EFFECT OF ENZYMATIC CHANGES IN VITAMIN D COMBINATION WITH LIV-52 AGAINST CARBON TETRACHLORIDE INDUCED LIVER DISEASE IN WISTAR RATS

ABSTRACT:

AIM: The present study was conducted to investigate the hepatoprotective effect of enzymatic changes in vitamin D combination with Liv-52 on CCl_4 induced liver disease 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 150-250g were used in this study. Liver disease associated animals were treated with Vitamin D and Liv-52 for 5 weeks. This study was conducted at Meenakshi Medical College Hospital and Research Institute, Kanchipuram, Tamil Nadu, India. **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

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 (Holbrook et al., 1991). And also an important ingredient in numerous industrial fluids, it was an effective metal degreaser and a element in fire extinguishers (Doherty et al., 2000). 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 (Wong et., 1998).

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(Hassanen et al., 2013). 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 (Hathaway et al. 1991). There is no proven human data clearly defining the relationship between CYP3A4 or CYP2E1 activity and CCl4 induced, animal studies have proved that CYP2E1 activity is positively correlated with the degree of CCl4- induced hepatotoxicity (Wong et al., 1998 & Dai et al. 2014).

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 (Subramonium et al., 1999). 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 prevent 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 related activities are down regulated.

Vitamin D is a group of sterols compound that has a hormone-like function, and it binds with intracellular receptor proteins, Vitamin D receptor (VDR) complex communicates with DNA in the nucleus of target cells and either selectively activates gene expression or particularly represses gene transcription. The main function of vitamin D in regulating calcium–phosphate levels, but vitamin D is now involved in a key modulator of the immune response to infection (Liu et al., 2006) and has been participating in cell division and proliferation and differentiation mechanisms. It is proved that vitamin D status is directly related to both innate and adaptive immune system, and the multifunctional role of vitamin D is under extensive study.

Vitamin D plays an important role in the immune system, and it will decrease inflammation and fibrosis (White et al., 2000). 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 (Petta et al., 2010). Almost 90% of macrophages are present in the liver hepatocyte (Sadeghi et al., 2006), 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 (Bilzer et al., 2006).

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 (Shelton et al., 2011). 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 (Saini et al., 1985). Liv.52 is known to upgrade the structural and functional efficiency of the liver by promoting xenobiotics catabolism and therefore it protecting from harmful food and medication toxins, 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 (Malik et al., 1979 & Dhumal et al. 1989).

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

MATERIAL AND METHODS:

Animal Care and Hosing

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 (temperature19 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. This research work was obtained and approved by the Institutional Animal Ethical Committee (REG No. 765/03/ca/CPCEA).

Experimental Protocol

The rats were split into six groups, and 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.

Blood and tissue collection for biochemical assay

After the experimental period of five weeks, all the rats were food-deprived overnight and anesthetized by exposing to diethyl ether and then sacrificed**.** 2 ml of the blood were collected from all the rats under ether-induced anaesthesia, into without anticoagulant dry test tube and supernatant was separated and 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 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 4ºC for one week, and used for biochemical assay.

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.

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.

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 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) when compared to the normal control group. But there was a significant decrease ($P<0.001$) of the AFP level in the vitamin D and liv-52 treated animals, when compared to drug induced control animals. However, treatment with combination of both vitamin D and liv-52 caused very much 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).

DISCUSSION:

Liver fibrosis is a consequence of many chronic liver diseases (Bataller et al., 2005), and oxidative stress has been implicated in its development (Poli et al., 2000). CCl4 is toxic and causes oxidative stress.

Lin et al. (2012) treated male rats with 2 ml/kgCCl4 for 12 weeks and reported that serum ALT, ALP, and GGT levels increased significantly compared to controls (Lin et al., 2002). Similarly, Motawi et al.(2011) reported elevated serum ALT, ALP, and GGT levels in male rats administered 0.5 mg/kgCCl4 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 (Muriel et al., 2001). Several literatures reported pretreatment with phenobarbital, acarbose, or natural products (such as s*alvia officinalis*) had been shown to potentiate the CYP2E1-mediated hepatotoxicity of CCl4 (Gruebele et al., 1996). 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) (Lang et al., 2003). 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 withCCl4 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 (Xin et al., 2017).

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 (Iruzubieta et al., 2014). However, in CLD patients of varying etiologies, this vitamin deficiency has been associated with increased fibrosis severity. Roth et al.

(2012) 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 (Rhee et al., 2013), and with the increasing severity of liver disease, the expression of hepatic cytokines also increased (Salum et al., 2012). In the previous study**,** rat lots were treated with vitamin D supplementation to prevent for 14 days. In addition, rats treated with CCl4 alone increased transaminase activity in serum. In the lots pre-treated with vitamin D, the results indicated a significant decreasing level in serum of ALT and AST when CCl4 was administrated. These results were confirmed by (Ning et al., 2015), 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. All concentrations of vitamin D (6, 12 and 24 μg/kg) tested in the present study reduced these parameters. 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 (Ning et al., 2015).

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 (Mehrotra et al., 1973). 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.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. 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.

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TABLES:

Table 1: Effect of vitamin D and Liv-52 on liver enzymes levels in the liver of control and experimental animals

Particulars	Group I (Control)	Group II (CCl4 Induced)	Group III (Vitamin D treated	Group IV (Liv-52 treated)	Group V (Both Vitamin D and Liv-52 treated)	Group VI (Both Vitamin D and Liv-52)
AST IU/L	70.68±4.71	112.1±6.78 [a*]	101.9±2.8 [b*]	83.05±7.75 [b*]	71.12±3.83 [b*]	70.9±4.78 [a]
ALT IU/L	22.2±2.27	62.65±4.09 [a*]	47.67±3.9 [b*]	34.54±3.37 [b*]	23.1±3.17	22.66±2.3

					b*	9 a
ALP IU/L	72.3±4.42	148.55±8.6 a*	118.7±5.4 b*	84.73±6.28 b*	73.0±4.58 b*	73.29±4.05 a
γ-GT IU/L	2.12±0.10	5.93±0.19 a*	4.89±0.12 b*	3.49±0.69 b*	2.26±0.36 b*	2.2±0.14 a

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:** * $p<0.001$

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

Particulars	**Group I (Control)**	**Group II (CCl4 Induced)**	**Group III (Vitamin D treated**	**Group IV (Liv-52 treated)**	**Group V (Both Vitamin D and Liv-52 treated)**	**Group VI (Both Vitamin D and Liv-52)**
AST IU/L	41.2±7.3	222.1±12.9a*	126.7±15.3b*	59.7±8.7 b*	47.1±6.4 b*	35.7±3.5a
ALT IU/L	22.2±6.8	205.0±6.3a*	137.4±10.9 b*	33.2±4.9 b*	27.1±5.9 b*	30.6±6.8a

ALP IU/L	112.3±17.6	230.2±11.7 [a*]	151.2±8.3 [b*]	125.3±7.0 [b*]	117.4±5.6 [b*]	114.0±10.5 [a]
γ-GT IU/L	2.1±0.65	4.1±0.9 [a*]	2.5±0.19 [b*]	2.4±0.37 [b*]	2.28±0.31 [b*]	2.19±0.45 [a]

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 Dand Liv-52 on liver marker levels in the serum of control and experimental animals

Particulars	**Group I (Control)**	**Group II (CCl4 Induced)**	**Group III (Vitamin D treated**	**Group IV (Liv-52 treated)**	**Group V (Both Vitamin D and Liv-52 treated)**	**Group VI (Both Vitamin D and Liv-52)**
AFP	0.52±0.06	24.4±2.48 [a*]	11.05±1.16 [b*]	10.1±1.07 [b*]	0.6±0.15 [b*]	0.55±0.14 [a]

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:** [*] $p<0.001$