



# Hepatoprotective effect of food preservatives (butylated hydroxyanisole, butylated hydroxytoluene) on carbon tetrachloride-induced hepatotoxicity in rat



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## ABSTRACT

Carbon tetrachloride (CCl<sub>4</sub>), a hepatotoxic agent is widely used to study the toxic mechanisms in experimental animals. This study was carried out to establish the hepatoprotective measures of food preservative antioxidants butylated hydroxyanisole and butylated hydroxytoluene (BHA, BHT) when mixed with food towards carbon tetrachloride (CCl<sub>4</sub>) intoxication (230 mg/kg b wt/rat/day) in rat. Biochemical markers like serum glutamate pyruvate transaminase (AST), serum glutamate oxaloacetate transaminase (ALT), alkaline phosphatase (ALP) and bilirubin content, antioxidant enzymes such as SOD, CAT, GPx, and malondialdehyde (MDA) as the end product of lipid peroxidation were measured. The results had shown the elevated level of AST (121.16%), ALT (124.68%), ALP (122.41%) and bilirubin content (57.14%) after CCl<sub>4</sub> treatment. Marked decrease of activity of antioxidant enzymes such as SOD (85.36%), CAT (67.47%), GPx (50.7%) had indicated that the ROS mediated toxicity and pretreatment of BHA and BHT restored the activity of these enzymes. High level of MDA content with reduced GSH value was also observed due to oxidative stress. The hepatic antioxidant status was restored with the food preservative (BHA, BHT) antioxidant treatment which had indicated the significant protective effect against CCl<sub>4</sub> induced hepatotoxicity and finally confirmed by histopathological studies.

## 1. Introduction

The liver is a vital organ, located in the upper right quadrant of the abdomen below the diaphragm and has a wide range of functions related to metabolism of carbohydrate, protein, lipid and xenobiotics which include gluconeogenesis, glycogenolysis, urea biosynthesis, production of plasma proteins and blood clotting factors, cholesterol biosynthesis, production of triglycerides and bile in addition to detoxification of various metabolites. Reactive oxygen species (ROS) resulting from oxidative stress (OS) are mainly by product of normal cellular metabolism. Altered cellular activities of electron transport system (ETS), cyclooxygenase, oxidases, peroxidases are main factors for production of increased amount of ROS due to an increased OS [1]. Liver is frequently exposed to a variety of xenobiotics, pesticides, organic solvents, anesthetics, and drugs. Carbon tetrachloride (CCl<sub>4</sub>) is a xenobiotic compound which produces hepatotoxicity in human beings and animals [2]. It appears in the environment specially in the water of industrial wastes from the manufacturing sector of chlorofluorocarbons,

dry cleaning fluids, fire extinguishing agents, etc [3]. Cytochrome p450 enzymes (mostly CYP2E1) of endoplasmic reticulum start its metabolism within the body and generate highly reactive trichloromethyl radical (CCl<sub>3</sub>·) which rapidly reacts with oxygen to form the highly reactive trichloromethylperoxy radical (CCl<sub>3</sub>OO·) [3]. The later molecule rapidly reacts with lipids (particularly PUFA) to form lipid peroxidation products. The free radical mediated lipid peroxidation is one of the main mechanisms of hepatic injury by CCl<sub>4</sub> [4,5]. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are GARS grade phenolic food preservative; are the structural analog of Vitamin E and most widely applied as synthetic antioxidants. They are mostly used in processed foods like, butter, meat, cereals, chewing gum, baked goods, snack foods, beer etc [6]. BHA, BHT have potential role to inhibit lipid peroxidation (LPO) and OS in many experimental models by restoring the cellular antioxidant enzyme status. Thus the present study was designed to investigate the hepato-protective effect of BHA and BHT on CCl<sub>4</sub> induced oxidative stress.

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## 2. Material and methods

### 2.1. Chemicals

Carbon tetrachloride, Tris buffer, Sodium chloride (NaCl), Triton-X 100, Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ), Dipotassium hydrogen phosphate ( $\text{K}_2\text{HPO}_4$ ), Ethylene diamine tetra acetate (EDTA), Sodium hydroxide (NaOH), Chloroform, Trichloro acetic acid (TCA), Thiobarbituric acid (TBA), Potassium hydroxide (KOH), Methanol, Alcohol, and other chemicals were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. 5', 5'-dithio (bis)-2- nitrobenzoic acid (DTNB), standard reduced glutathione (GSH), were procured from Sigma (St. Louis, MO, USA). All other chemicals were from Merck Ltd., SRL Pvt. Ltd., Mumbai and were of the highest purity grade available.

### 2.2. Selection of animals and care

The study was conducted on 24 healthy, Wister strain male albino rats (Supplied by CPSEA, Govt. of India registered firm) having a body weight of  $100 \pm 15$  g. These experimental animals were acclimatized in laboratory condition for period of 2 weeks prior to the experiment. Experimental animals were housed three rats/cage in a room with temperature  $22 \pm 2$  °C with 12–12 h dark–light cycles by the side of a humidity of  $50 \pm 10\%$ . Standard diet and water *ad libitum* were provided to them. The experimental animal care was provided according to the Guiding Principle for the Care and Use of Animals [7]. To carry out the experiments the rats were divided into four equal groups ( $n = 6/\text{gr}$ ) namely, Group I or control (feed normal diet with water *ad libitum*), Group II or  $\text{CCl}_4$  induced hepatic dysfunction (normal diet, water *ad libitum* + subcutaneous injection with ( $\text{CCl}_4$  at the conc. of 230 mg/kg body wt/rat/day diluted in olive oil) [8], Group III or  $\text{CCl}_4$  with pre-treatment by BHA [6] (pre-treated with 0.5 mg/kg BHA mixed with normal diet + subcutaneous injection with  $\text{CCl}_4$ ), and Group IV or  $\text{CCl}_4$  with pre-treatment by BHT [6] (pre-treatment of 0.8 mg/kg BHT along with normal diet + subcutaneous  $\text{CCl}_4$  injection).

#### 2.2.1. Sacrifice of animals and collection of blood and tissues

This experimental schedule for evaluation of protective function of selected antioxidants was continued for 28 days, after that the animals were sacrificed and blood was collected from the aorta, then liver and kidneys were collected for different biochemical and histological studies. The tissues were stored into  $-80$  °C until preparation of tissue homogenates. For histological examination, liver and kidneys were preserved in 10% neutral formaldehyde solution till processed.

#### 2.2.2. Separation of serum and homogenization of liver and kidney

Serum was separated by centrifugation ( $1500 \times g$ , 15 min) of blood samples and was kept ( $-80$  °C) [9] for the biochemical estimation of different parameters. Tissue homogenates were prepared through the following process; 1.5 g hepatic tissue was washed initially in 0.9% saline followed by immediate homogenization in the ice-cold buffer (0.25 M sucrose, 1 mM EDTA, and 1 mM Tris-HCl, pH 7.4) and then through centrifugation ( $600 \times g$ , 10 min at 4 °C). Later on, the supernatant was stored ( $-80$  °C) for the biochemical estimation of different parameters [10].

### 2.3. Biochemical determinations

#### 2.3.1. Biochemical markers of hepatotoxicity

Serum hepatic marker enzymes namely, serum glutamate oxaloacetate transaminases (AST), serum glutamate pyruvate transaminase (ALT) [11], alkaline phosphatase (ALP) [12], LDH [15], bilirubin [13], total protein [14] concentration were measured by using assay kits Sigma (USA). The extent of hepatocytes necrosis was determined by these activities as markers.

#### 2.3.2. Oxidative stress Profile

To evaluate the degree of cellular damage in hepatocytes, lipid peroxidation (LPO) in tissue homogenate was measured by estimating the formation of thiobarbituric acid reactive substances (TBARS). Tissue homogenate was mixed with 20% TCA (1.5 ml) and 1.34% TBA (1.5 ml) then boiled (30 min) and cooled, followed by an addition of 2.5 ml butanol. The mixture was centrifuged for 5 min in  $2000 \times g$ . Then optical density of the supernatant was measured at 535 nm. TBARS as malondialdehyde (MDA) content of the sample was calculated by using the molar extinction coefficient  $1.43 \times 10^{-3} \text{M}^{-1} \text{cm}^{-1}$  and expressed as nmol of MDA formed/mg protein [16].

The estimation of GSH from tissue homogenate was done by the method of Ellman. The reaction mixture contained 25% of TCA and then centrifugation ( $2,000 \times g$ , 15 min); supernatant was diluted to 1 mL with 0.2 M sodium phosphate buffer (pH 8.0) followed by addition of 2 mL DTNB (0.6 mM). After 10 minutes incubation at room temperature, the optical density of the yellow-colored complex formed by the reaction of GSH and DTNB (Ellman's reagent) was measured at 405 nm. The levels of GSH were expressed as  $\mu\text{g}$  of GSH/mg protein [17].

#### 2.3.3. Antioxidant Enzyme Profile

Activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) were assayed from hepatic tissue homogenate for assessment of cellular antioxidant enzyme status.

SOD activity of liver and kidney homogenate was determined from its ability to inhibit the auto-oxidation of pyrogallol according to Mestro and McDonald et al. [18]. The samples were measured at 420 nm at 25 °C for 3 min. SOD activity was expressed as unit/mg protein.

CAT activity of these tissue homogenate was measured by the method of Luck [19]. Catalase activity was calculated by using the molar extinction coefficient of  $43.6 \text{M}^{-1} \text{cm}^{-1}$  for  $\text{H}_2\text{O}_2$ . The level of CAT was expressed as unit/mg protein.

The GPx activity of liver and kidney homogenate was evaluated by the method of Paglia and Valentine [20]. Absorbance at 340 nm was recorded for 5 min. Values were expressed in nmol of NADPH oxidized to NADP in per min/mg protein by using the extinction coefficient of  $6.2 \times 10^3 \text{M}^{-1} \text{cm}^{-1}$  at 340 nm.

#### 2.3.4. Histological Study

Histological analysis of liver and kidney tissue of each experimental Group was performed by the method of Iranloye and Bolarinwa [21]. The animals were sacrificed and the tissues were immediately perfused in 0.9% saline and formalin, and were fixed for 7 days in 10% formaldehyde. After that, dehydration was carried out in ascending grade of alcohol (70%–100%). To remove the alcohol, the tissues were kept in xylene overnight. Then embedding and casting in paraffin wax with wooden block was completed and sectioning of  $5 \mu\text{m}$  thick was carried out by using a microtome. The sectioned tissues mounted on slides using a thin film of egg albumen smeared on each side. After deparaffinization by using xylene, the sections passed through alcohol, stained with haematoxylin-eosin, and mounted in neutral DPX medium. The slides were then evaluated for pathological changes under microscope.

### 2.4. Data analysis

The data were calculated and statistical analyses were done by using a statistical package, Origin 6.1, Northampton, Mass, USA. The statistically calculated data were expressed as mean  $\pm$  SEM,  $n = 6$ . Comparisons were done between the means of control and  $\text{CCl}_4$  administered group, by one way ANOVA,  $P < 0.05$ , level of significance.

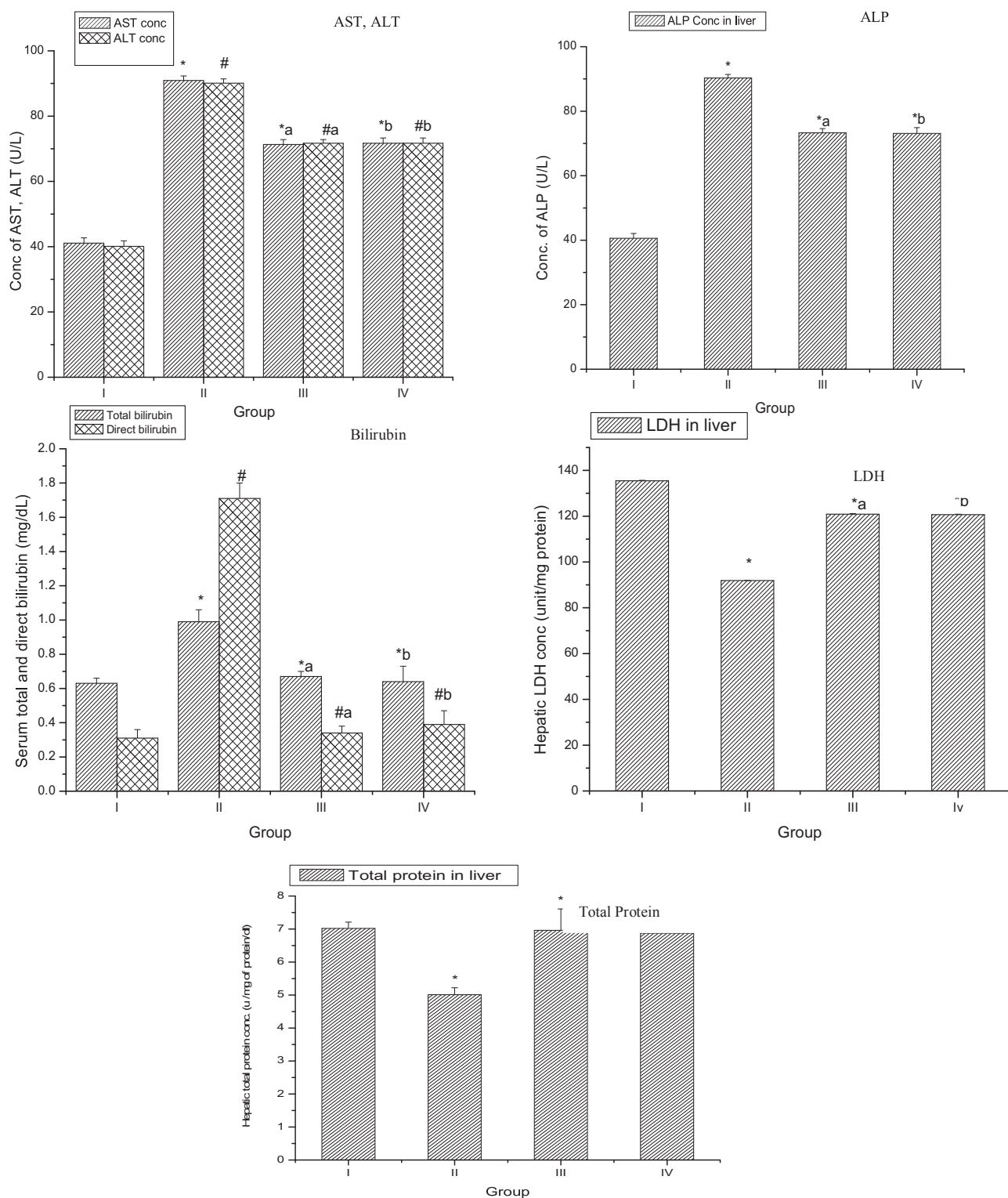


Fig. 1. Groupaphical presentation represents the AST, ALT, ALP, Total Protein, and LDH value of experimental groups. Values are expressed as mean ± SEM, n = 6. \*,# indicates significant difference (P < 0.05) compared to control Group. Group I: Control, Group II: CCl<sub>4</sub> treated, Group III: CCl<sub>4</sub> + BHA, Group IV: CCl<sub>4</sub> + BHT.

### 3. Result

#### 3.1. Biochemical markers of hepatotoxicity

In this present study, hepatotoxicity was started after administration of 230 mg/kg body wt/rat/day [8] followed by its metabolic activation. The mean value of serum AST, ALT, ALP, LDH, and total protein content of hepatic tissue had been increased significantly (p < 0.05) by

121.168%, 124.68% and 122.41%, 32.16%, 28.63% respectively in CCl<sub>4</sub> treated Group compared to control group (Fig. 1). Intoxication of CCl<sub>4</sub> showed a significant (P < 0.05) rise in serum bilirubin (total and direct) 57.14% and 45.16% respectively. However, pretreatment of BHA and BHT (Group III, IV) significantly (p < 0.05) recovered the level of serum enzymes like AST (73.47%, 78.80%), ALT (78.80%, 78.80%), ALP (80.54%, 80.54%), LDH (10.74%, 10.89%) respectively. Beside these, total protein in tissue homogenate (13.67%, 13.53%) as

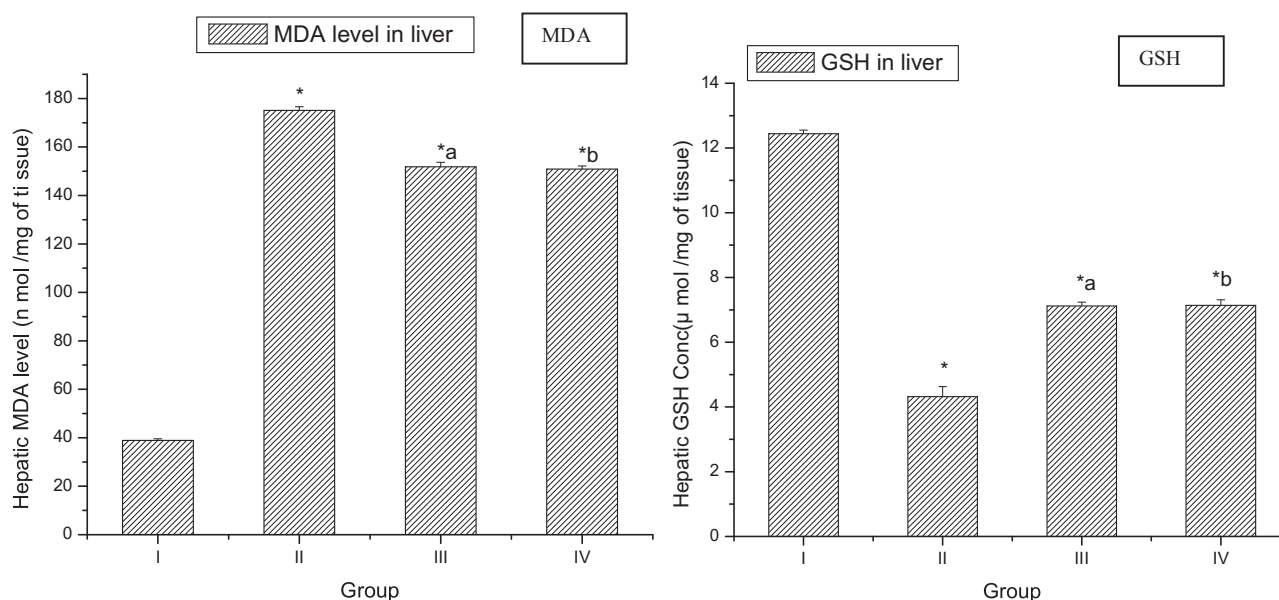


Fig. 2. Groupwise presentation represents the MDA and GSH value of experimental groups. Values are expressed as mean  $\pm$  SEM,  $n = 6$ . \*,# indicates significant difference ( $P < 0.05$ ) compared to control Group. Group I: Control, Group II: CCL<sub>4</sub> treated, Group III: CCL<sub>4</sub> + BHA, Group IV: CCL<sub>4</sub> + BHT.

well as serum bilirubin level (total, 1.34%, 1.58% and direct, 9.67%, 9.89%) had been improved in respect of CCL<sub>4</sub> treated Group (Fig. 1).

### 3.2. Oxidative stress marker

A marked increase (350.20%) in MDA levels as end product of lipid peroxidation was found in the hepatic tissue homogenate of CCL<sub>4</sub> intoxicated experimental rats when compared to the control group (Fig. 2). Supplementation of BHA, BHT on experimental animals had shown a significant ( $P < 0.05$ ) lowering effect on MDA levels (290.23%, 287.91%) more than CCL<sub>4</sub> treated group (Fig. 3). Significant reduction ( $p < 0.05$ ) of reduced glutathione (GSH) content by 65.27% had been seen in hepatic tissue of CCL<sub>4</sub> treated rats (Fig. 2) and synthetic antioxidant treatment had helped to restore the intracellular glutathione level by 42.76%, 42.60% (Fig. 2).

### 3.3. Antioxidant enzymes

Administration of CCL<sub>4</sub> had significantly diminished 65.27% of glutathione peroxidase (GPx) activity in hepatic tissue homogenate as compared to control group ( $p < 0.05$ ). Although, in antioxidant pretreated group, there was significant increase ( $p < 0.05$ ) in hepatic GPx levels (29.9%, 28.9%) in respect of CCL<sub>4</sub> treated Group (Fig. 3). Other detoxifying enzyme like superoxide dismutase (SOD) and catalase (CAT) had significantly ( $p < 0.05$ ) decreased by 85.36% and 67.47% respectively in hepatic tissue homogenate due to CCL<sub>4</sub> intoxication. Whereas, treatment of BHA, BHT were able to significant increase in SOD (42.19%, 42.68%) and CAT (13.73%, 14.63%) activity in liver tissue as compared to CCL<sub>4</sub> treated group (Fig. 3).

### 3.4. Histopathological examinations

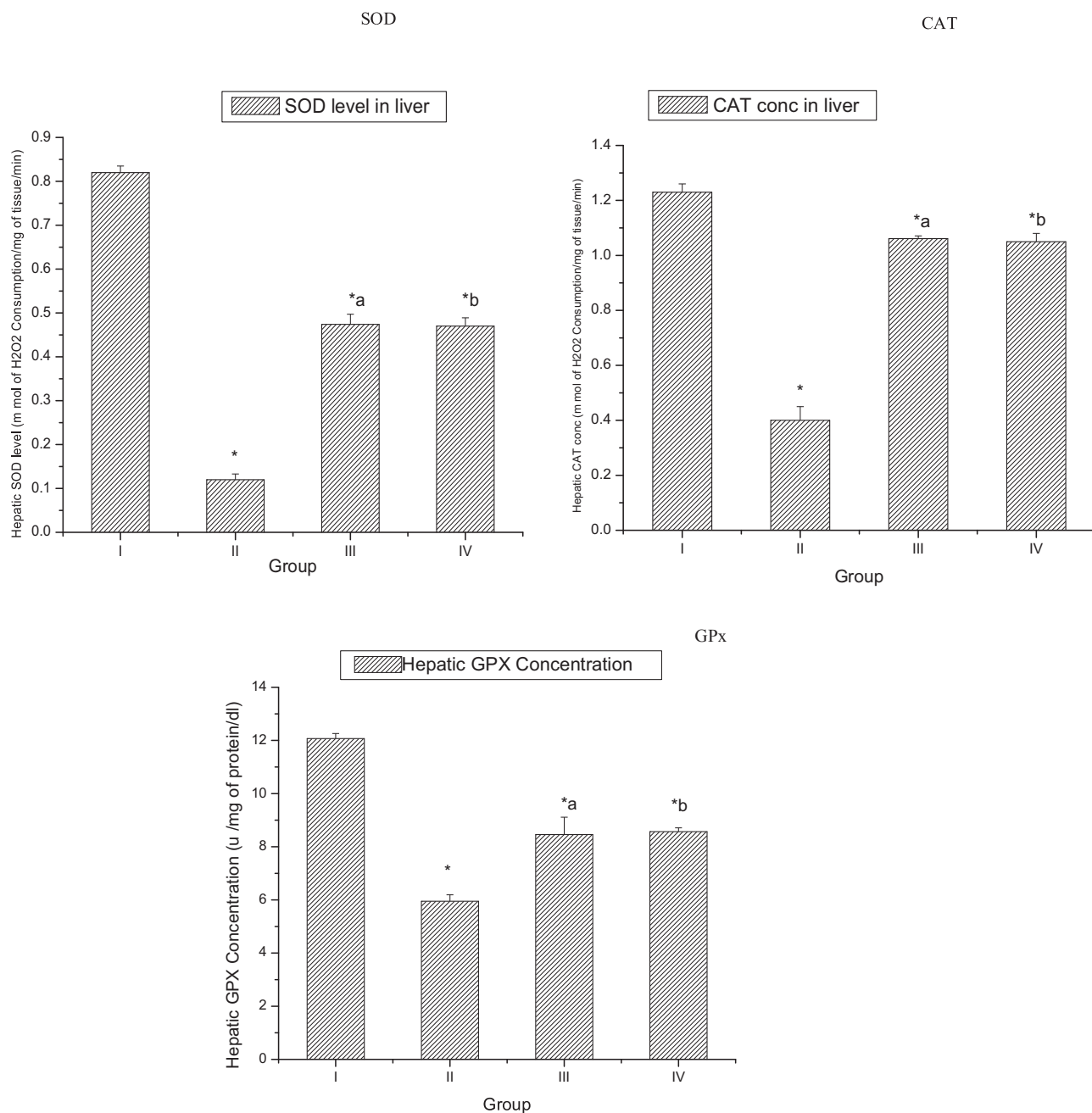
Marked changes had been observed in CCL<sub>4</sub> (230 mg/kg body wt/day) treated group compared to control group and BHA, BHT supplementary group. The histopathological studies had shown the accumulation of fat droplets, fatty degeneration, cellular necrosis, portal vein disruption after CCL<sub>4</sub> intoxication (Fig. 4, Table 1). Supplementation of synthetic antioxidants had minimized tissue toxicity (Fig. 4, Table 1). Histology of the liver sections of control animals showed normal hepatocellular architecture along with well preserved lobular pattern and visible central vein without any sign of necrosis. However, liver sections

of CCL<sub>4</sub> treated rat showed lipid accumulation, massive cellular necrosis, portal vein disruption which indicates loss of cellular architecture due to excessive intracellular lipid deposition.

## 4. Discussion

Carbon tetrachloride (CCL<sub>4</sub>) a lipophilic potent hepatotoxin can be able to cross the cell membrane and gets distributed throughout tissues soon after its exposure. Cytochrome P<sub>450</sub> starts biotransformation of CCL<sub>4</sub> and generates reactive metabolite trichloromethyl radical ( $\cdot\text{CCl}_3$ ); later is converted to trichloromethyl peroxyradical ( $\text{CCl}_3\text{OO}\cdot$ ) in presence of oxygen [22]. This highly reactive free radical causes cellular damage and the toxic effect can diminish by supplementation of hepatoprotective agents like natural antioxidants (Vit. C, Vit. E) or synthetic antioxidants (BHA, BHT). The efficacy of any hepatoprotective agents depend on their capacity of minimizing harmful effects and maintenance of intracellular physiological functions [6]. Our result had shown the elevated level of AST, ALT and ALP. The evidence of hepatic damage and determination of serum enzymes like AST, ALT and ALP was reported by several workers [8,6,23]. They reported that the level of increase of AST (120.29%, 70.3%, 155%), ALT (139.95%, 118.3%, 344.44%), and ALP (106.99%, 9.14%) varies respectively in rats (Sprague Dawley) after CCL<sub>4</sub> treatment and these findings are very similar to our observation. Reduction of serum transaminases close to normal levels after supplementation of mushroom extract, BHA and BHT as food antioxidant in CCL<sub>4</sub> treated groups suggested healing of hepatic parenchymal cells and regeneration of hepatocytes [6,2]. The other biochemical markers like LDH, bilirubin, and total tissue protein had estimated to evaluate liver performance and an increased level of these parameters had been shown in CCL<sub>4</sub> treated group. Previously, higher level of LDH, bilirubin, and total protein were also reported by [15,2,23,24]. Basically BHA and BHT are the synthetic analogues of Vit. E and their functions are very similar to the native vitamin. These molecules are converted to their respective radicals and stop the chain reaction during lipid peroxidation. Treatment of BHA, BHT protects the membrane of hepatocytes by scavenging the free radicals as well as LPO that leads to repair of hepatic tissue and prevents the leaching of intracellular transaminases to the serum. These food preservative antioxidants were being able to minimize disintegration of hepatic cells and rejuvenate hepatocytes.

The metabolic component of CCL<sub>4</sub>, ( $\cdot\text{CCl}_3$ ) had tended to increase



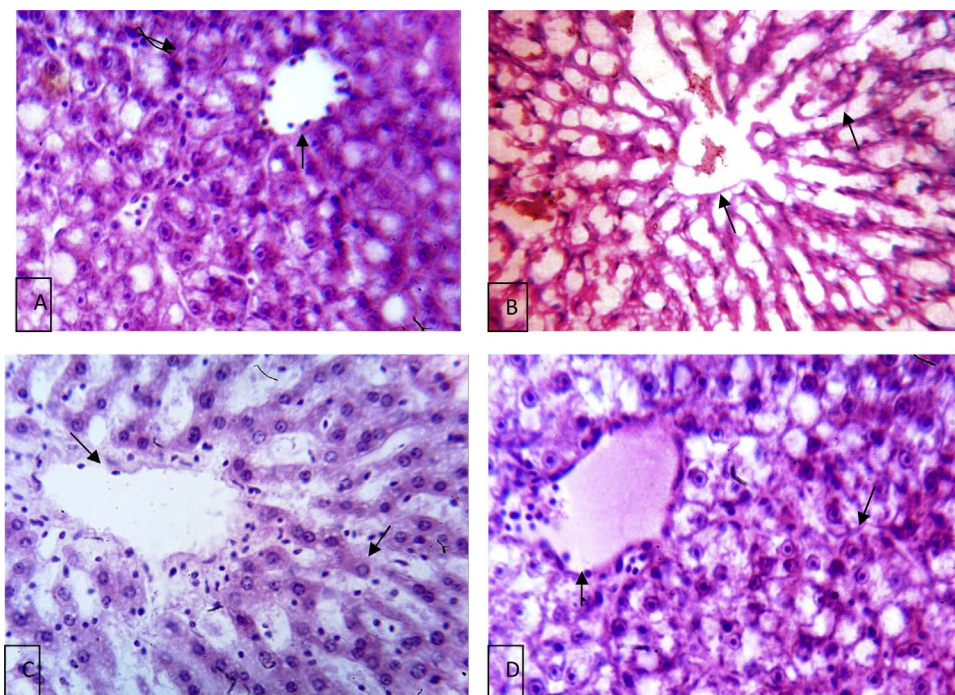
**Fig. 3.** Groupwise presentation represents SOD, CAT and GPx value of experimental group. Values are expressed as mean  $\pm$  SEM, n = 6. \*,# indicates significant difference ( $P < 0.05$ ) compared to control Group. Group I: Control, Group II: CCl<sub>4</sub> treated, Group III: CCl<sub>4</sub> + BHA, Group IV: CCl<sub>4</sub> + BHT.

MDA level as end product of lipid peroxidation [3] because  $\cdot\text{CCl}_3$  stimulates ROS generation and oxidative stress (OS) which promote lipid peroxidation (LPO) in hepatic cells [1]. LPO is the result of low antioxidant defenses and formation of excess amount of ROS. Intracellular antioxidant enzyme GPx plays an important role in protection of cells by detoxification reactions against LPO mediated OS [25]. The results of histological studies had revealed the LPO mediated membrane damage, lipid accumulation, alteration of cytoarchitecture and necrosis of the hepatic cells. Elgazar [6] had also shown the CCl<sub>4</sub> mediated changes of cytoarchitecture of the hepatic cells which were very similar to our results. He concluded that  $\cdot\text{CCl}_3$  increases the rate of lipid peroxidation as well as membrane damage in hepatocyte; the outcomes are lipid accumulation, fatty degeneration, inflammatory responses and necrosis. BHA, BHT are able to exert protective functions because antioxidants, synthetic or natural act as scavenging molecule against ROS, reduce the

rate of LPO as well as MDA formation and ultimately act as protective agents [26]. Other reports suggested that antioxidant rich food supplementation lowers the LPO level [27,28].

Cellular antioxidants always exert a defense mechanism against continuously producing ROS within the cell by scavenging the toxic molecules [29]. Cytosolic tripeptide glutathione, ubiquitously present in all cells acts as a key indicator of nonenzymatic intracellular redox homeostasis [30]. Our result had shown depletion of GSH level in experimental animals and its restoration by pretreatment of antioxidant. Earlier several researchers [8,1,6,26,23] reported that CCl<sub>4</sub> breaks the intracellular redox homeostasis and lowers the GSH level which also serves as a substrate for GPx [31]. In normal condition, GPx catalyzes oxidation of 2GSH to its oxidized state GSSG which is reduced back to GSH by GR (glutathione reductase) and eventually replenishes the cellular GSH level. The improvement of intracellular GSH level was also





**Fig. 4.** Histological structure of liver tissue Section A- Group I: Control, Section B- Group II: CCl<sub>4</sub> treated, Section C- Group III: CCl<sub>4</sub> + BHA, Section D- Group IV: CCl<sub>4</sub> + BHT.

**Table 1**

Liver histological changes in different experimental groups (The Ishak system (a six-point scale for fibrosis stage) was applied for scoring hepatotoxicity. Control, Group II: CCl<sub>4</sub> treated, Group III: CCl<sub>4</sub> + BHA, Group IV: CCl<sub>4</sub> + BHT.

Parameters	Group I	Group II	Group III	Group IV
Cellular necrosis	0	6	0	0
Hepatocyte degeneration	0	6	0	0
Portal vein disruption	0	4	0	0
Lipid Accumulation	0	5	1	1

possible by supplementation of antioxidant rich foods in animal model [32,33].

Detoxifying enzymes like SOD, CAT, GPx help to control the formation of ROS such as superoxide radicals ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radical ( $OH^{\cdot}$ ) within the cell [34]. Previously, Tirkey et al. [23] reported that CCl<sub>4</sub> intoxication reduced the endogenous antioxidant enzymes like SOD, CAT, GPx. The present study had indicated the marked reduction of antioxidant enzymes (SOD, CAT, GPx) in hepatic tissue and pretreatment of BHA, BHT re-established the activity of these enzymes to their near normal value. Several studies had demonstrated the fall of antioxidant enzymes (SOD, CAT, GPx) activity in liver, [35] kidney and brain [36,37] following CCl<sub>4</sub> exposure. While other reports revealed that exogenous supplementation of antioxidants enhanced GSH, GPx, SOD, CAT level in experimental rat intoxicated with CCl<sub>4</sub> [38,6]. In normal cells, reactive species are constantly formed which are scavenged by intracellular antioxidant enzymes [29]. In fact SOD converts the superoxide radicals ( $O_2^{\cdot-}$ ) to hydrogen peroxide ( $H_2O_2$ ) via dismutation and later is removed by the activity of CAT and GPx. Any impairment in this pathway will disturb the intracellular redox homeostasis; resultant is excess LPO and other detrimental effects [39,40]. Cellular antioxidants resist ROS generation while, antioxidant enzymes enhance metabolic detoxification and protect the tissue [41–43].

Actually CCl<sub>4</sub> was converted to more toxic radical  $\cdot CCl_3$  through the enzymatic reaction of a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzyme CYP450 (specifically, CYP2E1), which was further metabolized to  $\cdot OOCCL_2$  [3]. Later was a potent free radical, produced severe oxidative stress.  $\cdot OOCCL_2$  reacted with

polyunsaturated fatty acids of membrane lipids and bound with their unsaturated units. This reaction converted the membrane lipids (RH) to lipid macro radical ( $ROO^{\cdot}$ ) in presence of oxygen and continuously operated the chain reactions of lipid peroxidation. Finally,  $ROO^{\cdot}$  was modified to hydroperoxide (ROOH) or endoperoxide followed by malondialdehyde. The effects of severe OS and LPO emerged as a result of decreased of several OS related markers like SOD, CAT, GPx from their respective standards. These cellular antioxidants were categorized to two groups: I) preventive antioxidants like catalase, peroxidases which reduced the rate of chain reactions by reacting with ROOH and II) chain breaking antioxidants SOD, Vit E or its analogue like BHA, BHT which interfered with chain propagation. SOD trapped the superoxide free radicals in aqueous phase while BHA and BHT trap the  $ROO^{\cdot}$  in lipid phase. Thus the present study had indicated that supplementation of BHA, BHT successfully ameliorated degenerative changes due to CCl<sub>4</sub> induced hepatic toxicity. Finally it is concluded that dietary intake of these antioxidants with food formulations may be beneficial for patients with hepatic dysfunction.

#### Conflict of interest statement

The authors declare that there are no conflicts of interest.

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