliary epithelial cells and other non-parenchymal cells [12].

When vitamin D level is adequate, the intracellular oxidative stress related compounds are downregulated mainly reduction in acellular response to a molecule due to a decrease in the number of receptors on the cell surface. Low concentrations of serum vitamin D fail to subdue oxidative stress conditions, augment intracellular oxidative damage and the rate of apoptosis. Vitamin D as well upregulates the expression of GPX that converts the ROS molecule H₂O₂ to water [13]. Vitamin D also affects the generation of glutathione via activation of the enzyme glucose-6-phosphate dehydrogenase which downregulates nitrogen oxide, a strong precursor for generating ROS that converts O₂⁻ to H₂O₂ and upregulates superoxide dismutase (SOD). These vitamin D-related actions collectively reduce the burden of intracellular ROS.

Liv.52 is an herbal hepatoprotective formulation introduced in 1955 and has been sold worldwide and has been recognized by thousands of health professionals [14]. Liv.52 is known to upgrade the structural and functional efficiency of the liver by promoting xenobiotics catabolism and therefore it protecting liver from harmful food and medication toxins, which maintaining healthy levels of liver enzymes and markers. Mechanistically, Liv.52 is known to protect hepatocellular membrane damage by lowering lipid peroxidation. This drug is widely used in many countries for patients with hepatic disorders [15,16].

In this regard, a current study in rats proved that the active metabolite of vitamin D and herbal products of liv-52 as well as combination of both effectively reduce the liver enzymes and markers levels in the in-vivo model.

2. MATERIALS AND METHODS

2.1 Animal Care and Housing

Adult male Albino Wistar rats weighing 150–250 g were used in the study. After veterinary examination for good health and suitability for the study, the rats were acclimatized to laboratory conditions for seven days before the treatment. During acclimatization, animals were observed daily. Rats were housed under standard laboratory conditions (temperature 19 to 25 °C), relative humidity between 30 and 70%, and with 12 hours light and 12 hours dark cycle. Rats were housed in standard polysulfone cages (size: Length 425 x Breadth 266 x Height 185 mm and 6 rats per cage) with stainless steel top grill having facilities for standard food and water ad libitum.

2.2 Experimental Protocol

The rats were split into six groups, each consisted of six rats. Group I was the control, Group II-induced CCl₄ (1 mL/kg b.w., 50% CCl₄ in olive oil) two days in a week for five weeks, Group III, CCl₄ + Vitamin D at dose levels of 500 IU/kg b.w., daily for five weeks. Group IV, CCl₄ + Liv-52 at dose level of 1 mL/kg b.w., daily for five weeks. Group V, CCl₄ + Vitamin D + Liv-52 with CCl₄ daily for five weeks (as above). Group VI, treated with vitamin D and Liv-52 at dose levels of 500 IU and 1 mL/kg b.w., without intoxication with CCl₄, respectively.

2.3 Blood and Tissue Collection for Biochemical Assay

After the experimental period of five weeks, all rats were food-deprived overnight and anesthetized by exposing to diethyl ether and then sacrificed. 2 ml of the blood were collected from all rats under ether-induced anaesthesia, into dry test tube without anticoagulant and supernatant was separated and was used for biochemical assays. Liver tissue was...
immediately taken out and washed in saline and patted dry and weighed. Around 100 mg tissue from the liver was taken and homogenized with motor driven Teflon coated homogenizer (Model: Inco, Orign: Ambala, India) in ice-cold 0.1M Tris-HCl buffer pH 7.4 to obtain 10% homogenate. After packing, the remaining cells were removed by washing solution using isotonic saline to remove the buffy coat. And then, 4 ml of packed cells were washed thrice with isotonic Tris-HCl buffer 0.1M pH 7.4. Haemolysis was performed by pipetting out the washed red blood cell suspension into polypropylene centrifuge tubes, which contained hypotonic buffer (Tris– HCl buffer 0.015 M, pH 7.2). Erythrocyte ghosts were sedimented using a high speed refrigerated centrifuge at 20,000 x g for 40 minutes. The supernatants were separated, stored at 4°C for one week, and used for biochemical assay.

2.4 Drugs and Chemicals

CCl₄, Vitamin D was purchased from Sigma chemical, Liv-52 was purchased from Himalaya Drug Company, and other chemicals were purchased from SRL chemicals.

2.5 Statistical Analysis

The data were expressed as mean ± SD. The statistical analysis of the experimental data was carried out using Statistical Package for Social Sciences (SPSS) for Windows version 21.0 software, one-way ANOVA method and the group mean were compared by Duncan’s Multiple Range Test (DMRT). Statistical probability P <0.05 was considered to be significant.

3. RESULTS

The effect of vitamin D and Liv-52 on rats induced by CCl₄ is shown in Table 1. The levels of Aspartate amino transaminase (AST), Alanine amino transaminase (ALT), Alkaline phosphate (ALP), and Gama-Guttural transaminase (γ-GT) were taken as an index for hepatotoxicity induced by CCl₄. Liver marker enzymes such as ALT, AST, ALP and γ-GT were analyzed for the control and experimental animals. In the group II CCl₄ treated animals, showed the level of marker enzymes were significantly elevated (P<0.001) when compared to the normal group I animals. But there was a significant decrease (P<0.001) of the enzyme level in the vitamin D and liv-52 treated animals when compared to drug induced control animals. However, treatment with a combination of both vitamin D and liv-52 caused a significant reduction (P<0.001) of the liver marker enzymes compared to hepatotoxic bearing animals. There was no significant difference in the liver marker enzymes levels between the control rats and the control rats treated with vitamin D and liv-52 combination (G-VI).

Table 2 shows that the group II CCl₄ treated animals showed that, the level of marker enzymes was significantly elevated (P<0.001) compared to the normal group I animals. But there was a significant decrease (P<0.001) of the enzyme level in the vitamin D and liv-52 treated animals when compared to drug induced control animals. However, treatment with a combination of both vitamin D and liv-52 caused much significant reduction (P<0.001) of the liver marker enzymes compared to hepatotoxic bearing animals. There was no significant difference in the liver marker enzymes levels between the control rats and the control rats treated with vitamin D and the liv-52 combination (G-VI).

The result showed in the Table 3, Carbon tetrachloride (CCl₄) administration caused significant higher (P<0.001) in the serum AFP level as compared to the normal control group. There was a significant decrease (P<0.001) of the AFP level in the vitamin D and liv-52 treated

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Group I (Control)</th>
<th>Group II (CCl₄ Induced)</th>
<th>Group III (Vitamin D treated)</th>
<th>Group IV (Liv-52 treated)</th>
<th>Group V (Both Vitamin D and Liv-52 treated)</th>
<th>Group VI (Both Vitamin D and Liv-52 treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST IU/L</td>
<td>70.68±4.71</td>
<td>112.1±6.78</td>
<td>83.05±7.75</td>
<td>71.12±3.83</td>
<td>70.9±4.78</td>
<td></td>
</tr>
<tr>
<td>ALT IU/L</td>
<td>22.2±2.27</td>
<td>62.65±4.09</td>
<td>47.67±3.9</td>
<td>34.54±3.37</td>
<td>23.1±3.17</td>
<td>22.66±2.39</td>
</tr>
<tr>
<td>ALP IU/L</td>
<td>72.3±4.42</td>
<td>148.55±8.6</td>
<td>84.73±6.28</td>
<td>73.0±4.58</td>
<td>73.29±4.05</td>
<td></td>
</tr>
<tr>
<td>γ-GT IU/L</td>
<td>2.12±0.10</td>
<td>5.93±0.19</td>
<td>4.89±0.12</td>
<td>3.49±0.69</td>
<td>2.26±0.36</td>
<td>2.2±0.14</td>
</tr>
</tbody>
</table>

Each value is expressed as mean ±SD for six rats in each group. a: as compared with Group I, b: as compared with Group II, Statistical significance: *P<0.001.
animals, when compared to drug induced control animals. However, treatment with combination of both vitamin D and liv-52 caused significant reduction (P<0.001) of the AFP level when compared to hepatotoxic bearing animals. There was no significant difference in the AFP level between the control rats and the control rats treated with vitamin D and liv-52 combination (G-VI).

4. DISCUSSION AND CONCLUSION

Liver fibrosis is a consequence of many chronic liver diseases [17], and oxidative stress has been implicated in its development [18]. CCl4 is toxic and causes oxidative stress.

Lin et al. [19] treated male rats with 2 ml/kgCCl4 for 12 weeks and reported that serum ALT, ALP, and GGT levels were significantly increased, when compared to controls (Lin et al., 2002). Similarly, Motawi et al. [20] reported elevated serum ALT, ALP, and GGT levels in male rats administered 0.5 mg/kg CCl4 for six weeks.

Current clinical evidence suggests that the liver is the main target organ of acute and chronic CCl4 toxicity. CCl4 is metabolized and activated by multiple cytochromes P450 enzymes, such as CYP2B1, CYP2B2, and CYP2E1. Among these, CYP2E1 is a major cytochromes contribution to CCl4 activation [21]. Several literatures reported pretreatment with phenobarbital, acarbose, or natural products (such as Salvia Officinalis) had been shown to potentiate the CYP2E1-mediated hepatotoxicity of CCl4 [22]. Vitamin D is investigated to induce the expression of CYP2B6 and CYP3A through activation of the VDR, the pregnane X receptor, and the constitutive androstane receptor (CAR) [23]. Hepatic CYP2E1 expression level was not changed by pretreatment with Vitamin D3. These finding suggest that CYPs are not primary mediators of the Vitamin D3 potentiation of CCl4 toxicity.

Several studies reported that rats treated with CCl4 were associated with changes in biomarkers of hepatic function, which were indicated by elevated transaminases (AST, ALT), bilirubin, and ALP levels and decreased albumin and total protein levels [24].

Because the liver plays a significant part in Vitamin D pleiotropic functions and metabolism, the question is whether vitamin D deficiency is a contributor to liver dysfunction or a consequence of liver disease [25]. However, in CLD patients of varying etiologies, this vitamin deficiency has been associated with increased fibrosis severity. Roth et al. [26] showed that the existence of vitamin D deficiency affects the progression of liver fibrosis in nonalcoholic fatty liver disease with a slightly (non-significant) effect on liver function tests.

The increasing evidence suggests that the circulating concentration of vitamin D was negatively associated with the risk of liver disease [27], and with the increasing severity of

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### Table 2. Effect of vitamin D and Liv-52 on liver enzymes levels in the serum of control and experimental animals

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Group I (Control)</th>
<th>Group II (CCl4 Induced)</th>
<th>Group III (Vitamin D treated)</th>
<th>Group IV (Liv-52 treated)</th>
<th>Group V (Both Vitamin D and Liv-52 treated)</th>
<th>Group VI (Both Vitamin D and Liv-52)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST IU/L</td>
<td>41.2±7.3</td>
<td>222.1±12.9*a</td>
<td>126.7±15.3</td>
<td>59.7±8.7</td>
<td>47.1±6.4</td>
<td>35.7±3.5</td>
</tr>
<tr>
<td>ALT IU/L</td>
<td>22.2±6.8</td>
<td>205.0±6.39*b</td>
<td>137.4±10.9</td>
<td>33.2±4.9</td>
<td>27.1±5.9</td>
<td>30.6±6.8</td>
</tr>
<tr>
<td>ALP IU/L</td>
<td>112.3±17.6</td>
<td>230.2±11.7*a</td>
<td>151.2±8.3</td>
<td>125.3±7.0</td>
<td>117.4±5.6</td>
<td>114.0±10.5</td>
</tr>
<tr>
<td>y-GT IU/L</td>
<td>2.1±0.65</td>
<td>4.1±0.9*a</td>
<td>2.5±0.19</td>
<td>2.4±0.37</td>
<td>2.28±0.31</td>
<td>2.19±0.45</td>
</tr>
</tbody>
</table>

Each value is expressed as mean ±SD for six rats in each group. a: as compared with Group I, b: as compared with Group II. Statistical significance: *p<0.001, NA-Not significant

### Table 3. Effect of vitamin D and Liv-52 on liver marker levels in the serum of control and experimental animals

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Group I (Control)</th>
<th>Group II (CCl4 Induced)</th>
<th>Group III (Vitamin D treated)</th>
<th>Group IV (Liv-52 treated)</th>
<th>Group V (Both Vitamin D and Liv-52 treated)</th>
<th>Group VI (Both Vitamin D and Liv-52)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFP</td>
<td>0.52±0.06</td>
<td>24.4±2.48*a</td>
<td>11.05±1.16</td>
<td>10.1±1.07</td>
<td>0.6±0.15</td>
<td>0.55±0.14</td>
</tr>
</tbody>
</table>

Each value is expressed as mean ±SD for six rats in each group. a: as compared with Group I. Statistical significance: *p<0.001

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liver disease, the expression of hepatic cytokines also increased [28]. These results were confirmed by [29], who showed that serum ALT and AST activity were extremely elevated in rats of the diabetic group (DM) when in the vitamin D group, 1,25-(OH)₂D₃ treatment significantly lowered serum activity of ALT and AST compared with the DM group. These vitamin D concentrations can contribute to liver protection. It was reported that 1,25-(OH)₂D₃ has protective effects on the liver of DM rats by modulating inflammation and lipid metabolism [29].

The Ayurvedic formulation of Liv.52 exhibits potent hepatoprotective properties against chemically induced hepatotoxicity. It restores the functional efficiency of the liver by protecting the hepatic parenchyma and promoting hepatocellular regeneration. The antiperoxidative activity of Liv.52 prevents the loss of functional integrity of the cell membrane, maintains the cytochrome P-450 enzyme system and lipid membrane [30]. Liv.52 is known to improve the functional efficiency of the liver by promoting detoxification and thus protecting from harmful food and medication toxins, maintaining healthy levels of liver enzymes. Liv.52 is also known to support the liver's normal ability to burn fat and maintain the body's metabolic homeostasis.

The present study proved that the levels of liver enzymes such as AST, ALT, ALP, and γ-GT were significantly decreased in vitamin D and Liv-52 treated animals when compared with CCl₄ induced animals. The levels of AFP were decreased in vitamin D, and Liv-52 treated animals when compared with CCl₄ induced animals. The combination drugs were more hepatoprotective effect when compared with the individual.

DISCLAIMER
The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

NOTE
The study highlights the efficacy of “Ayurvedic” which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

CONSENT
It is not applicable.

ETHICAL APPROVAL
This research work was approved by the Institutional Animal Ethical Committee (REG No. 765/03/ca/CPCEA).

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COMPETING INTERESTS
Authors have declared that no competing interests exist.

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