HEPATOPROTECTIVE POTENTIAL OF METHANOLIC EXTRACT OF *VETIVERIA ZIZANIOIDES* ROOTS AGAINST CARBON TETRACHLORIDE-INDUCED ACUTE LIVER DAMAGE IN RATS

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Vetiveria zizanioides roots possess anthelmintic, antimicrobial and *in-vitro* antioxidant activity. The aim of present study was to evaluate the hepatoprotective potential of methanolic extract of *Vetiveria Zizanioides* roots (MEVZ) against CCl_4-induced acute liver damage in rats. Animals were pretreated with MEVZ (300 and 500 mg/kg, p.o) and silymarin (200 mg/kg, p.o) respectively 30 min prior to CCl_4 (0.5 ml/kg, i.p) ingestion for 7 days. The effects of MEVZ were assessed directly by liver histology and by serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphate (ALP), total and direct bilirubin (TBL & DBL), lactate dehydrogenase (LDH), gamma glutamyl transferase (GGT), total protein (TP), liver malondialdehyde (MDA) and reduced glutathione (GSH). Also Serum Interleukin (IL-6), IL-1β and Tumor necrosis factor-α (TNF-α) were measured by enzyme linked immunosorbent assay (ELISA). CCl_4-induced acute liver damage offers a protection in MEVZ treated rats and its effects are comparable with standard drug silymarin. The data indicated that ALT, AST, ALP, TBL, DBL, LDH, GGT, MDA, IL-6, IL-1β and TNF-α level were significantly increased and decreased in GSH and TP levels in CCl_4 control. We found significantly reversal in all above parameters in treated groups. Our results clearly suggest hepatoprotective potentials of MEVZ mediated through attenuation of TNF-α and Il-6 mediated pathways, indicates it as a novel herbal drug for the prevention of acute liver damage.

1. Introduction

Liver is the largest organ for metabolism and detoxification of various components enter into the body. It is involved in wide range of functions and hence it is exposed to toxic substances and drugs absorbed from the intestine. Apart from the toxins and drugs [1], viral infections (hepatitis A, B, C, D, etc.) cause damage to the hepatocytes.

Carbon tetrachloride (CCl_4)-induced liver damage is widely used model for hepatoprotective drug screening [2-3]. The acute liver damage of CCl_4 occurs by its biotransformation to trichloromethyl free radical (CCl_3*) or trichloroperoxyl radical ($CCl_3O_2{}^{*}$) produced by the mixed-function cytochrome P450 oxygenase system of the endoplasmic reticulum, which causes oxidative stress and membrane damage [4]. These free radicals cause lipid peroxidation which results in liver damage and enhances formation of inflamed tissues. The merit of this CCl_4 model is that it can develop hepatitis within a few hours, which specifically leads to

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necrosis and fatty liver, similar things what happens in acute hepatitis. Meanwhile, following an inflammatory response caused by resident inflammatory cells, CCl_4-induced acute liver damage also involves an intricately regulated process of hepatocyte regeneration when the dosage of CCl_4 is below lethal level which would lead to irreversible liver damage [5-6].

Liver diseases remain one of the serious health problems and the Indian traditional system of medicine, especially Ayurveda, have put forward a number of medicinal plants and their formulations for liver disorders. In this modern age, it is very important to provide scientific proof to justify the various medicinal uses of herbs. Herbal drugs are prescribed widely even when their biologically active components are unknown because of their effectiveness, fewer side effects and relatively low cost [7].

Vetiveria zizanioides Linn. root (Commonly known as: Ushira, Family: Poaceae), is a perennial herb which found throughout the plains and lower hills of India, particularly on the river banks and in rich marshy soil. The plant used as digestive, carminative, stomachic, constipating, haematinic, expectorant, antispasmodic, antiasthmatic, antigout [8]. It possesses various pharmacological activities such as anthelmintic [9], antimicrobial [10], diuretic [11] and *in-vitro* antioxidant activity [12]. Recently, in our recent study *Vetiveria zizanioides* was demonstrated to possess hepatoprotective action against ethanol intoxication in rats [13]. It contains a wide variety of phytoconstituents such as flavonoids, terpenoids, glycosides and volatile oils [13]. Plant derived natural products such as flavonoids, terpenoids, glycosides, volatile oils, steroids, saponins, alkaloids and tannins [14-21] have received considerable attention in recent years due to their diverse pharmacological properties including antioxidant and hepatoprotective activity.

Silymarin, a standardized extract obtained from seeds of *Silybum marianum*, is widely used in treatment of liver diseases of varying origins [22]. Seeds of *S. marianum* have been shown to treat liver and gall bladder disorders, including hepatitis, cirrhosis and jaundice and to protect the liver against poisoning from chemicals, environmental toxins, snake bites, insect stings, mushroom poisoning and alcohols [23]. Due to its proven hepatoprotective and antioxidant properties, silymarin is being used in the current study as a standard drug for comparison with of methanolic extract of *V. zizanioides* [24]. The aim of this study is to assess whether *V. zizanioides* could prevent acute liver damage induced by CCl_4 in rats and to investigate the possible mechanism of its protective role.

2. Materials and methods

2.1. Plant material and extraction

The plant material *V. zizanioides* was collected from forest area of Jodhpur, Rajasthan, India. The plant was identified and authenticated by Dr A. S. Reddy, Department of Biosciences, Sardar Patel University, Vallabh Vidyanagar, Gujarat, India where a voucher specimen (No. MP-2) was kept for future reference. The powder of *V. zizanioides* roots was defatted with petroleum ether (60-80°C), and cold extracted with methanol. The methanol crude extract (10.5 % yields) was obtained by evaporation using Rotavapour ® (BÜCHI, Switzerland) under reduced pressure. The dry methanol extract was stored in cool and dry place which further used for the evaluation of hepatoprotective activity. All the test and standard suspensions were prepared in the distilled water.

2.2. Animals

Studies were carried out using either sex Wistar albino rats (200-250 g). They were obtained from the animal house, Anand pharmacy college (APC), Anand, India. The animals were grouped and housed in polyacrylic cages (38 × 23 × 10 cm) with not more than six animals per cage and maintained under standard laboratory conditions; temperature (22 ± 2°C), relative humidity (55 ± 5 %) with dark and light cycle (12/12 h). They were allowed free access to standard pellet diet (Amrut feed, Sangli, India) and water *ad libitum*. The rats were acclimatized to laboratory condition for 10 days before commencement of experiment. Animal studies were

approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and conducted according to the regulations of Institutional Animal Ethics Committee (Protocol no. 7004).

2.3. Drugs and Chemicals

CCL_4 was purchased from S. D. Fine Chem. Ltd. (Mumbai). Silymarin was obtained as a gift sample from Micro labs, Bangalore, India. Serum AST & ALT, ALP, Bilirubin kits were procured from Span Diagnostics, Surat, India. While LDH, GGT kits were procured from Coral Clinical Systems, Goa, India. Enzyme-linked immunosorbent assay (ELISA) kits for IL-1β, IL-6 and TNF-α from R and D system (Minneapolis, MN, United States). Trichloroacetic acid was purchased from Merck India Ltd, Mumbai, India. Thiobarbituric acid and Di thiobis Nitro Benzoic Acid were purchased from Himedia, Mumbai, India. All other chemicals and reagents used were of analytical grade.

2.4. Acute Toxicity Studies

Rats were divided into three different groups (n = 6) and assigned either as vehicle (distilled water, p.o, 5 ml/kg), low and high dose of Methanolic extract of *V. zizanioides* (MEVZ) at 3 g/kg and 5 g/kg, p.o. respectively. The rats were not fed overnight prior to the treatments. After treatments, the rats were observed for toxicity symptoms and behavioural changes for a period of 48 hr. The observations continued up to day 14. Then, the rats were sacrificed after overnight fasting on day 15. Livers and kidneys were excised for gross necropsy and histopathological examination. There was no lethality in any of the groups. One tenth of the maximum dose of the extract tested for acute toxicity was selected for evaluation of hepatoprotective activity, i.e., 300 & 500 mg/kg [25].

2.5. Experimental design

2.5.1. Carbon tetrachloride-induced liver damage in rats [26]

Animals were randomly divided into five groups six of each. All animals except normal control group were intoxicated with CCL_4 (0.5 ml/kg/d, i.p. for 7 d). Group I (Control) received only distilled water and Group II (CCL_4 control) received CCL_4 (0.5 ml/kg/d, i.p. for 7 d). Group III (MEVZ-300+ CCL_4) and Group IV (MEVZ-500+ CCL_4) were pretreated with MEVZ at a dose of 300, 500 mg/kg and Group V (Silymarin-200+ CCL_4) silymarin at a dose 200 mg/kg/d, p.o., respectively 30 min prior to CCL_4 ingestion for 7 days.

2.5.2. Effects of MEVZ on Serum Biomarkers

At end of the treatment, fasting blood samples were obtained under light anesthesia via cardiac puncture and allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 2500 rpm at 4 ºC for 15 min and used for the estimation of various serum biomarkers like Alanine and Aspartate aminotransferase (ALT & AST) [27], alkaline phosphatase (ALP) [28], total and direct bilirubin (TBL & DBL) [29], lactate dehydrogenase (LDH) [30], gamma glutamyl transferase (GGT) [31], total protein (TP) [32], Interleukin (IL-6), IL-1β and Tumor necrosis factor-α (TNF-α) [33].

2.5.3. Effects of MEVZ on Oxidative stress parameters

After collection of blood samples, the rats were sacrificed by light ether anesthesia and their livers were excised, rinsed in ice cold normal saline, followed by 0.15 M Tris-HCl (pH 7.4) blotted dry and weighed. A small 10% (w/v) portion of the Liver was homogenized in chilled Phosphate buffered saline (50 mM, pH 7.4) using a Potter Elvehjehm Teflon homogenizer. The homogenate obtained was centrifuged in a cooling centrifuge at $1,000{\times}g$ for 10 min at 4°C to

remove nuclei and unbroken cells. The pellet was discarded and portion of supernatant was again centrifuged at $12,000 \times g$ for 20 min at 4°C obtain a post-mitochondrial supernatant which was used for enzyme analysis [34]. The contents of malondialdehyde (MDA) [35], reduced glutathione (GSH) [36] were estimated spectrophotometrically using above post-mitochondrial supernatant.

2.5.4. Histopathological studies

A Small piece of liver were fixed in 10% neutral buffered formalin and subsequently embedded in paraffin. A transverse section of 5 μm was cut from each sample and stained with haematoxylin and eosin. Histopathological assessment (light microscopy) was performed on randomized sections of liver [37].

2.5.5. Statistical Analysis

The experimental results were expressed as Mean ± SEM for six animals in each group. All parameters were analyzed statistically using one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls multiple comparisons test using Graph Pad prism 5.0 software. Data were considered statistically significant at $P < 0.05$.

3. Results and discussion

The hepatotoxic agent CCl_4 induces selective toxicity to the liver cells due to metabolic activation and this maintains them with semi normal metabolic function. It also causes functional and morphological changes in the cell membrane which may lead to hepatic cell death [38]. There was no morbidity and mortality observed throughout the study. *Vetiveria Zizanioides* was found not toxic to the experimental rats up to the high dose of 5 g/kg.

The hepatic cells consist of higher concentrations of AST, ALT, LDH and GGT in cytoplasm and AST in particular exists in mitochondria [39]. Due to the damage caused to hepatic cells, the leakage of enzymes in plasma [40] causing an increased levels of hepatospecific enzymes in serum. The elevated serum enzyme levels like AST, ALT, LDH and GGT are indicative of cellular leakage and functional integrity of cell membrane in liver [41]. In our study when rats treated with CCl_4 showed significant ($P < 0.01$) liver damage and it was well indicated by increased levels of hepatospecific enzymes like ALT, AST, ALP, LDH and GGT in serum. Treatment with two different doses of MEVZ (300 and 500 mg/kg) decreased the levels of hepatospecific enzymes significantly ($P < 0.01$) compared to CCl_4 group, which was comparable with standard drug, silymarin [Table 1].

A high concentration of bilirubin in serum is an indication for increased erythrocyte degeneration rate [42]. Due to the liver injury caused by the hepatotoxin, there is a defective excretion of bile by the liver which is reflected in their increased levels of bilirubin in serum [43]. A significant ($P < 0.01$) elevation in TBL and DBL was observed in CCl_4 group compared to control. Treatment with two different doses of MEVZ (300 and 500 mg/kg) decreased the levels of TBL and DBL significantly ($P < 0.01$) compared to CCl_4 group [Table 1].

The TP levels will be depressed in hepatotoxic conditions due to defective protein biosynthesis in liver [44]. The CCl_4 intoxication causes disruption and disassociation of polyribosomes on endoplasmic reticulum and thereby reducing the biosynthesis of protein. In our study the TP levels were decreased significantly ($P <0.01$) in CCl_4 group. Treatment with MEVZ significantly ($P < 0.01$) increased the TP levels by protecting the polyribosomes in Table 1.

The increase in MDA or decrease in GSH levels indicates the lipid peroxidation. MDA is one among the end products produced by the decomposition of Omega-3 and Omega-6 poly-unsaturated fatty acids [45]. GSH play a vital role in the defence mechanism of tissue against the reactive oxygen species [46]. The significant ($P < 0.01$) increase in MDA and decrease in GSH levels were observed in CCl_4 groups compared to control. Treatment with MEVZ at 300 and 500 mg/kg reverse the above changes which were altered by CCl_4. The effects were comparable with the standard group silymarin. This also suggests the defensive antioxidant potential of MEVZ against the reactive oxygen species generated by CCl_4 [Table 1].

Table 1: Effect of methanolic extract of V. Zizanioides on various parameters

Biochemical Parameters	I Control	II CCL$_4$ control	III MEVZ-300+ CCL$_4$	IV MEVZ-500+ CCL$_4$	V Silymarin-200+ CCL$_4$
AST (IU/L)	33.5 ±4.7	168.33 ±14.9 [a]***	93.33±5.4 [b]**	75.67 ±4.9 [b]**	51.67±4.4 [b] ***
ALT (IU/L)	28.17±2.1	185.00±14.9 [a] ***	123.33±7.7 [b]**	104.5±4.0 [b]***	58.33±3.2 [b]***
ALP (KAU/dl)	7.18 ±0.7	95.90± 5.8 [a]***	41.62± 2.2 [b]**	32.52± 2.7 [b]***	15.13±1.6 [b]***
TBL (mg/dl)	0.36±0.04	1.68±0.1 [a]**	0.84 ±0.07 [b]*	0.78 ±0.06 [b]**	0.46 ±0.07 [b]**
DBL (mg/dl)	0.14 ±0.02	0.89 ±0.04 [a]**	0.59±0.03 [b]*	0.43 ±0.03 [b]**	0.29±0.02 [b]**
LDH (U/L)	426.07±12.6	1752.6±152.2 [a] ***	862.13±54.6 [b]**	685.48±80.6 [b]**	491.05±22 [b]***
GGT (U/L)	39.08±2.9	187.10±9.08 [a] ***	103.88±5.5 [b]**	94.81±5.4 [b] **	65.09±8.3 [b]***
TP (mg/ml)	7.69±0.8	3.02±0.4 [a]***	5.75±0.7**	6.99±0.6 [b]**	7.50±0.6 [b]**
MDA (nm/mg protein)	6.53±1.0	32.19±1.6 [a]***	15.17±1.9 [b]**	11.21±1.08 [b]***	12.07±1.09 [b]***
GSH (nm/mg protein)	11.81±0.2	5.26±0.2[a]***	8.76±1.1[b]*	9.60±0.6 [b]**	10.44±0.6 [b]***

Data were expressed as Mean ± S.E.M and analyzed using one-way ANOVA followed by Student-Newman-Keuls multiple comparisons test. Six rats were used in each group. 'a' CCl$_4$ control group was compared with control. 'b' MEVZ and Silymarin treated groups were compared with CCl$_4$ control group. Statistical significance was considered as *P<0.05, **P<0.01 and ***P<0.001.

To find the underlying mechanism, we evaluated the effects of MEVZ on the serum level of certain key cytokines tightly related to inflammation and cell proliferation. IL-1β, IL-6 and TNF-α, as acute phase proteins, are considered to be the special biomarkers that affect inflammatory status [47]. IL-1β plays a major role in inflammation, usually leading to tissue destruction. Serum IL-1β can increase dramatically during different inflammatory and non-inflammatory processes [48]. In our study serum IL-1β was found to be elevated in CCl$_4$ control group, whereas MEVZ treated group resulted in significant attenuation of the elevation [Figure 1]. The decreased level of inflammatory cytokines may explain the accelerated liver regeneration observed in MEVZ administrated group.
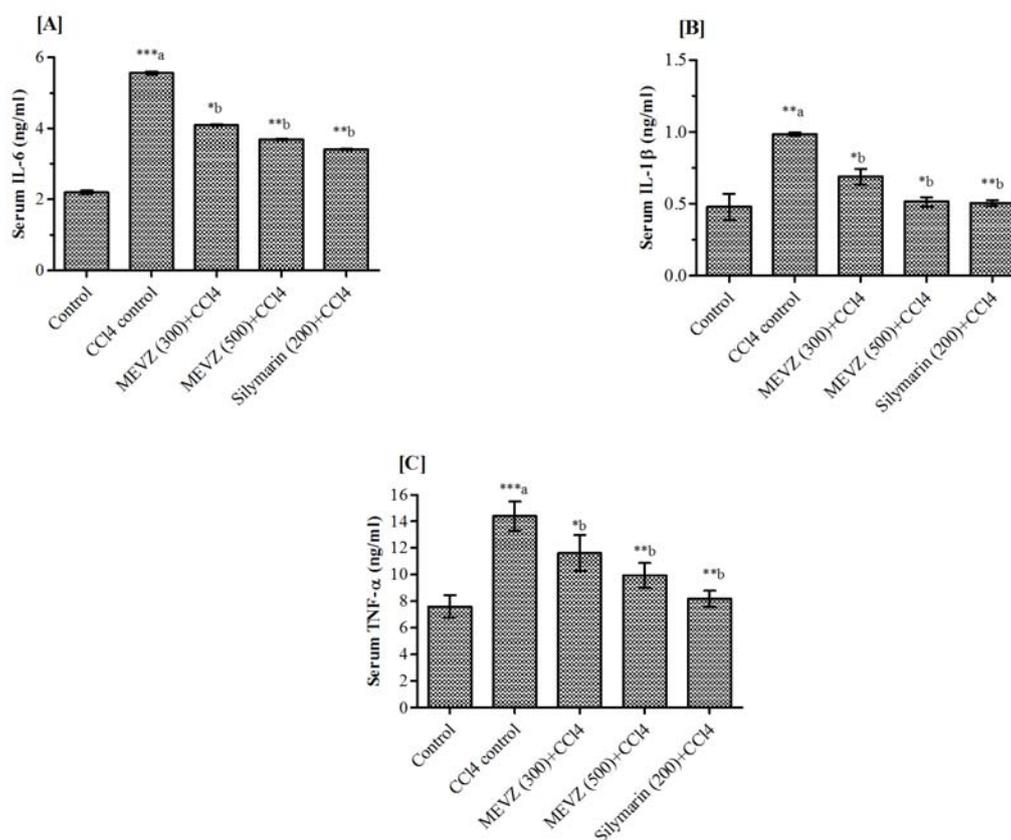
Fig. 1. Levels of interleukin-6, interleukin-1β and tumor necrosis factor-α in serum of control, CCl₄ control and treatment groups.

Data were expressed as Mean ± S.E.M and analyzed using one-way ANOVA followed by Student-Newman-Keuls multiple comparisons test. Six rats were used in each group. 'a' CCl₄ control group was compared with control. 'b' MEVZ and Silymarin treated groups were compared with CCl₄ control group. Statistical significance was considered as $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$

IL-6 and TNF-α expression has been identified as attractive targets for liver regeneration. The release of TNF-α, as a pro-inflammatory mediator in liver apoptosis, is also linked to cytotoxicity induced by CCl₄ [49-50]. Kupffer cells produce TNF-α in rapid response to tissue injury, which then up-regulates the expression of IL-6. TNF-α and IL-6 together activate the neighboring hepatocytes, leading to signal transducer and activator of transcription and the production of several other proteins that are shared within the growth-factor-mediated pathway network. CCl₄-induced acute liver damage could activate hepatic non-parenchymal cells (including Kupffer cells and stellate cells) and increase the production of TNF-α and IL-6 [51]. In our study, we found that TNF-α and IL-6 level in serum were elevated significantly in CCl₄ group as compared to the control group, whereas MEVZ treated group resulted in significant attenuation of the elevation [Fig. 1]. The effects were comparable with the standard group silymarin.

The histopathological studies are direct means for assessing the protective effect of MEVZ. The areas of necrosis, ballooning fatty degeneration of hepatocytes and clusters of inflammatory cells were observed in the CCl₄ group compared to control group. The group of animals treated with MEVZ and silymarin showed a recovery with only inconspicuous necrosis, very few inflammatory cells and fatty materials were present The results of the histopathological studies supported and well correlated with data obtained from evaluation of the serum biomarkers and stress parameters. The photomicrographs of the liver sections were given in Fig 2.
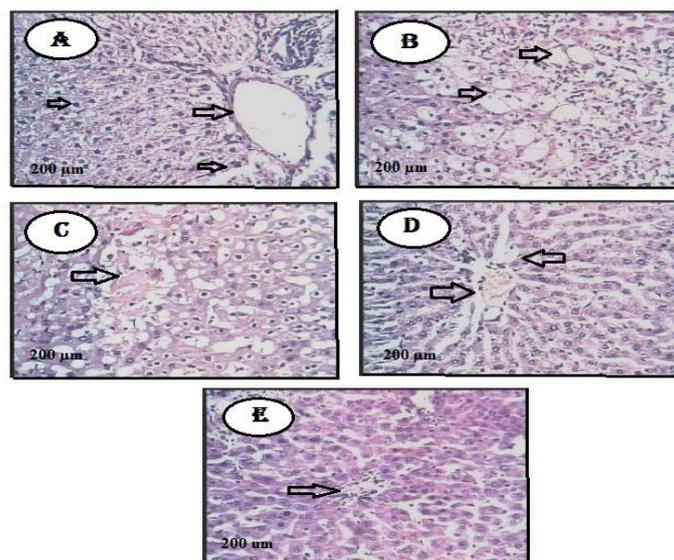
Fig 2: Photomicrograph of Rat Liver Obtained From Different Groups.

A: Control: *Normal liver architecture,* ***B: CCl₄ control:*** *Necrosis, ballooning fatty degeneration of hepatocytes and few clusters of inflammatory cells.* ***C: MEVZ-300+ CCl₄ control:*** *Moderate necrosis, inflammatory cells and fatty materials.* ***D & E: MEVZ-500+ CCl₄ control & Silymarin-500+ CCl₄ control:*** *Only inconspicuous necrosis, very few inflammatory cells and fatty materials.*

4. Conclusion

The acute toxicity study showed that *V. zizanioides* was not toxic to the experimental rats up to an oral dose of 5 g/kg body weight. Furthermore, the results of this study demonstrate that *V. zizanioides* has a hepatoprotective activity upon CCl₄-induced liver damage in rats comparable to the effects of silymarin, a standard drug used to treat liver diseases. Preliminary phytochemical analysis showed the presence of phytoconstituents such as flavonoids, terpenoids, glycosides and volatile oils which may be responsible for its hepatoprotective activity due to its well known antioxidant potential. All these results support the possibility of *V. zizanioides* being a therapeutic potential for acute liver damage, and accelerates liver regeneration by regulating the TNF-α and IL-6 mediated pathways. These results can be useful as a starting point of view for further applications of this plant or its constituents in pharmaceutical preparations after performing clinical researches.

Competing Interests
The authors declare that they have no competing interests.

Authors Contributions
MP and TG have performed experimental designed, literature search and animal treatment. MP and PS have carried out biochemical and statistical analysis as well as interpretation of the data. VT and PS participated in histopathological investigation. MP and SAR involve in writing of the manuscript. TG and PS have review and edited manuscript.

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