Effects of Butylated Hydroxytoluene and Butylated Hydroxyanisole Against Hepatotoxicity Induced by Carbon Tetrachloride in Rats

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Abstract: Butylated Hydroxytoluene (BHT) and butylated Hydroxyanisole (BHA) are synthetic phenolic compounds commonly used as antioxidant food preservatives. The main objective of the present study was to investigate the protective effect of BHT and BHA against carbon tetrachloride - (CCL$_4$) hepatotoxicity in rats. The experiment was performed on 42 mature rats distributed into 6 equal groups. One group was kept as control, while the second was rendered hepatotoxic by S/C injection of CCL$_4$ during the last week of experiment. The remaining 4 groups were pretreated with BHA at 0.25 and 0.5mg/kg^{-1} and BHA at 0.4 and 0.8 mg/kg^{-1} given orally for 4 weeks and co-administered CCL$_4$ in the last week. On the next day, the rats were euthanized by ether and blood was collected for estimating serum liver enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP), total cholesterol, (TC) and triglycerides (TG). Tissue lipid peroxidation product malondialdehyde (MDA), reduced glutathione (GSH) and antioxidant enzymes glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) were determined. Histopathology of liver was also performed. The pretreatment with BHT and BHA significantly decreased the elevated serum levels of AST, ALT, ALP, TC and TG in CCl$_4$-intoxicated rats. It also decreased the elevated MDA content and increased the low content of GSH and the activity of GPx, SOD and CAT enzymes in liver of hepatotoxic rats. These biochemical alterations were accompanied by amelioration of degenerative changes seen in liver of intoxicated rats. The effect of BHT and BHA against CCl$_4$-induced liver damage was attributed to their antioxidant properties. In conclusion, the use of BHT and BHA in food formulations may be beneficial to patients who suffer from liver diseases due to oxidative stress.

Key words: Butylated Hydroxytoluene · Butylated Hydroxyanisole · Hepatoprotection · Lipid peroxidation · CCL$_4$ - intoxicated rats

INTRODUCTION

Butylated Hydroxytoluene (BHT) and butylated Hydroxyanisole (BHA) are synthetic monocyclic phenolic compounds. They are commonly used in many food formulations as food preservatives for their antioxidant properties. The toxic effects of BHT and BHA were reviewed by Kahl and Kappus [1] who concluded that concentrations of both BHT and BHA which used in foods, drugs and cosmetics are harmless and their toxic effects only may take place after high dosage and long-term treatment. The pretreatment with BHT and BHA produced anticancer activity via decreased DNA-2-aminofluorene adducts in liver, bladder and leukocytes [2]. The later author concluded that both BHT and BHA

decreased N-acetylation of carcinogens and formation of DNA-carcinogen adducts in vivo. BHA and BHT are safe as food preservatives when added to foods at recommended concentrations and produce no cancer hazard. Furthermore, BHA and BHT may be anticarcinogenic at current levels of food additive use [3]. The mechanism of anticarcinogenic effect of BHT and BHA appear to be possibly through interception of the reactive chemical species of the carcinogen [4]. In addition, BHT and BHA were found to have the potential to inhibit lipid peroxidation and oxidative rancidity in cooked and frozen pork patties [5].

Liver is the key organ of metabolism and excretion. It is often exposed to a variety xenobiotics and therapeutic agents. The hepatotoxin carbon tetrachloride

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(CCl$_4$) is frequently used to induce liver fibrosis in animal models [6]. CCl$_4$ is a selective hepatotoxic chemical agent which produces reactive free radicals that initiate cell damage through two mechanisms of covalent binding to the membrane proteins and causing lipid peroxidation [7]. Production of free reactive radicals by reductive metabolites of CCl$_4$ is believed to increase lipid peroxidation which is associated with hepatic cell damage and leads to liver cirrhosis and fibrosis [8]. Previous studies revealed that the toxic effects of CCL$_4$ can be partially prevented by antioxidant compounds such as Silymarin [9], α- tocopherol [7], Salvianolic acid [10] and Diphenyl diselenide [11]. Therefore, the aim of the present study was to investigate the hepatoprotective effect of butylated Hydroxytoluene and butylated Hydroxyanisole against acute liver injury induced by CCL$_4$ in rats.

MATERIALS AND METHODS

Rats: Forty two adult male rats of Sprague Dawley strain weighing 155 -160 g b.wt. and 12-14 weeks old were used in the study. The rats were obtained from the Laboratory Animal Colony, Helwan, Egypt. The animals were housed under hygienic conditions in plastic cages which contain wood shavings. The animals were kept at a room temperature of 25 ± 2°C with relative humidity of 50-60% and on 12 h light / 12 h dark cycle in Laboratory Animal House at Agricultural Research Center, Giza, Egypt. The rats were provided free access of basal diet and water. The rats were allowed to acclimatize to the laboratory environment for 7 days before start of the experiment.

Preparation of Basal Diet: Basal diet was prepared according to Reeves *et al.* [12]. It consists of 20 % protein, 10 % sucrose, 4.7% corn oil, 2% choline chloride, 1% vitamin mixture, 3.5 % salt mixture and 5% fibers. The remainder was corn starch up to 100 %.

Chemicals: Butylated Hydroxytoluene (BHT) and butylated Hydroxyanisole (BHA) were supplied from Sigma Chemical Company (St. Louis, MO, USA). Both chemicals were dissolved in corn oil as a solvent as they are insoluble in water.

Carbon tetrachloride (Ccl$_4$) was obtained from El-Gomhoryia Company for Chemical Industries, Cairo, Egypt as 10 % solution packed in white plastic bottles. It was diluted with corn oil before use (1:1, Volume: Volume).

Experiment and Grouping of Rats: The experiment was carried out on 42 mature male rats randomly distributed into 6 equal groups. Group (I) was fed on basal diet and kept as non treated control (vehicle, corn oil), while group (II) was subcutaneously injected with CCL$_4$ at 1.5 ml.kg^{-1}/rat/day during the last week of the experiment (4 weeks) to induce acute liver injury as described by Wasser *et al.* [13]. Groups (III), (IV), (V) and (VI) were pretreated with BHT at 0.25 and 0.5 mg.kg^{-1} and BHA at 0.4 and 0.8 mg.kg^{-1}, respectively and treated with CCL$_4$ the same as group (II). The selected doses of BHT and BHA were calculated for the rat from acceptable daily intake (ADI) in man according to conversion table of Paget and Barnes [14]. These doses are corresponding to ADI and double ADI in man. On the next day from last administration, blood samples were collected to separate the serum which was kept frozen till biochemical analyses. Half of liver tissues was taken in ice bags and frozen till preparation of liver homogenates. The other halfs of livers were preserved in 10% neutral formaldehyde solution till processed for histopathological examination. The experiment was carried out according to guidelines and rules for animal experimentation which approved by the Institutional Animal Care and Use Committee, National Research Centre, Animal Care Unit, Dokki, Egypt.

Blood Sampling: Blood samples were collected by puncture of retro-orbital plexus of veins using microcapillary tubes and withdrawn into dry plastic centrifuge tubes. The samples were kept at room temperature for 15 minutes then centrifuged at 5000 RPM for 10 min. for separating the serum which kept in a refrigerator at - 8°C till used for biochemical analysis.

Biochemical Analyses: Serum liver enzymes AST and ALT [15], ALP [16], TC [17] and (TG) [18] were chemically estimated. Serum levels of AST, ALT, ALP, TC and TG were determined using specific kits purchased from Biodiagnostics Company, Dokki, Egypt. Measurements were performed using spectrophotometer (model T80, UV/visible, double beam, UK).

Preparation of Liver Homogenate: One gram of each frozen liver tissue was washed in ice- cooled 0.9% NaCl solution and homogenized in 1.15% solution of potassium chloride and 50 mM potassium phosphate buffer solution (pH 7.4) to yield a 10% (W/V) homogenate. Homogenization was performed using Sonicator, 4710 Ultrasonic Homogenizer (Cole-Parmer Instrument Co., USA). The homogenate was centrifuged at 4000 x g for 5 min. at 4°C. The supernatants were collected and used for biochemical analysis.

Analytical Procedures: The content of reduced glutathione (GSH) in liver homogenate was determined chemically [19] and the lipid peroxide (MDA) content was also determined [20]. Tissue glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) enzymes were chemically determined [21, 22, 23], respectively.

Histological Procedure: The other half of livers were taken and fixed in 10 % neutral formalin solution. The fixed specimens were then trimmed, washed and dehydrated in ascending grades of alcohol. These specimens were then cleared in xylene, embedded in boxes containing paraffin, sectioned using microtome at 4-6 microns thickness and stained with Hematoxylin and Eosin (H&E). The stained liver sections were examined microscopically according to Carleton [24].

Statistical Analysis: Data were expressed as mean ± standard errors (S.E.) using computerized SPSS program (version 15). Differences between means in different groups were tested for significance using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test according to Snedecor and Cochran [25]. The differences were considered significant at level $P<0.05$.

RESULTS

The obtained results showed that subcutaneous injection of CCL_4 to rats significantly ($P < 0.05$) increased levels of AST, ALT and ALP liver enzymes in the serum. The pretreatments with the small and large doses of BHT and BHA for 4 weeks significantly ($P < 0.05$) decreased the elevated serum levels of liver enzymes in CCL_4-intoxicated rats as recorded in Table 1.

Administration of CCL_4 to rats significantly ($P < 0.05$) elevated serum levels of total TC and TG. The pretreatments of rats with BHT and BHA in the tested doses for 4 weeks significantly ($P < 0.05$) lowered the elevated levels of TC and TG in the serum of hepatotoxic rats as shown in Table 2.

It is clear from Table 3 that CCL_4 significantly ($P < 0.05$) increased the content of lipid peroxidation product malondialdehyde (MDA) and decreased the content of reduced glutathione (GSH) in the liver homogenates of the treated rats. The pretreatments of rats with both doses of BHT and BHA significantly ($P < 0.05$) reduced the elevated content of MDA and increased the low level of GSH in liver homogenates of CCL_4-intoxicated rats.
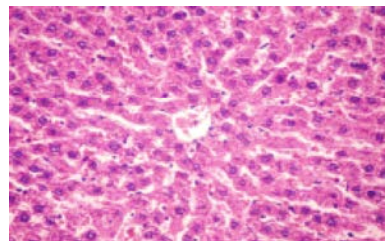

Fig. 1: Liver section of a control rat showing normal architecture with normal central veins, portal tract, hepatocytes and sinusoids. (H &E x 400)
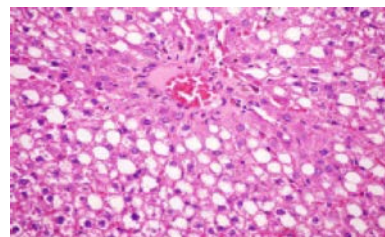

Fig. 2: Liver section of rat given CCL_4 showing fatty degeneration (fat droplets) of hepatocytes and congestion (H &E x 400)
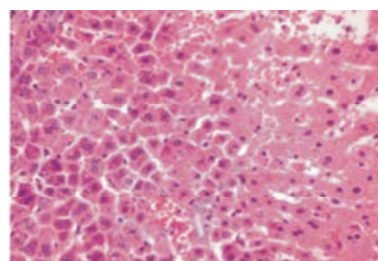

Fig. 3: Liver section of rat given CCL_4 showing coagulative necrosis of hepatocytes (H &E x 400)
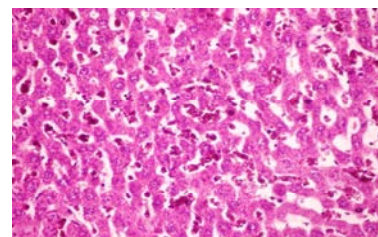

Fig. 4: Liver section of a rat pretreated with small dose (0.25mg/kg) of BHT showing mild focal necrosis. (H &E x 400)

Fig. 5: Liver section of a rat pretreated with large dose (0.5 mg/kg) of BHT showing almost normal architecture of hepatic lobule. (H &E x 400)

Table 1: Effect of BHT and BHA on serum levels of AST, ALT and ALP in CCL_4-intoxicated rats

	Parameters		
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Groups and Treatments	AST (U/L)	ALT (U/L)	ALP (U/L)
Group I (Normal control) (0.5 ml corn oil)	58.6±1.3[d]	35.5±1.4[d]	87.5±2.6[c]
Group II CCL4 (1.5 ml/kg^{-1})	99.8±2.3[a]	77.5±1.6[a]	95.5±1.4[a]
Group III BHT (0.25 mg/kg^{-1})	81.3±2.4[b]	68.7±2.2[b]	85.6±1.6[b]
Group IV BHT (0.50 mg/kg^{-1})	78.8±2.6[c]	64.5±2.6[c]	76.8±2.3[c]
Group V BHA (0.40 mg/kg^{-1})	84.5±1.8[b]	69.5±1.7[b]	87.7±2.4[b]
Group VI BHA (0.80 mg/kg^{-1})	86.6±2.4[b]	65.5±1.9[c]	78.3±1.5[c]

Means ± SE with different letters superscripts in the same column are significant at $P < 0.05$.

n=7 rats

Table 2: Effect of BHT and BHA on serum levels of TC and TG in CCL_4-intoxicated rats

	Parameters	
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Groups	TC (mg/dL)	TG (mg/dL)
Group I (Normal control) (0.5 ml corn oil)	92.98±2.4[c]	52.43±1.5[c]
Group II CCL4 (1.5 ml/kg^{-1})	124.17±2.8[a]	89.60±3.4[a]
Group III BHT (0.25 mg/kg^{-1})	118.50±3.2[b]	69.50±2.1[b]
Group IV BHT (0.50 mg/kg^{-1})	115.90±3.5[b]	65.40±3.2[b]
Group V BHA (0.40 mg/kg^{-1})	122.25±4.1[b]	78.50±2.4[b]
Group VI BHA (0.80 mg/kg^{-1})	120.25±4.1[b]	76.50±2.4[b]

Means ± SE with different letters superscripts in the same column are significant at $P < 0.05$.

n=7 rats

Table 3: Effect of BHT and BHA on contents of MDA and GSH in liver homogenates of CCL_4-intoxicated rats

	Parameters	
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Groups	MDA (nmoles/mg tissue)	GSH (μmol/mg tissue)
Group I (Normal control) (0.5 ml corn oil)	19.70±0.14[c]	77.50±0.86[c]
Group II CCL4 (1.5 ml/kg^{-1})	38.64±0.17[a]	49.15±2.16[a]
Group III BHT (0.25 mg/kg^{-1})	24.83±0.24[b]	66.36±3.78[b]
Group IV BHT (0.50 mg/kg^{-1})	27.50±0.25[b]	73.15±3.79[b]
Group V BHA (0.40 mg/kg^{-1})	26.67±0.27[b]	64.25±0.86[b]
Group VI BHA (0.80 mg/kg^{-1})	24.67±0.17[b]	67.15±0.86[b]

Means ± SE with different letters superscripts in the same column are significant at $P < 0.05$.

n=7 rats

Table 4: Effect of BHT and BHA on activities of GPx, SOD and CAT enzymes in liver homogenates of CCL_4- intoxicated rats

	Parameters		
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Groups and Treatments	Gpx (U/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)
Group 1 (Normal control) (0.5 ml corn oil)	18.7±1.34[c]	85.49±1.86[c]	68.49±4.86[c]
Group II CCL4 (1.5 ml/kg^{-1})	35.6±1.67[a]	94.15±2.16[a]	88.15±2.16[a]
Group III BHT (0.25 mg/kg^{-1})	25.8±2.14[b]	86.56±2.78[b]	79.56±3.78[b]
Group IV BHT (0.50 mg/kg^{-1})	22.5±1.40[b]	85.15±2.79[b]	75.15±3.79[b]
Group V BHA (0.40 mg/kg^{-1})	28.2±1.35[b]	84.50±1.86[b]	78.50±1.86[b]
Group VI BHA (0.80 mg/kg^{-1})	26.2±1.22[b]	83.50±1.86[b]	76.50±1.86[b]

Means ± SE with different letters superscripts in the same column are significant at $P < 0.05$
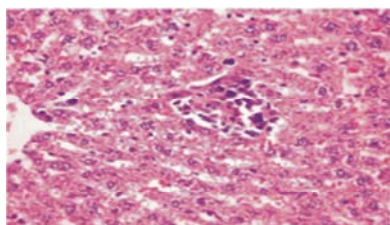
n=7 rats.

Fig. 6: Liver section of a rat pretreated with large dose (0.8 mg/kg) of BHA showing hepatocellular infiltration with few leukocytes. (H &E x 400)

Subcutaneous injection of CCL_4 to rats significantly (P < 0.05) increased activities of glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) antioxidant enzymes in liver of CCl_4- intoxicated rats. Rats pretreated with both doses of BHT and BHA normalized activities of GPx, SOD and CAT enzymes in liver homogenates as recorded in Table 4.

Histopathological examination of liver sections of normal rats revealed normal architecture of hepatic lobules with normal central vein, portal tract, hepatocytes and sinusoids as shown in Fig. 1. Subcutaneous injection of CCL_4 to rats caused fatty degeneration (fat droplets) as shown in Fig. 2 and coagulative necrosis (Fig. 3) of hepatocytes. Examination of liver sections of the rats pretreated with the small dose $(0.25 mg/kg^{-1})$ of BHT showed only mild focal necrosis (Fig. 4). The liver of rats pretreated with the large dose $(0.5 mg/kg^{-1})$ of BHT showed almost normal architecture of hepatic lobule (Fig. 5). The pretreatment of rats with the large dose $(0.8 mg kg^{-1})$ of BHA caused only hepatocellular infiltration with few leukocytes as illustrated in Fig. 6.

DISSCUSION

Butylated Hydroxytoluene (BHT) and butylated Hydroxyanisole (BHA) are two commonly used safe antioxidant food preservatives. The main objective of present study was to investigate the hepatoprotective effect of BHT and BHA at small and large doses (equal to ADI and double ADI) against CCL_4- induced hepatic damage in rats. Lipid peroxidation and activities of tissue antioxidant enzymes were also carried out to evaluate the use of BHT and BHA as antioxidant food preservatives. Results of the present study showed that subcutaneous injection of CCL_4 to rats induced alterations in both serum and tissue biomarkers of hepatotoxicity. These alterations included significant increases in serum levels of AST, ALT and ALP enzymes and in TC and TG levels. There were also significant increases of lipid peroxidation

byproduct malondialdehyde (MDA) and the activity of antioxidant enzymes (GPx, SOD and CAT) with a decrease of reduced glutathione (GSH) content in liver of hepatotoxic rats. These serum biochemical alterations were accompanied by degenerative changes as fatty degeneration and inflammatory leukocytes infiltration in the liver.

It is well known that CCL_4 is a selective hepatotoxic chemical agent commonly used for induction of hepatotoxicity in laboratory animal models [6]. The reported hepatotoxic effect of CCl_4 in the current study was similar to that demonstrated in previous studies [26], [27] and [28]. The mechanism of hepatotoxicity of CCl_4 was attributed to its direct cytotoxic on the hepatocytes causing loss of hepatic cellular enzymes into the extracellular fluids including serum [26]. The most remarkable histopathological characteristics of CCl_4 hepatotoxity were fatty degeneration, inflammatory reactions and necrosis [27]. The histopathological lesions induced by CCl_4 have been thought to result from the formation of reactive intermediate free radical (CCl^{3+}) after metabolism of CCL_4 by the mixed function cytochrome P450 in hepatic endoplasmic reticulum [27]. The reactive free radicals (CCl^{3+}) of CCL_4 initiated cell damage through two mechanisms of covalent binding to the cell membrane proteins and via inducing lipid peroxidation [7]. The extent of hepatic damage by CCL_4 was assessed by the increased levels of cytoplasmic enzymes ALT, AST and ALP, thus lead to leakage of large quantities of these enzymes into the blood circulation [28].

In the present study, the pretreatment of hepatotoxic rats with BHT and BHA significantly decreased the elevated serum levels of AST, ALT and ALP enzymes and in TC and TG levels. It also decreased the elevated tissue lipid peroxide MDA content and increased the low content of GSH and the activity of GPx, SOD and CAT enzymes in the liver of hepatotoxic rats. These serum biochemical alterations were accompanied by amelioration of histopathological degenerative changes (fatty liver and necrosis) which seen in liver of CCl4- intoxicated rats.

The results of this study denoted that both BHT and BHA induced hepatoprotective effect. This effect was in agreement with that demonstrated by Gowri-Shankar *et al.* [28]. The mechanism(s) of hepatoprotection of BHT and BHA could be attributed to their antioxidant activity [29] which maintains the integrity of the cell membrane of the liver. In addition, Courtois *et al.* [30] reported that BHT and BHA normalized the abnormal intracellular events involved in fat absorption and it maintained the cellular antioxidant activity near baseline values and

prevented lipid peroxidation. The inhibition of lipid peroxidation by BHT and BHA was previously demonstrated by Sasse *et al.* [5].

The reported hypocholesterolemic effect of BHT and BHA, in the current study, was similar to the previously demonstrated by Gowri-Shankar *et al.* [28]. The previous findings confirmed the result reported in the present study that BHT and BHA inhibited tissue lipid peroxidation in CCL_4 - intoxicated rats. The results of the present study seem to bear out the postulation that BHT and BHA protect the liver through their inhibitory action on lipid peroxidation [3, 7] and /or via their antioxidant properties [29]. Furthermore, previous studied revealed that the toxic effects of CCL_4 can be partially prevented by antioxidant compounds such as α- tocopherol [7], salvianalic acid [10], butylated Hydroxytoluene [30] and diphenyl diselenide [11]. However, the antioxidant activity of BHT and BHA that had been demonstrated in this study was similar to that reported in previous studies [28, 29, 30, 31, 32].

In conclusion, the current results showed that the pretreatment with BHT and BHA ameliorated CCL_4-induced signs of hepatotoxicity in rats. The hepatoprotective effect was evident from normalization of serum biomarkers of hepatotoxicity and alleviation of fatty liver and degenerative changes seen in CCL_4- intoxicated rats. Both BHT and BHA also exhibited good antioxidant activity in hepatotoxic rats. Therefore, this study suggests that dietary intake of BHT and BHA with food formulations may be beneficial for patients who suffer from liver diseases due to oxidative stress.

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