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ANTIOXIDANT, HEPATORENAL AND MUTAGENIC EFFECTS OF BUTYLATED HYDROXYTOLUENE

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SUMMARY

The antioxidants, biochemical, and mutagenic effects of butylated hydroxytoluene (BHT) in doses of 3 and 30 mg/kg b.wt. were studied in normal and CCl4-intoxicated rats. The oral LD_{50} of BHT in rats was 1740 mg/kg b.wt. No significant changes of almost all of the studied serum and tissue parameters including bone marrow cells compared to the control groups indicating the safety of this compound. CCl4- intoxicated animals showed significant elevations in serum alanine aminotransferases (ALT), aspartate aminotransferases (AST) and alkaline phosphatase (AP) activities, serum bilirubin, cholesterol, creatinine, urea and tissue lipid peroxides (LP) concentrations. In contrast, significant reduction was observed in tissue reduced glutathione concentration (GSH). Oral administration of BHT concurrently with CCl4 for 4 weeks significantly returned all the biochemical parameters to toward their normal values confirming safety and excellent antioxidant effect of BHT.

Key words: antioxidants, BHT, Liver and kidney function, glutathione, chromosomal aberrations, tissue peroxides

INTRODUCTION

Butylated hydroxytoluene (BHT) is a phenolic antioxidant that prevents rancidity of fats and oils in food by protecting against lipid oxidation (Wessling, 2001). BHT may protect against toxicity in some cases but may potentiates toxicity in others (Powell and Connolly, 1990). There are reports that BHT inhibits formation of some types of tumors but it also can increase the risk of cancer in some cases (Preat et al., (1986). BHT protects against carbon tetrachloride poisoning (Freyschuss et al., 2001). There is some evidence that it slows aging in mice. Large number of data have been published concerning the efficacy of BHT as

antioxidant, however little information concerning their pharmacologic and toxicological effects are available. There is still question as to whether antioxidants in the form of dietary supplements counteract the effects of increased numbers of free radicals in the body. This study was conducted to investigate the antioxidant effects of different doses of butylated hydroxytoluene on the liver and kidney functions. Moreover, possible mutagenic effect was studied in albino rats.

MATERIAL AND METHODS

Materials:

Chemicals:

Butylated hydroxytoluene (BHT) and thiobarbituric acid (TBA) was supplied in a pure powder from Merck (Germany). Reduced glutathione (5,5'-dithio-bis (2-nitrobenzoicacid)) (DTNB) was obtained from Cayman Chemical Company, Michigan, USA. Diphenyl picryl hydrazyl (DPPH) was obtained from Sigma Co., Germany. Pyrogallol was obtained from Loba Chemie, Mumbai, India. Colchicine was obtained from Sigma-Aldrich, Germany. Other reagents were obtained in pure form or of analytical grade

Animals and grouping:

I. Determination of LD50 of BHT:

Oral median lethal dose (LD_{50}) of BHT was determined in albino rats as described by Behrens and Karber (1970) Doses of 1000, 1500, 2000, 2500, 3000, 3500 and 4000 mg/kg b.wt. were given orally to groups of 6 rats. Treated animals were observed for possible mortalities or any toxic symptoms.

II. Hepatorenal and antioxidants effect:

One hundred and two adult female Swiss albino rats weighing 100 - 120 g. provided from the breeding unit of the National Research Center (Giza, Egypt) were used throughout this study. Animals were fed on commercial standard pellets and tap water was supplied ad- libitum. Thirty six adult female Swiss albino rats housed in steel mesh cages were randomly divided into 6 equal groups. Rats of group I (Normal control) received pure corn oil at a dose of 0.3ml/100g. b. wt. daily for 4 weeks. Animals of group II (CCl_4- intoxicated) received 0.5 ml/kg b. wt. of CCl_4 50% in corn oil (v/v) twice a week for 4 weeks (Ogawa et al., 1992). Animals of Group III received a daily intra-gastric dose of BHT (3.0 mg/kg b. wt., low dose, FAO/WHO, 1967) throughout the experimental period, while those of group IV received the same dose of BHT in addition to CCl_4 as described before. Animals of Group V received a daily intra-gastric dose of BHT (30.0 mg/kg b. wt., high dose) throughout the experimental period, while those of group VI received the same dose of BHT in addition to CCl_4 as described before. Doses of BHT were calculated from the human acceptable daily intake (ADI) and converted to rats according to surface area (Paget and Barnes, 1964).

Blood samples

Blood samples (3ml) were collected every 2 weeks from the retro-orbital plexus of each rat into dry centrifuge tubes and left to clot at room temperature. Serum samples were obtained after centrifugation at 1500 rpm for 10 min, and then kept in clean epindorf tubes at -20°C until analysis. Biodiagnostic kits were used for determination of serum ALT and AST activities (U/ml, Reitman and Frankel, 1957), ALP activity (mg/dl, Belfield and Goldberg, 1971), serum cholesterol Level (mg/dl, Allain et al.,(1974), serum total bilirubin (mg/dl, Walter and Gerade, 1970), serum urea nitrogen (Fawcett, 1960) and serum creatinine (Slot, 1965).

Tissue sampling:

From each animal two pieces from the liver and kidney tissues were taken on spot in ice cold saline. One part of the liver or the kidney tissue was added to 4 parts of the ice cold normal saline (0.9%) and homogenated and then centrifuged at 4000 rpm for 5 min using a cooling centrifuge. The supernatant was used for determination of reduced glutathione (GSH) and lipid peroxides concentration.

Determination reduced glutathione (GSH):

The reduced glutathione GSH content in liver and kidney homogenates (20%) was determined by the colorimetric method of Ellman (1959) and modified by Bulaj et al., (1998). The level of reduced glutathione (umol/g tissue) was measured from a standard curve prepared for GSH (0.1-1 umol/ml), phosohate buffer and Ellmanís reagent (0.1 ml of each concentration: 1.8: 0.1 ml respectively) against absorbance at 412 nm

Determination of lipid peroxidation concentration:

The lipid peroxides content in the liver and kidney homogenates (20%) was determined by the colorimetric method of Ruiz-Larea et al., (1994). The level of lipid peroxides was expressed as thiobarbituric acid (TBA) values in nmol/g tissue.

In vitro Scavenging activity using Electron Spin Resonance spectrophotometry:

Radical DPPH scavenging capacity of BHT was determined by an electron spin resonance spectrometry method (ESR), using the stable 2,2-diphenyl-1-picryhydrazyl radical (Yu et al., 2002). ESR analysis was conducted using ELEX sys.500 operated at X-band frequency.

In vitro Scavenging activity against DPPH radical using Spectrophotometry

The Scavenging activity of BHT against different concentration (200, 400 ,600, 800, 1000, 3000 5000 mg/ml) of BHT was (23.7, 31.3340.8, 47.9 53.5, 68.59 and 70.2 %) respectively. The scavenging activity of the standard pyrogallol was 10(

% at all time intervals. The scavenging activity of BHT increases with time and concentration. The inhibition of oxidation was indicated by decreasing the colour of the DPPH solution.

III. Mutagenic studies:

Effect of BHT on chromosomal aberration in bone marrow cells was tested in two groups of rats. Animals of Group I were given corn oil 0.3ml/100g b. wt. for 3 months and kept as normal control. Animals of Group II were given the acceptable daily intake (ADI) of butylated hydroxytoluene (3.0 mg/kg b. wt) (FAO/WHO, 1967) for 3 months. All animals were sacrificed at the end of the experiments. Smears of bone marrow were stained Geimsa stain and examined microscopically.

Statistical analysis:

Differences between means was tested for significance by the ANOVA followed by LSD.

RESULTS

I. LD_{50} for butylated hydroxytoluene:

After oral administration of rats with toxic doses, the observed symptoms were cyanosis of mucous membranes, ataxia, general depression, shallow and rapid respiration, loss of reflexes, diarrhoea and finally convulsions and death. The oral LD_{50} of BHT in rats was calculated as 1740 mg/kg b.wt. No deaths were recorded in doses of 1200 mg/kg b.wt. or lower while oral administration of 2200 mg/kg b.wt. killed all the animals. Post mortem findings revealed the presence of congested heart and lungs, cyanosed mucous membranes and inflamed intestinal membranes.

II. Effect on hepatorenal functions:

Oral administration of BHT in a dose of 3.0 or 30 mg/kg b.wt. for 2 or 4 weeks did not affect the serum ALT, or AST activity as compared to that in the control rats. BHT in a dose of 3.0 for 2 or 4 weeks did not affect the serum alkaline phosphatase activity, however the large dose (30 mg/kg b.wt.) significantly ($P < 0.05$) decreased the serum alkaline phosphatase activity. Oral administration of CCl_4 twice a week for either 2 or 4 weeks significantly increased serum ALT, AST and AP activity as compared to normal control BHT in a dose of 3.0 or 30 mg/kg b.wt. given concurrently with CCl_4 significantly ($p \leq 0.05$) decreased serum ALT, AST and AP activity as compared too CCl_4 only treated group (Table 1).

Oral administration of BHT in doses of 3 or 3 mg/kg b. wt. daily for 2 or 4 weks significantly ($p \leq 0.05$) decreased serum cholesterol but serum bilirubin was not affected as compared to control group. Oral administration of CCl_4 twice a week for either 2 or 4 weeks significantly ($p \leq 0.05$) increased serum cholesterol and serum bilirubin compared to normal control. Oral administration of BHT (3mg/kg b.wt. or 30mg/kg b.wt.) conc

rently with CCl4 significantly ($p \leq 0.05$) decreased serum cholesterol and bilirubin compared to CCl4 treated group (Table 2) .

BHT (3.0 or 30 mg/kg b.wt. daily for 2 or 4 weeks) did not affect serum urea or serum creatinine compared to control group. Oral administration of CCl4 twice a week for either 2 or 4 weeks significantly increased serum urea and serum Creatinine as compared to normal control. BHT (3.0 mg/kg b.wt.) given concurrently with CCl_4 significantly ($p \leq 0.05$) decreased serum urea but not creatinine as compared to CCl_4 only treated group (Fig. 3). The large dose (30mg/Kg) significantly ($p \leq 0.05$) decreased both serum urea and serum creatinine (table 3).

III. Antioxidant effects:

Effect on liver and kidney reduced glutathione (GSH):

Oral administration ofo BHT in doses of 3.0 or 30 mg/kg b. wt. daily for 4 weeks did not affect liver or kidney glutathione as compared to control group. Oral administration of CCl_4 twice a week foor 4 weeks significantly ($p \leq 0.05$) decreased liver and kidney glutathione as compared to normal control. BHT (3 or 30 mg/kg b.wt.) given concurrently with CCl4 significantly ($p \leq 0.05$) increased liver and kidney glutathione as compared to CCl_4 only treated group (Table 4).

effect on liver and kidney lipid peroxides (LP):

Oral adminsitration of BHT in a dose of 3.0 mg/kg b.wt. daily for 4 weeks did not affect liver LP compared to control group . On the other hand oral administratioon of BHT in doses of 30 mg/kg b. wt. daily for 4 weeks significantly ($p \leq 0.05$) decreased liver LP. Oral administration of CCl4 twice a week for 4 weeks significantly increased liver LP as compared to normal control. Oral administration of BHT in a dose of 3.0 mg/kg b.wt. concurrently with CCl4 significantly ($p \leq 0.05$) decreased liver LP compared to CCl4 only treated group . (Table 4). Oral administration of BHT 3 mg/kg b. wt. daily for 4 weeks did not significantly affect kidney LP compared to control group Oral administration of CCl_4 twice a week for weeks significantly ($p \leq 0.05$) increased kidne peroxides as compared to control groups. Oral administration of BHT (3 or 30 mg/kg b.wt.) concurrently with CCl4 significantly ($p \leq 0.05$) decreased kidney LP compared to CCl4 only treate group (Table 4).

Scavenging activity against diphenyl picryl hydrazyl (DPPH) radical using Electron Sp Resonance Spectrophotometry (ESR)

When the BHT was added to the system, the s nal intensity of DPPH decreased because of competition reaction between DPPH and the sc engers. The % of inhibition of BHT was 70.19 Results are shown in figure 1.

Scavenging activity against DPPH radical ing Spectrophotometry

The Scavenging activity of BHT against of di

ent concentration (200, 400 ,600, 800, 1000, 3000, 5000 mg/ml) of BHT was (23.7, 31.3340.8, 47.9, 53.5, 68.59 and 70.2 %) respectively. The scavenging activity of the standard pyrogallol was 100 % at all time intervals. The scavenging activity of BHT increases with time and concentration. The inhibition of oxidation was indicated by decreasing the colour of the DPPH solution. Results are shown in figure (2).

Mutagenic effect:

BHT (3.0 mg/kg b.wt. for three months) did not significantly change the number of chromosomal aberrations (table 5) which were examined as chromosome break in BHT treated group (Fig. 3).

Table 1: Effect of BHT (3.0 or 30 mg/kg) given daily alone or concurrently with CCl_4 (twice a week for 4 weeks) on serum ALT, AST and AP activity (mean ± SE, n=6),

Groups	ALT (U/ml).		AST (U/ml).		AP (mg/dl)	
	2w	4w	2w	4w	2w	4w
Control	29.3±1.1	30.3±1.0	38.17±0.9	42.7±1.8	102.4±2.2	96.4±2.2
CCl_4 $^{\$}$	99.6±2.6*	166.3±3.7*	126.8±2.2*	208.2±1.7*	202.0±2.*	285.6±9.*
BHT(LD $^{\$}$	27.5±1.1	28.9±1.3	37.1±1.4	41.9±1.4	97.8±1.2	95.6±1.24
BHT (LD) +CCl_4 $^{@}$	77.3±13.9*	158.2±3.5*	109.0±2.1*	162.7±2.4*	114.2±4.2*	177.9±1.1*
BHT HD $^{\$}$	27.4±0.9	29.6±1.3	37.2±1.8	36.65±1.6	65.1±0.8*	82.2±3.2*
BHT (HD) + CCl_4 $^{@}$	92.3±4.2*	154.9±3.5*	83.1±1.1*	157.5±5.5*	98.0±2.0*	172.4±1.8*

$^{\$}$ compared with control, $^{@}$ compared with CCl_4, * $P < 0.05$
LD (Low dose) = 3.0 mg/kg b.wt., HD (High dose) = 30 mg/kg b.wt.

Table 2: Effect of BHT (3 or 30 mg/kg b.wt.) given daily alone or concurrently with CCl_4 (twice a week for 4 weeks) on serum cholesterol and bililirubin (mg/dl, mean ± SE)

Groups	cholesterol		Billirubin	
	2w	4w	2w	4w
Control	48.72±2.98	53.92±2.88	0.56±0.02	0.56±0.01
CCl_4 §	152.54±3.99*	162.42±2.96*	1.53±0.03*	1.66±0.05*
BHT(LD §	47.25±1.65	51.36±1.35*	0.52±0.02	0.52±0.02
BHT (LD) + CCl_4 @	96.34±2.46*	81.6±1.53*	1.51±0.01*	1.52±0.05*
BHT HD §	42.12±0.99*	42.51±1.75*	0.5±0.02	0.5±0.02
BHT (HD) + CCl_4 @	90.83±2.08*	65.8±1.77*	1.28±0.02*	1.44±0.02*

§ compared with control, @ compared with CCl_4, * $P < 0.05$
LD (Low dose) = 3 mg/kg b.wt. , HD (High dose) = 30 mg/kg b.wt.

Table 3: Effect of BHT in a dose of 3.0 or 30 mg/kg b.wt. daily given alone or concurrently with CCl_4 given twice a week for 4 weeks on serum urea (mg/dl)

Groups	Serum urea		Serum creatinine	
	2w	4w	2w	4w
Control	45.56±0.88	48.33±1.45	0.36±0.01	0.33±0.02
CCl_4 §	85.11±1.75*	109.36±3.35*	1.31±0.03*	1.74±0.05*
BHT LD §	44.16±0.83	47.45±1.69	0.34±0.02	0.32±0.01
BHT LD +CCl_4@	59.37±0.68*	76.57±1.61*	1.2±0.06	1.69±0.01
BHT HD §	42.95±1.33	45.90±2.45	0.35±0.01	0.3±0.02
BHT HD + CCl_4 @	54.53±0.79*	72.26±1.17*	0.91±0.02*	1.63±0.02*

§ compared with control, @ compared with CCl_4, * $P < 0.05$
LD (Low dose) = 3.0 mg/kg b.wt., HD (High dose) = 30 mg/kg b.wt.

Table 4: Effect of BHT in a dose of 3.0 or 30 mg/kg b.wt. daily given alone or concurrently with CCl_4 given twice a week for 4 weeks on liver and kidney glutathione and lipid peroxide (mean ± SE, n=6)) .

Groups	Glutathione (*U*mol/g tissue		lipid peroxide (nmol/g tissue)	
	Liver	Kidney	Liver	Kidney
Control	17.8±0.75	21.11±0.77	225.03±1.43	300.13±3.17
CCl_4[§]	6.46±0.26*	3.05±0.26*	306.43±2.79*	408.25±2.88*
BHT (LD[§])	17.5±0.68	20.14±1.29	223.29±1.32	295.6±3.33
BHT LD + CCl_4[@]	13.92±0.69*	12.79±0.14*	241.02±2.97*	355.22±3.80*
BHT (HD[§])	18.03±0.23	23.03±0.31	217.48±1.83*	256.23±5.32
BHT HD + CCl_4[@]	15.06±0.36*	13.2±0.34*	222.33±1.14*	289.74±3.37*

[§] compared with control, [@] compared with CCl_4 , * $P < 0.05$
LD (Low dose of BHT) = 3.0 mg/kg b.wt.., HD (High dose of BHT) = 30 mg/kg b.wt.

Table 5: Effect of ADI doses of BHT in a dose of (3.0 mg/kg b. wt.) daily for 3 months on bone marrow.

	No. of examined metaphase	Total damage							
		Dicentric chromosome	Douple minute	Chromatid break	Chromosome break	Acentric fragment	No.	%	Mean ± S.E
control	500	2	1	3	3	3	13	2.6	1.08± 0.29
BHT	500	-	6	1	3	6	17	3.2	1.42±0.29

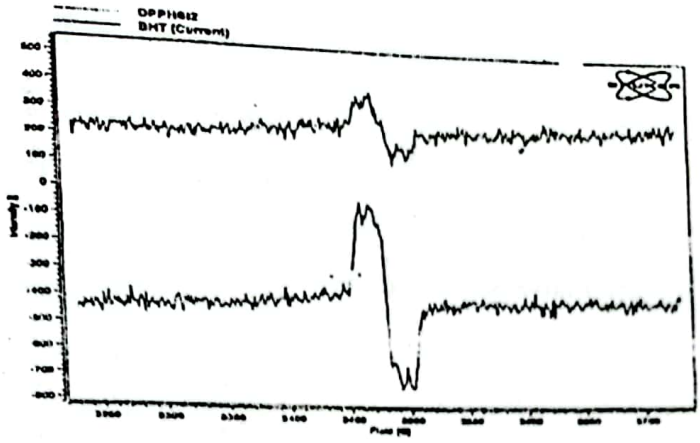

Figure 1: The scavenging effect of BHT using electron spin resonance spectrophotometry

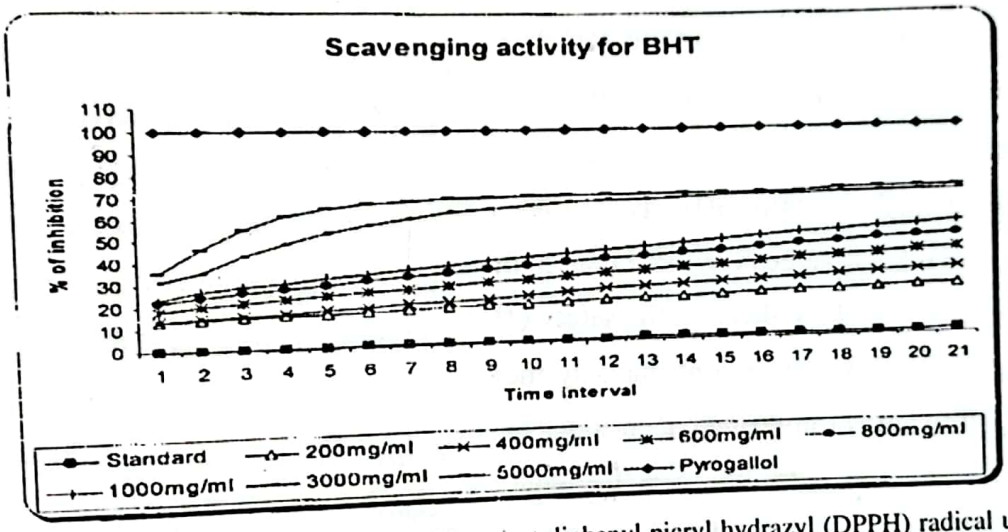

Figure 2: Scavenging activity of BHT against diphenyl picryl hydrazyl (DPPH) radical using Spectrophotometry.

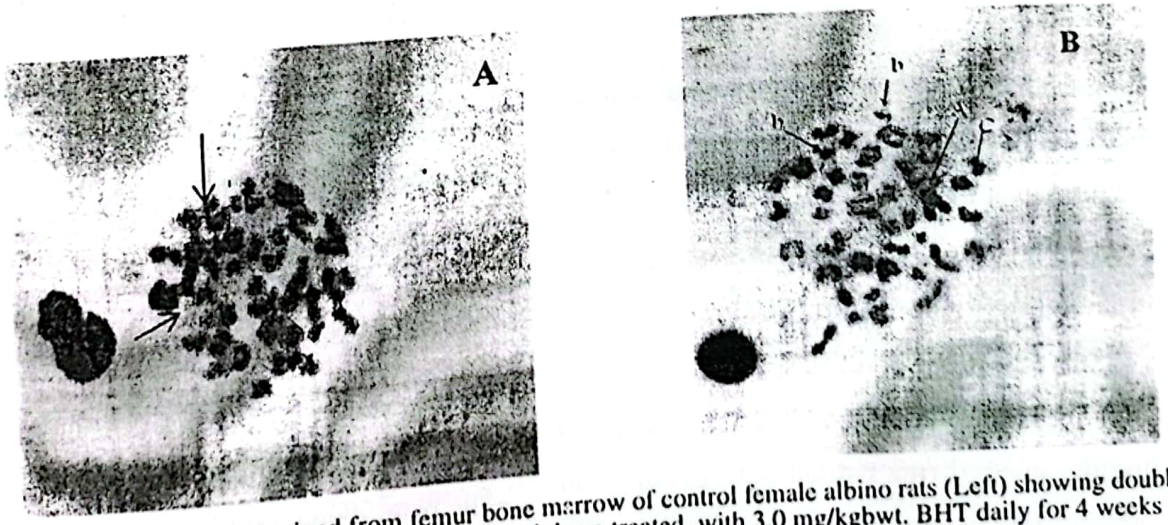

Fig. 3: Metaphase spread obtained from femur bone marrow of control female albino rats (Left) showing double minute (a) and acentric fragment (b). and those treated with 3.0 mg/kgbwt. BHT daily for 4 weeks showing double minute (a), acentric fragment (b) and chromosome-break(c)

DISCUSSION

Data of the present work clearly demonstrated the antioxidant effect with minimal adverse effects of butylated hydroxytoluene (BHT). The median lethal dose (LD50) of BHT (1740 mg/kg b.wt.) reported in this study was lower than those reported in mice (2000 mg/Kg bwt) and in rabbits (2100-3200 mg/Kg bwt. by Lubin and Machlin, (1982). The toxicity of BHT is due to three toxic metabolites namely quinine methide (QM), hydroxyl.tertiary.butyl analog (BHT-OH) and the hydroxide-quinone methide (QM-OH), (Bolton and Thompson, 1991). Such values are more than 500 the acceptable daily intake indicating the safety of BHT. Safety of BHT was also confirmed by the absence of significant chromosomal aberrations in the bone marrow compared with control groups. Similar observations were recorded by Brand et al., (1982).

CCl_4 significantly decreases liver and kidney GSH. The effect of CCl_4 on kidney was much marked than liver (GSH level was 3.05 µ mol/g in kidney compared to 6.46 µmol/g in liver). Similar observation was reported by Letteron et al., (1990). The toxic effect of CCl_4 results from its reductive dehalogenation by microsomal P450 into trichloromethyl free radical (CCl_3) which in turn add molecular oxygen to form trichloromethyl peroxyl radical (CCl_3O). Abstraction of hydrogen atom from unsaturated lipids by such radical creates carbon-centered lipid radicals. These lipid radicals quickly add molecular oxygen to form peroxyl radical, thereby initiating the process of lipid peroxidation and leads to depletion of glutathione in the tissues. These results are in harmony with these of Purucker et al., (1995) and Naveen et al., (2005).

Oral administration of BHT alone in either low or high doses did not affect both liver and kidney glutathione in rats. The protective effect of BHT against CCl_4 induced hepatorenal damage was indicated by maintaining glutathione toward normal values. The protective effect of BHT against CCl_4 induced depletion of tissue GSH may be due to inhibition of lipid peroxidation by scavenging of free radicals, and induction of enzymes systems involved in the biotransformation of xenobiotic (Shertzer and Sainsbury, 1991). A comparable study was done by Ip and Ko (1996) who reported that BHT in a dose of 3mmol/Kg/day X 3 orally in mice did not produce any protective effect on CCl4-induced GSH depletion. This difference could be due to the difference in the dose of BHT or the species in his experiment where mice were used.

The protective effect of BHT may also be due its ability to scavenge free radicals thus protecting cells from damage induced by xenobiotics such CCl_4 (Shertzer and Sainsbury,1991). Comparable study was done by Yamamoto et al, (1995) wh

stated that feeding BHT 0.2% in 1 month diet increase thiobarbituric acid reactive substances concentration.nm. Similar observations were reported by Nagababu and Lakshmaiah (1994) who reported a decrease in lipid peroxides when used against microsomal mixed function oxidase mediated peroxidation using Fe+≥-ADP-.NA.DPH and cumene hydroperoxide (CumOOH). The scavenging activity of BHT was also confirmed by the in vitro experiments using electron spin resonance (ESR) and the scavenging activity against DPPH radical. The protective effect of BHT is also indicated by the observed decreased activity of liver enzymes (ALT and AST), decreased serum cholesterol, bilirubin, urea and creatinine compared to CCl_4 only treated group. In conclusion , this extensive study points to the potential antioxidant activity of BHT which strongly contributes to their significant hepato-renal protective effect Our findings suggest that presence of BHT as food additives even if they are added 10 times as ADI is very safe and can protect against liver and kidney diseases.

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تأثير البيوتيلاتيد هيدروكسيتولوين المضاد للأكسدة والواقى للكبد والكليتين والمطفر

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تم دراسة تأثير البيوتيلاتيد هيدروكسيتولوين كمضاد للأكسدة وتأثيره على مكونات الدم الكيميائية وكذل التأثير المحدث للطفرات فى جرعات ٣ و ٣٠ مجم / كجم فى الفئران الطبيعية والفئران التى تم حقنها برابع كلوريد الكربون وقد أثبتت النتائج أن الجرعة النصف مميتة لمركب البيوتيلاتيد هيدروكسيتولوين هى ١٧٤٠ مجم / كجم من وزن الجسم ولم يكن له أى تأثير معنوى على مكونات الدم الكيميائية ولا يوجد له تأثير على خلايا نخاع العظام مقارنة بالمجموعة الضابطة مما يدل على على أن هذا المركب له درجة أمان عالية وقد كانت الحيوانات المحقونة برابع كلوريد الكربون بها إرتفاع معنوى فى الإنزيمات الناقلة للأمين وكذلك إنزيم الفوسفاتيز القاعدى وكذا صبغة البيليروبين والكوليستيرول والكرباتينين واليوريا وكذلك إنزيم بيروكسيد الدهون إلى أنه أدى إلى إنخفاض معنوى فى الجوتاثيون المختزل. وقد أثبتت التجارب أن إعطاء مركب البيوتيلاتيد هيدروكسيتولوين مع رابع كلوريد الكربون لمدة ٤ أسابيع قد أدى إلى عودة معظم هذه القياسات فى إتجاه معدلاتها الطبيعية مما يدل على أن هذا المركب له تأثير مضاد للأكدة وواقى للكبد بدرجة ملوحوظة وليس له أثار سلبية بالجرعات المستخدة .