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Restorative effect of (5E, 13E)-5,13-Docosadienoic acid on carbon tetrachloride induced oxidative stress in rats

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ABSTRACT

Objective: To evaluate the restorative effect of (5E, 13E)-5,13-Docosadienoic acid on carbon tetrachloride induced oxidative stress in rats. **Methods:** Wistar strain male albino rats, weighing 180-200 g/bw were selected for the study. Rats were divided into four groups. Group I animals were served as normal control. Group II was administered with corn oil (3 ml/kg, *i.p.*) as vehicle control. Group III was given single dose (29th day) of CCl_4 in corn oil (1:1 v/v, 3 ml/kg, i.p.). Groups IV was treated with (5E, 13E)-5,13-Docosadienoic acid (DA) (6 mg/kg body weight) for 28 days and given single dose of (29th day) CCl_4 in corn oil (1:1 v/v, 3 ml/kg, i.p.). Six hours after CCl4 intoxication, the experimental animals were sacrificed. The blood samples were collected. Liver was excised immediately and immersed in physiological saline. **Results:** The lipid peroxidation was initiated in CCl4 intoxicated rats which is evidenced by thiobarbituric acid (TBARS) and diminution of GSH content in liver. Super oxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), vitamin C and E in CCl4 intoxicated rats retrieved towards near normalcy. After treating with DA which significantly altered (P<0.001) serum marker enzyme level and antioxidant level near normal against CCl_4 treated rats. **Conclusions:** It was observed that the entire variable tested i.e., SOD, CAT, GPx, reduced glutathione, vitamin C and E recorded a significant decline on CCl_4 treatment. However, treatment with DA restored the levels to near normal value, suggesting the therapeutic effect of DA to counter the oxidative stress.

1. Introduction

The rabid metabolic nature of CCl_4 highly induces the toxicity when it is administrated into living things. CCl_4 is bio transformed by the cytochrome P450 is a isoenzyme in endoplasmic reticulam to convert CCl4 into trichloromethyl radical (CCl_3•) in the liver after the initiation of lipid peroxidation. CCl_3• reacting with oxygen of cellular proteins and lipids produce a trichloromethyl peroxyl radical which attacks rabidly lipid membrane of endoplasmic reticulam than trichloromethyl free radical. It leads to reduced glutathione, accumulation of triacyl glycerol, Ca^{2+} and Na^{2+} influx and finally cell swelling in mitochondria which allows the mitochondrial membrane damage, reduced carbonylation of protein, loss of enzyme activity and cell death [1-3]. These result in changes of structure of the endoplasmic reticulum and other membrane, and loss of glucose-6-phosphatase activation, leading to liver damage [4-6].

The cells of living things continuously reacting with oxygen leads to produce free radicals which are responsible for carrying out of any biochemical process [7]. The most common Reactive oxygen species (ROS) include superoxide (O_2•-) anion, hydrogen peroxide (H_2O_2), peroxyl (ROO•) radicals, and reactive hydroxyl (OH•) radicals. The nitrogen-derived free radicals are nitric oxide (NO•) and peroxy nitrite anion (ONOO•). ROS have been a major factor for many diseases including arthritis, carcinogenesis, aging and acquired immunodeficiency syndrome [8].

The non-beneficial action of reactive oxygen species is nullified by broad class of protective agents called antioxidants it prevents the oxidative damage by reacting over the free radicals. Vitamin C, E and reduced glutathione etc., which are called as non-enzymatic antioxidents playing a greater role in the protection of tissues damage against free radical mediated damage. The enzymatic antioxidants (SOD, CAT, GSHPx) also involves to invading the free radicals [9,10]. The plant derived compounds which

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are having natural antioxidant properties may cure the cell damage. The plants are producing a lot of antioxidants to treat many diseases like diabetic, cancer and oxidative stress. So the plants are creating a novel way to produce a new compound with antioxidant activity to protect free radical induced damage in various experimental models [11]. The plant isolates protect the endogenous antioxidant defence system from ROS devastated actions by neutralizing it. The disease prevention action of these plant isolates is gaining the immense importance [12-14]. (5E, 13E)-5,13-Docosadienoic acid is the major leaf and root bulb chemical compound of the Hybanthus enneaspermus. (5E, 13E)-5,13-Docosadienoic acid has not been extensively worked out. Hence in the present study, an attempt has been made to create an animal model with oxidative stress using CCl4 and evaluate the therapeutic efficacy of (5E, 13E)-5,13-Docosadienoic acid.

2. Materials and methods

2.1. Chemicals

Carbon tetrachloride, thiobarbituric acid, 2,4-dinitro phenyl hydrazine, (5E, 13E)-5,13-Docosadienoic acid and glutathione were purchased from sigma chemicals, (Sigma Aldrich chemical Pvt. Ltd., Bangalore, Karnataka, India). All other reagents and chemicals used in this study were of analytical grade with high purity.

2.2. Animals

Wistar strain male albino rats, weighing 180-200 g were selected for the study. The animals were housed individually in polypropylene cages under hygienic and standard environmental conditions (28±2 ℃, humidity 60-70 %, 12 h light/dark cycle). The animals were allowed a standard feed and water ad libitum. They were acclimatized to the environment for one week prior to experimental use. All the animal experiments were duly approved by the Institutional Animal Ethics Committee (743/03/abc/CPCSEA dt 3.3.03) Guidelines (IAEC).

2.3. Determination of optimum dosage of (5E, 13E)-5,13-Docosadienoic acid

Initially, dose determination was conducted with (5E, 13E)-5,13-Docosadienoic acid.

Mice administered with (5E, 13E)-5,13-Docosadienoic acid at five different doses (2, 4, 6, 10 and 12 mg/kg body weight) to determine the optimum dosage. The dose of 6 mg showed significant (P<0.001) alteration in the activities of pathological marker enzymes such as aspartate transaminase (AST), Alanine transaminase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) in liver tissues, and the histological observations evidenced that (5E, 13E)-5,13-Docosadienoic acid effectively rescues the hepatocytes from CCl_4 induced oxidative damage without disturbing its cellular metabolic function and structural integrity. Hence, the dose of 6 mg/kg BW was chosen in this study.

2.4. Induction of oxidative stress

Oxidative stress through hepatic injury was created by intraperitoneal injection of CCl4 in corn oil (1:1 v/v, 3 ml/kg) [15]. The control animals received vehicle alone through intraperitoneal injection.

2.5. Experimental protocol

Rats were divided into four groups with six animals in each group. Group I animals were served as normal control. Group II was administered with corn oil (3 ml/kg, i.p.) as vehicle control. Group III was given single dose (29th day) of CCl4 in corn oil (1:1 v/v, 3 ml/kg, i.p.). Groups IV was treated with (5E, 13E)-5,13-Docosadienoic acid (6 mg/kg body weight) for 28 days and given single dose of (29th day) CCl4 in corn oil (1:1 v/v, 3 ml/kg, i.p.). Six hours after CCl4 intoxication, the experimental animals were sacrificed. The blood was collected with EDTA as anticoagulant. Serum was separated by centrifugation. Liver was excised immediately and immersed in physiological saline. It was suspended in 10% (w/v) ice-cold 0.1 M phosphate buffer (pH 7.4) and cut into small pieces. The required amount was weighed and homogenized using a Teflon homogenizer. Tissue homogenate and serum were used for the estimation of various biochemical parameters.

2.6. Biochemical analysis

Serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) activities were measured according to the method described by Reitman and Frankel [16]. MDA released from endogenous lipoperoxides, reflecting the lipid peroxidation process, were assayed in liver as described by Beuge and Aust [17]. The activities of antioxidant enzymes Cu/Zn SOD, Catalase and Glutathione peroxidase were determine by the methods of Kakkar et al., Beers and Sizer, Rotruck [18-20] respectively. The levels of non-enzymatic antioxidants such as GSH, Vitamin C and E were estimated by the method of Moron et al., Omaye *et al*., and Baker *et al*. [21-23] respectively. The protein content was estimated by the method of Lowry's *et al*. [24].

2.7. Histopathological studies

A portion of the liver was cut into two to three pieces of approximately 6 mm^3 size and fixed in phosphatebuffered 10% formaldehyde solution. After embedding in paraffin wax, thin sections of 5 lm thickness of liver tissue were cut and stained with haematoxylin-eosin. The thin sections of liver

were made into permanent slides and examined [25] under high resolution microscope with photographic facility and photomicrographs were taken.

2.8. Statistical analysis

Values were expressed as mean ± SD for six rats in the each group and statistical significant differences between mean values were determined by one way analysis of variance (ANOVA) followed by the Tukey's test for multiple comparisons [26]. Statistical analysis carried out by MS-Windows based graph pad Instat software (Graph Pad Software, San Diego, CA, USA) 3 version was used. A value of $P<0.001$ was considered statistically significant.

3. Results

The hybanthus ennespermus contains rich percentage of (5E, 13E)-5,13-Docosadienoic acid. Significant ($p<0.001$) rise was observed in the activities of liver marker enzymes Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) in serum (Table 1 and Figure 1) of group III CCl4 intoxicated rats as compared to group I and II control animals. Group IV CCl4 intoxicated rats treated with (5E, 13E)-5,13-Docosadienoic acid significantly reduced the release of these diagnostic marker enzymes into the systemic circulation as compared with group III rats. The concentration of MDA was significantly higher in liver of CCl4 treated rats, as compared to normal and vehicle control animals (Table 2 and Figure 2). These constituents were found to attain a near normal level in liver of CCl4 + (5E, 13E)-5,13-Docosadienoic acid treated rats. The activities of SOD, CAT and GPx recorded a significant decline in CCl4 administered rats, when compared with normal controls (Table 2 and Figures 3, 4, 5 and 13) . In CCl_4 + (5E, 13E)-5,13-Docosadienoic acid treated rats, the activities of these enzymes level is restored .The GSH , Vitamin C and E contents in liver of Group III animals showed (Table 2) a significant decline when compared with controls. But in Group IV animals these contents were found to attain near normalcy (Figures 6, 7, 8 and 13)

Table 1

Activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) content in serum of normal and experimental rats

Groups	Group I	Group II	Group III	Group IV
AST	45.30 ± 2.40	48.47 ± 2.72	133.29 ± 3.83[a]	52.12 ± 2.10 [b]
ALT	37.19 ± 2.20	43.61 ± 2.55	129.84 ± 3.45[a]	45.24 ± 2.32 [b]

Results were expressed as Mean ± SD for 6 animals. Values expressed: ALT and AST-μmol pyruvate liberated/h/liter in serum; MDA - nmol/dl; Reduced glutathione- mg/dl; [a]$P<0.001$ significantly different compared with Group I & II control animals. [b]$P<0.001$ significantly different compared with Group III animals.

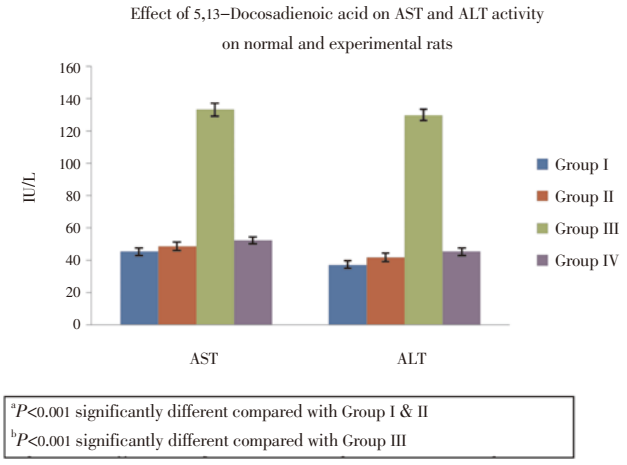

Figure 1. Effect of (5E, 13E)-5,13-Docosadienoic acid on AST and ALT activity on normal and experimental rats.

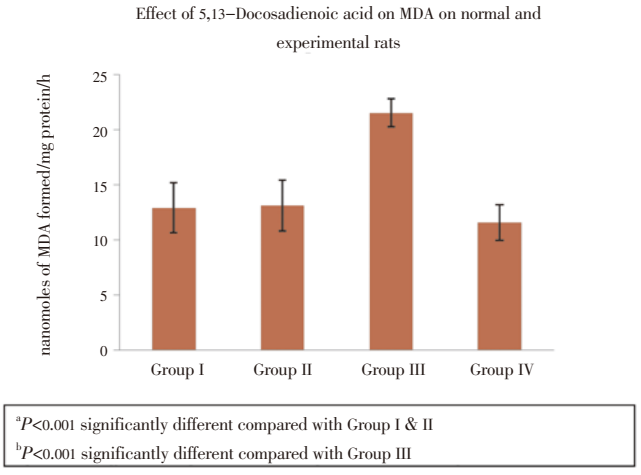

Figure 2. Effect of (5E, 13E)-5,13-Docosadienoic acid on liver MDA in control and experimental rats.

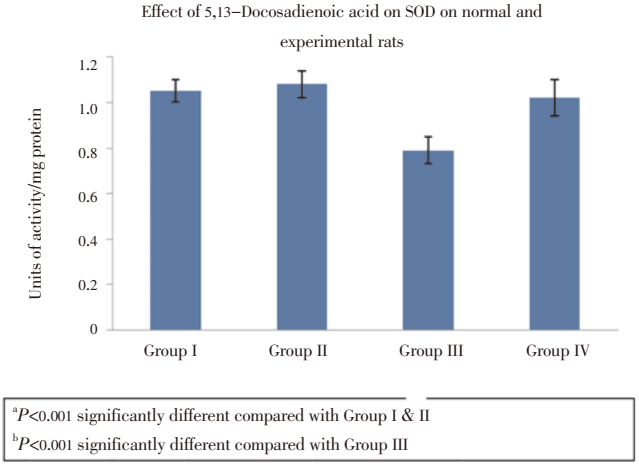

Figure 3. Effect of (5E, 13E)-5,13-Docosadienoic acid on liver SOD in control and experimental rats.

Table 2

Activities of MOD, SOD, CAT, GPX and GSH, vitamin C and vitamin E content in liver of normal and experimental groups of rats.

Groups	Group I	Group II	Group III	Group IV
MDA	12.9± 2.25	13.11± 2.32	21.51± 1.26[a]	11.59± 1.61[b]
SOD	1.05 ± 0.05	1.08 ± 0.06	0.79 ± 0.06[a]	1.02 ± 0.08[b]
CAT	4.25 ±0.05	4.27 ± .06	2.12 ± 0.03[a]	4.24 ± 0.05[b]
GPX	6.89 ± 0.2	6.91 ± 0.3	3.97 ± 0.6[a]	6.95 ± 0.6[b]
GSH	5.13 ± 0.73	5.89 ± 0.86	1.57 ± 0.56[a]	6.10 ± 0.94[b]
Vitamin C	0.75 ± 0.10	0.83 ± 0.12	0.52 ± 0.02[a]	0.73 ± 0.09[b]
Vitamin- E	5.08 ± 0.41	5.35 ± 0.62	3.50 ± 0.27[a]	5.00 ± 0.41[b]

Results were expressed as Mean ± SD for 6 animals. Values expressed: MDA-nanomoles of MDA formed/mg protein/h; SOD- Units of activity/mg protein; CAT- μmoles of H2O2 decomposed/mg protein/min; GSH- μg/mg protein; GPX - μgrams of glutathione utilized/min/mg protein; Vitamin C and Vitamin E - μmol/mg tissue. aP< 0.001 significantly different compared with Group I & II control animals. bP< 0.001 significantly different compared with Group III animals.

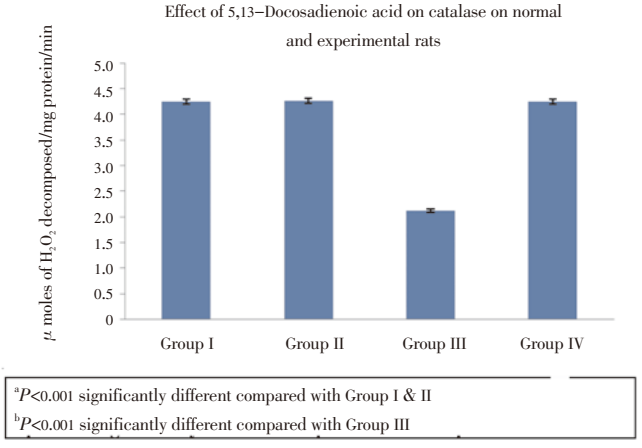

Figure 4. Effect of (5E, 13E)-5,13-Docosadienoic acid on liver catalase in control and experimental rats.

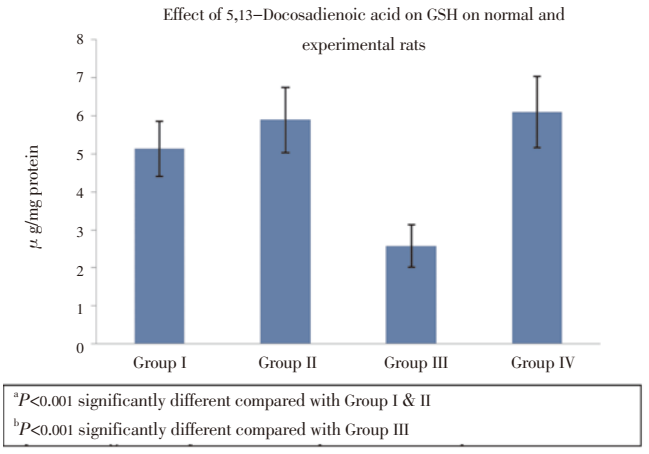

Figure 6. Effect of (5E, 13E)-5,13-Docosadienoic acid on liver GSH in control and experimental rats.

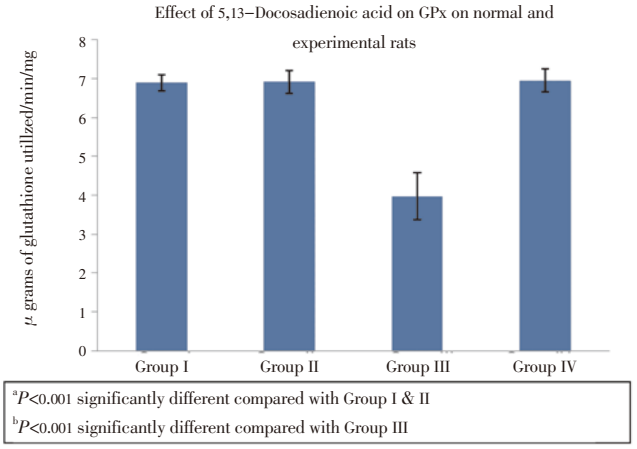

Figure 5. Effect of (5E, 13E)-5,13-Docosadienoic acid on liver GPx in control and experimental rats.

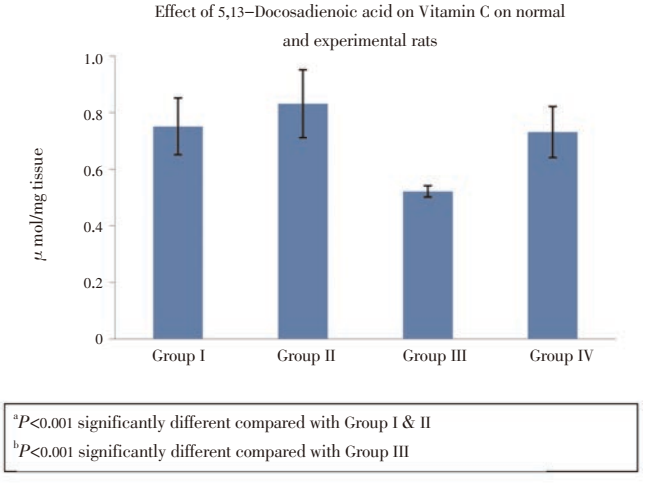

Figure 7. Effect of (5E, 13E)-5,13-Docosadienoic acid on liver Vitamin C in control and experimental rats.

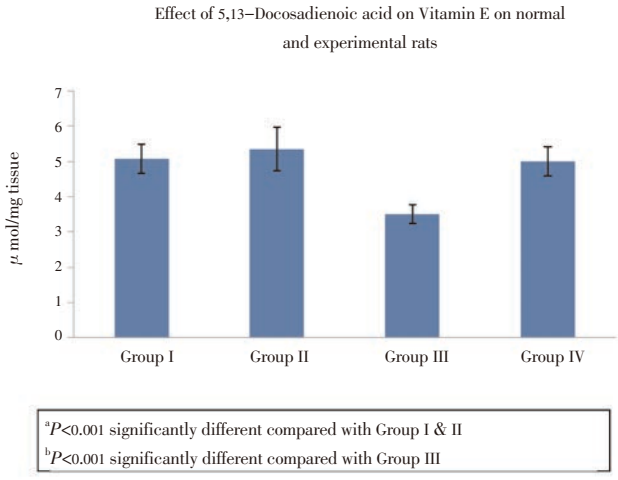

Figure 8 . Effect of (5E, 13E)-5,13-Docosadienoic acid on liver Vitamin E in control and experimental rats.

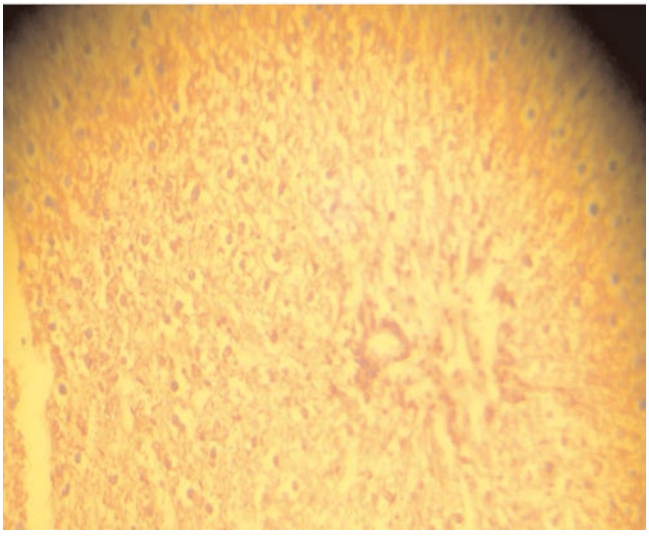

Figure 9. Photomicrograph of control rat liver section showing well brought central vein, hepatic cells with preserved cytoplasm and prominent nucleus at H & E X100.

3.1. Histopathological observations

The histopathological observation basically support the results obtained from serum enzyme assays. Histology of the liver sections of normal control animals (Group I) showed normal hepatic cells with well-preserved cytoplasm, prominent nucleus and nucleolus and well brought out central vein (Figure 9). The rat liver section vehicle control (Group II), showing moderately brought central vein, hepatic cells with preserved cytoplasm and prominent nucleus (Figure 10) The liver sections of CCl4-intoxicated rats showed (Group III) massive fatty changes, necrosis, ballooning degeneration, and broad infiltration of the lymphocytes and kupffer cells around the central vein and the loss of cellular boundaries (Figure 11). CCl4-induced group was more severe than the other groups. The histological architecture of liver sections (Group IV) of rats treated with (5E, 13E)-5,13-Docosadienoic acid (6 mg/kg) showed (Figure 12) a more or less normal lobular pattern with a mild degree of fatty change, necrosis and lymphocyte infiltration almost comparable to the normal control groups.

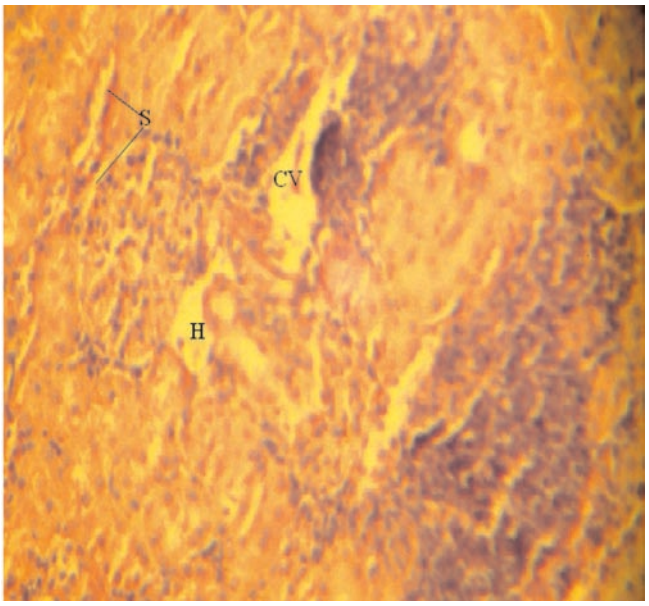

Figure 10. Photomicrograph of rat liver section vehicle control, showing moderately brought central vein, hepatic cells with preserved cytoplasm and prominent nucleus at H & E X100.sinosoidal space(S), hepotocytes(H) and central vein(CV).

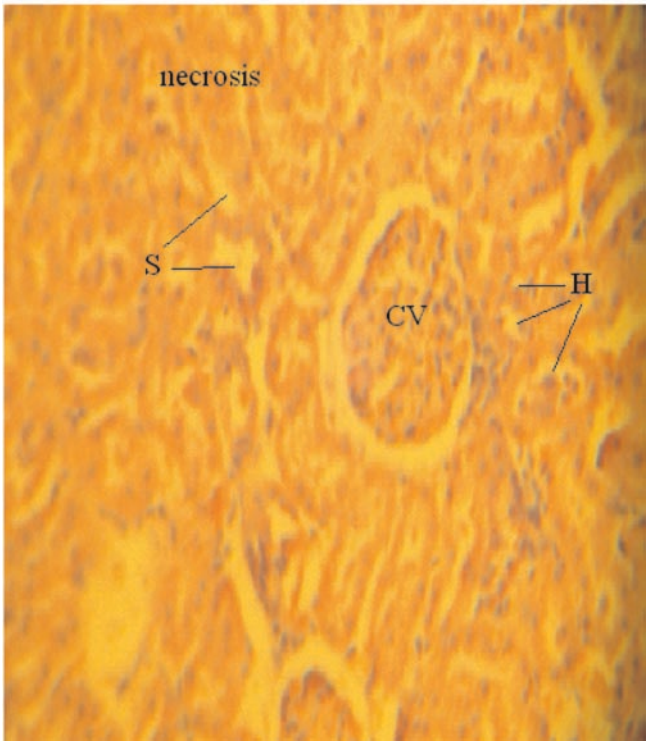

Figure 11. Photomicrograph of rat liver section with CCl4 treatment showing marked steatosis of the hepatocytes with ballooning degeneration & distended portal vein, mild periportal fibrosis and necrosis at H & E X100.

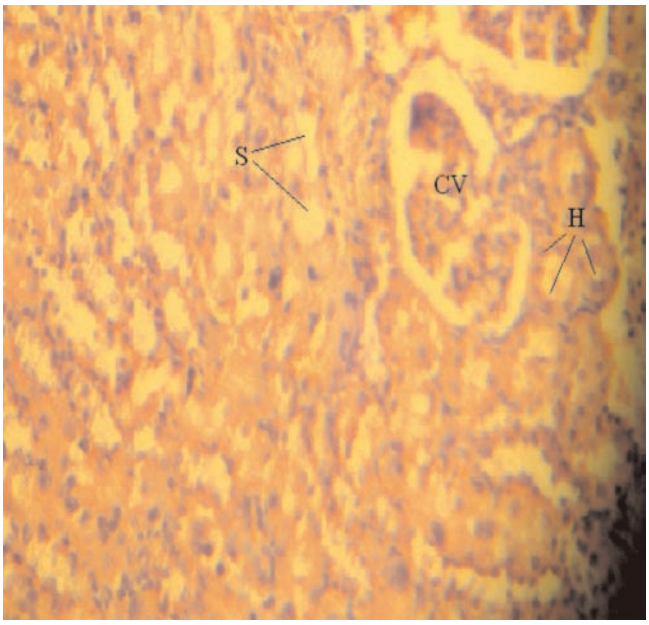

Figure 12. Photomicrograph of rat liver section of CCl4 + (5E, 13E)-5,13-Docosadienoic acid (6 mg/kg b wt), showing moderately regeneration in hepatocellular architecture at H & E X100.

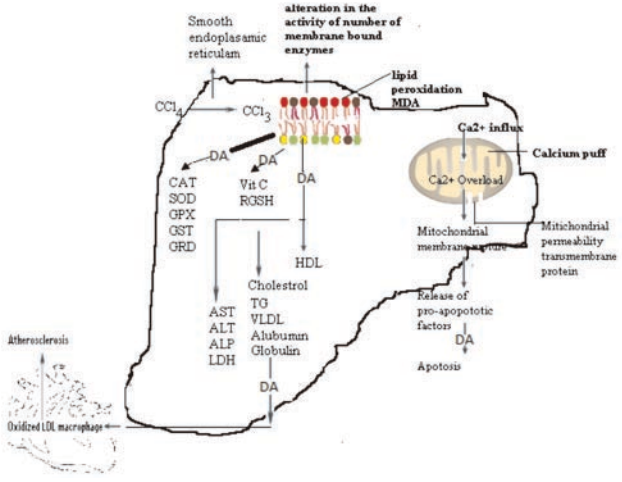

Figure 13. Restorative effect of (5E, 13E)-5,13-Docosadienoic acid(DA) on CCl_4 induced rats.

4. Discussion

Many experimental studies report that the involvement of oxidative stress in pathogenesis leads to several chronic diseases such as diabetes, cancer and cardiac disease [27]. The cells are reacting with oxygen so it is indispensable for maintaining life sometimes it leads to generate the most aggressive agents called as reactive oxygen species (ROS) are highly toxic to cells. The ROS induces a host of disorders in body due to its high reactivity resulting in tissue damage and necrosis in many instances [28]. The LPO is the main factor to cause the CCl_4-induced liver injury by free radical derivatives of CCl_4. Thus the generation of free radicals inhibition is important in the protection against CCl_4-induced hepatopathy [29]. CCl4 mediated oxidative stress was taken here as the experimental model for hepatotoxicity and oxidative stress studies.

The organ diseases are diagnosed by measurement of number of non-functional plasma enzymes characteristics. The release of enzyme depends on the intracellular concentration of the enzymes, degree of cellular damage and mass of affected tissues . The liver, heart, muscles and blood cells containing SGPT and SGOT. During liver cell damage the transaminases are released into blood stream where they can be measured. They are therefore index of liver injury [30]. Administration of (5E, 13E)-5,13-Docosadienoic acid to CCl4 intoxicated rats restored the level of SGOT and SGPT offering the maximum hepatoprotection with respect to different liver marker enzymes. It confirms that the (5E, 13E)-5,13-Docosadienoic acid is the majorly presenting chemical compound of Hybanthus enneaspermus which gives liver protection .

The lipid peroxidation studies are getting much attention in recent years due to its role in disease processes. LPO contains polyunsaturated fatty acids which easily affects the membrane lipids. It has been implicated in the pathogenesis of a number of diseases and clinical conditions [31]. Malondialdehyde (MDA) a commonly used biomarker of lipid peroxidation, which arises from the breakdown of lipid peroxyl radicals is one of the indicators of oxidative stress. Measured levels of MDA can be considered a direct index of oxidative injuries associated with lipid peroxidation [32]. In this context a marked increase in the concentration of MDA indicates oxidative stress in CCl_4 intoxicated rats when compared to control rats. Administration of (5E, 13E)-5,13-Docosadienoic acid significantly decreased the level of MDA and it demonstrates the reduction of oxidative stress in (5E, 13E)-5,13-Docosadienoic acid and CCl4 intoxicated rats.

GSH is a major non-protein thiol in living organism, which act against xenobiotics and neutralize the reactive oxygen species. GSH status disturbance in the biological system has been reported to lead to serious consequences [33]. Decline in GSH (Table 1) in the liver of CCl_4 intoxicated rats, and its subsequent return towards near normalcy in CCl_4 and (5E, 13E)-5,13-Docosadienoic acid treated rats reveal antioxidant effect of (5E, 13E)-5,13-Docosadienoic acid. Yibin Feng et al., 1987 explained the possible mechanism of hepatoprotective properties of drugs include the prevention of GSH depletion and destruction of free radicals [34].

The cell membrane damage is protected from oxidative stress by the combined action of Ascorbate (Vitamin C) and lipophilic antioxidant α-tocopherol [35]. Ascorbate convert the tocopherol radical to tocopherol [36]. In the present study, significantly decreased level of vitamin C and α-tocopherol in CCl_4 intoxicated rats, demonstrating the increased free radical accumulation in CCl_4 administered rats(Figures 6

and 7) are shown in glutathione level may contribute to the decrease in ascorbate as well tocopherol concentration. Supplementation of (5E, 13E)-5,13-Docosadienoic acid to CCl4 intoxicated rats improved vitamin C and α-tocopherol level as compared to control rats (Table 2), which may be due to increase in the GSH in (5E, 13E)-5,13-Docosadienoic acid treated rats improve the recycling of vitamin C and α-tocopherol. SOD suppresses the action of ROS derived from the peroxidative process of xenobiotics in liver tissues. In our study, the MDA and oxidative stress elicited by CCl_4 intoxication have been nullified due to the effect of (5E, 13E)-5,13-Docosadienoic acid. This observation perfectly agrees with those of Lin et al. [37] CAT plays an important role in antioxidant defense system. Suppressed action of this enzyme results in enhanced sensitivity to free radical-induced cellular damage [15]. In our study, decline (Table 2) in the activity of this enzyme in CCl_4 administered rats. Administration of (5E, 13E)-5,13-Docosadienoic acid increases the activities of catalase in CCl_4 induced oxidative stress rats to prevent the accumulation of excessive free radicals and protects the liver from CCl_4 intoxication. This observation agrees with those of Fatma *et al* [38]. GPx is a seleno-enzyme present in the cytosol and mitochondria. Its main role is preventing the oxidative DNA damage. In our study, GPx level was decreased in CCl_4 administered rats. The decreased GPx activity suggests that the (5E, 13E)-5,13-Docosadienoic acid have efficient protective mechanism in response to ROS. And also, these findings indicate that (5E, 13E)-5,13-Docosadienoic acid may be associated with decreased oxidative stress and free radical-mediated tissue damage.

5. Conclusion

It was observed that the entire variable tested i.e., SOD, CAT, GPx, reduced glutathione, vitamin C and E recorded a significant decline on CCl_4 treatment. However, treatment with (5E, 13E)-5,13-Docosadienoic acid restored the levels to near normal value, suggesting the therapeutic effect of (5E, 13E)-5,13-Docosadienoic acid to counter the oxidative stress. It can be suggested that the compound (5E, 13E)-5,13-Docosadienoic acid exhibit a greater action against CCl_4 induced oxidative stress and possessed anti-lipid peroxidative and antioxidant activities. This indicates that the lipid peroxidation and oxidative stress elicited by CCl4 intoxication has been nullified due to the effect of (5E, 13E)-5,13-Docosadienoic acid present in Hybanthus enneaspermus.

Conflict of interest statement

We declare that we have no conflict of interest.

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