Protective Effect of *Mesona procumbens* against *tert*-Butyl Hydroperoxide-Induced Acute Hepatic Damage in Rats

GOW-CHIN YEN,* CHI-TAI YEH, AND YEN-JU CHEN

Department of Food Science, National Chung-Hsing University,
250 Kuokuang Road, Taichung 40227, Taiwan

The protective effect of Hsian-tsao (*Mesona procumbens* Hemsl.) and its active compounds on liver damage was evaluated using the model of *tert*-butyl hydroperoxide (*t*-BHP)-induced acute hepatic damage in rats. Male Sprague−Dawley rats (200 ± 10 g) were orally pretreated with a water extract of Hsian-tsao (WEHT) (0.1, 0.5, and 1.0 g/kg) or caffeic acid (0.1 g/kg of body weight) for 13 days before a single dose of *t*-BHP (0.2 mmol/kg, intraperitoneally) to each animal, and the rats were sacrificed 18 h later by decapitation; blood samples were collected for the assays of serum biochemical values. The livers were excised from the animals and assayed for oxidative injury, antioxidant enzyme, and pathological histology. The result showed that the oral pretreatment of WEHT (0.1, 0.5, and 1.0 g/kg) or caffeic acid (0.10 g/kg) before *t*-BHP (0.2 mmol/kg) treatment significantly lowered the serum levels of the hepatic enzyme markers (alanine aminotransferase, aspartate aminotransferase, and lactate dehydrogenase) and reduced oxidative stress of the liver by evaluation of malondialdehyde, glutathione, 8-hydroxy-2′-deoxyguanosine, glutathione peroxidase, and glutathione reductase. The histopathological evaluation of the rat livers showed that WEHT and caffeic acid reduced the incidence of liver lesions including cloudy swelling, pyknosis, and cytolysis induced by *t*-BHP in rats. On the basis of the results of this study, it can be speculated that *M. procumbens* protects liver against *t*-BHP-induced hepatic damage in rats.

KEYWORDS: *tert*-Butyl hydroperoxide; *Mesona procumbens*; hepatic damage; caffeic acid

INTRODUCTION

Reactive oxygen species (ROS) act as subcellular messengers in such complex cellular processes as mitogenic signal transduction, gene expression, and regulation of cell proliferation (*1*). Excessive production of ROS may, however, lead to oxidative stress, loss of cell function, and ultimately apoptosis or necrosis (*2*). Antioxidants, which can neutralize free radicals, may be of central importance in the prevention of some forms of cancer (*3*). Therefore, the intake of oxygen radical scavengers (antioxidants) found in vegetables and fruits may be a good strategy for cancer prevention (*4*). It is generally assumed that the active dietary constituents contributing to these protective effects are antioxidant nutrients such as α-tocopherol and β-carotene and/or plant antioxidants such as flavonoids and polyphenols.

The herb *Mesona procumbens* Hemsl., called Hsian-tsao in China, is consumed as a herbal drink and jelly-type dessert in the Orient. It is also used as a herbal remedy in the folk medicine of China and is effective against heat-shock, hypertension, diabetes, liver disease, and muscle and joint pains. Many compounds, such as sterol compounds, stigmasterol, β-sitosterol, tripterpene compounds, oleanolic acid, and ursolic acid, have

been isolated from Hsian-tsao (*5*). Some studies have indicated that oleanolic acid and ursolic acid showed many biological effects including hypoglycemia, anti-inflammatory, and hepatoprotective effects and relief of acute and chronic hepatitis (*6*). In our previous study, we found that phenolic compounds extracted from Hsian-tsao significantly contributed to the antioxidant activity and free radical scavenging effects (*7*). Yen et al. (*8*) indicated that that UV-C- and/or H_2O_2-induced DNA damage in human lymphocytes is significantly reduced by the water extract of Hsian-tsao (WEHT). Recently, WEHT was found to exhibit strong antimutagenic effects that were correlated with its phenolic and ascorbic acid contents (*9*). Therefore, the caffeic acid, which is the major compound in Hsian-tsao, showed the highest antioxidant activity (*10*). However, its bioactivity is uncertain in the in vivo systems.

tert-Butyl hydroperoxide (*t*-BHP) is widely applied to investigate the mechanism of cell injury initiated by oxidative stress (*11*). It can be metabolized to free radical intermediates by cytochrome P-450 (hepatocyte) or hemoglobin (erythrocytes), which in turn can initiate lipid peroxidation, affect the cell integrity, and mediate DNA damage (*12*, *13*). These phenomena are similar to the oxidative stress occurring in the cell and/or tissue. Oxidative stress is considered to play a prominent role in the cause of many diseases, for example, inflammation, aging, and cancer (*14*, *15*).

* Author to whom correspondence should be addressed (telephone 886-4-2287-9755; fax 886-4-2285-4378; e-mail gcyen@dragon.nchu.edu.tw).

To understand the antioxidant action of Hsian-tsao and its active compound (caffeic acid, CA) in the living system, we used *t*-BHP to induce oxidative damage in rat liver to investigate the potential protective effects of Hsian-tsao and caffeic acid on hepatic damage in rats.

MATERIALS AND METHODS

Materials and Chemicals. Dried Hsian-taso (*M. procumbens* Hemsl.) was purchased from a local market in Taichung, Taiwan. CA, *t*-BHP, β-nicotinamide adenine dinucleotide phosphate (β-NADPH), perchloroacetic acid (PCA), butylated hydroxytoluene (BHT), deoxy-guanosine (dG), 8-hydroxy-2′-deoxyguanosine (8-OHdG), sodium acetate−ammonium acetate, glutathione (GSH), glutathione reductase (GR), sodium chloride, 1,1,3,3-tetramethoxypropane (TMP), thiobarbituric acid (TBA), 2,4-dinitrofluorobenzene (DNFB), sodium bicarbonate, and kits for aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) were purchased from Sigma Chemical Co. (St. Louis, MO). Iodoacetic acid (IAA) and solvents were purchased from E. Merck Co. Ltd. (Darmstadt, Germany). Tris and protein assay kit were from Bio-Rad Laboratory Ltd. (Watford, Herts, U.K.).

Preparation of WEHT. The dried Hsian-tsao was cut into small pieces and ground into a fine powder in a mill (RT-08, Rong Tsong, Taichung, Taiwan). A Hsian-tsao sample (10 g) was extracted with boiling water (200 mL) for 2 h. The extracts were filtered through Whatman no. 1 filter paper, and the filtrate was freeze-dried to a powder form. The yield of freeze-dried residue corresponded to 15.2% of the original dry weight. The CA content in extracts isolated form Hsiantsao (*M. procumbens Hemsl*) was 17.2 mg/g of lyophilized powder. The deep brown extract powder was dissolved directly in 0.9% saline for further tests.

Animal Treatment. Male Sprague−Dawley rats (200 ± 10 g) were used for the experiments. The rats were provided with food and water ad libitum and divided into five groups (six rats/group). To study the protective effect against *t*-BHP-induced acute hepatic damage, WEHT (1.0, 0.5, and 0.1 g/kg) or CA (0.1 g/kg of body weight (bw) of CA dissolved in 0.9% saline buffer solution) was given daily by gavage to the animals for 13 consecutive days. The control group received vehicle only (0.9% saline solution). On the 13th day, *t*-BHP (0.2 mmol/kg of bw) was injected (intraperitoneally, ip) into each animal, and 18 h later the rats were killed by decapitation. Blood samples were immediately collected in tubes, kept at room temperature for 1 h, and centrifuged at 1000*g* for 10 min to obtain serum. Serum was stored at −20 °C until it was assayed for serum ALT, AST, and LDH. The livers were excised from the animals and assayed for malondialdehyde (MDA) formation, 8-OHdG level, reduced glutathione/glutathione disulfide (GSH/GSSG) ratio and pathological histology according to the procedures described below. All experimental procedures involving animals were conducted in accordance with National Institutes of Health (NIH) guidelines. This experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of the National Chung Hsing University, Taichung, Taiwan.

Hepatotoxicity Assessment. Hepatic enzymes (AST, ALT, and LDH) were used as the biochemical markers for the early acute hepatic damage. The serum activities of AST and ALT were determined according to the colorimetric method of Reitman and Frankel (*16*).

Lipid Peroxidation Assay. Rats were killed by decapitation, and the abdomens of the animals were opened and perfused by injection of ice-cold saline. Liver tissues were removed promptly and placed immediately on ice. Approximately 100 mg of liver was homogenized in ice-cold 20 mM Tris−HCl (pH 7.4) to produce a tissue homogenate. The homogenate was centrifuged at 2500*g* for 30 min at 4 °C. Aliquots of the homogenate were collected and immediately tested for lipid peroxidation. The lipid peroxidation product, MDA, was assayed according to an improved thiobarbituric acid fluorometric method at 555 nm with excitation at 515 nm using 1,1,3,3-tetramethoxypropane as a standard (*17*). The protein concentration was determined using a standard commercial kit (Bio-Rad Laboratory Ltd.) with bovine serum albumin as a standard. The results were expressed as MDA formation per milligram of protein.

Pathological Histology. Immediately after removal, liver and kidney tissues were fixed in 10% buffered formaldehyde and processed for histological examination with the conventional methods and staining with hematoxylin and eosin (H&E). The liver pathology was scored as described by French et al. (*18*) as follows: 0 = no visible cell damage; 1 = focal hepatocyte damage on <25% of tissue; 2 = focal hepatocyte damage on <25−50% of the tissue; 3 = extensive, but focal, hepatocyte lesion; 4 = global hepatocyte necrosis. Pathology was scored in a blind manner by one of the authors and by an outside expert in rodent liver pathology. The morphology of any lesions observed was classified and registered (*19*).

Assay of Glutathione Peroxidase (GPx). The GPx activity was determined spectrophotometrically according to the method of Mohandas et al. (*20*). The following solutions were pipetted into a cuvette: 0.1 mL of homogenate and 0.8 mL of 100 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 unit/mL GSH reductase, and 1 mM GSH. This mixture was preincubated for 5 min at 37 °C. Thereafter, the overall reaction was initiated by adding 0.1 mL of 2.5 mM H_2O_2. Enzyme activity was calculated by the change of the absorbance value at 340 nm for 5 min. The nonenzymic reaction rate was correspondingly assayed by replacing the homogenate sample with potassium phosphate buffer. GPx activity could be expressed as nanomoles of NADPH per minute per milligram of protein.

Assay of Glutathione Reductase (GR). The GR assay monitored the oxidation of NADPH consumed in the reduction of glutathione disulfide (GSSG) by the change in absorbance at 340 nm (*21*). The following solutions were pipetted into a 1 cm spectrophotometric cuvette: 0.1 mL of homogenate and 0.9 mL of 0.10 M phosphate buffer, pH 7.0, containing 1 mM $MgCl_2 \cdot 6H_2O$, 50 mM GSSG, and 0.1 mM NADPH. This mixture was preincubated for 5 min at 37 °C. GR activity was calculated by the change of the absorbance value at 340 nm for 5 min; GR activity was expressed as nanomoles of NADPH per minute per milligram of protein.

GSH/GSSG Ratio. Liver tissue was removed for the GSH assay according to the method of Reed et al. (*22*). In brief, 100 μL of 100% perchloric acid (PCA) was added to the liver homogenates (400 μL) as a precipitation of protein. After centrifugation (at 15000 rpm for 15 min at 4 °C), the pellet was dissolved in 1 N NaOH for protein content analysis. The supernatants (200 μL) were decanted into other centrifuge tubes and treated with 20 μL of a fresh aqueous solution of iodoacetic acid (IAA; 100 mM dissolved in 0.2 mM *m*-cresol purple) and then neutralized (pH was adjusted to 10) with an excess of sodium bicarbonate (dry powder, ∼20 mg). The mixture was allowed to react using a wave rotor for 15 min in the dark at room temperature to form the *S*-carboxymethyl derivative of GSH. Then, 0.2 mL of 3% 2,4-dinitroflurobenzene (DNFB) was added to the reaction mixture and allowed to react in the dark at room temperature for 8 h to form the *N*-(2,4-dinitrophenyl) derivatives of *S*-carboxymethyl-GSH and GSSG. The mixture was centrifuged (at 15000 rpm for 15 min), and an aliquot of the supernatant was used for the determination of GSH and GSSG by HPLC analysis. The HPLC system consisted of a Hitachi L-6200 pump, a Hitachi D-2500 chromatointegrator, a Hitachi L-4200 UV−vis detector, and a 3-aminopropyl column (200 × 4 mm, 5 m). The mobile phase consisted of (A) water/methanol (1:4, v/v) and (B) 2 M sodium acetate (pH 4.6)/methanol (36:64, v/v). The 3-aminopropyl column was eluted by a 30 min gradient from 75% (A) to 5% and then isocratically maintained for 15 min at a flow rate of 1.2 mL with UV detection at 365 nm. The retention times for GSH and GSSG were 31.7 and 40.6 min, respectively.

Measurement of 8-OHdG Levels. Liver tissue was homogenized and lyed with lysis buffer containing Pronase E (Merck) and butylated hydroxytoluene (*23*). DNA was immediately digested to the deoxy-nucleosides by DNase I (Boehringer Mannheim), nuclease P1 (Sigma), and alkaline phosphatase as described by Frenkel (*24*). The nucleosides obtained were analyzed by HPLC equipped with UV and electrochemical detectors for the presence of deoxyguanosine (dG) and 8-OHdG, respectively (*25*). The amount of 8-OHdG was expressed as the number of 8-OHdG for every 10^5dG in DNA.

Statistical Analysis. The values of mean and standard deviation (mean $\pm$ SD) and 95% confidence intervals (CI) of means to verify

Mesona procumbens against Hepatic Damage

J. Agric. Food Chem., Vol. 52, No. 13, 2004 **4123**

Table 1. Relative Liver and Kidney Weights of *tert*-Butyl Hydroperoxide (*t*-BHP)-Treated Rat with or without Gavage with Water Extract of Hsian-tsao (WEHT) or Caffeic Acid (CA) for 13 Days

	relative organ wt[a] (g/100 g of bw)	
treatment	liver	kidney
control (0.9% NaCl)	3.5 ± 0.4	0.9 ± 0.1
t-BHP (0.2 mmol/kg)	3.4 ± 0.2	0.8 ± 0.1
CA: 0.1 g/kg of bw + *t*-BHP	3.4 ± 0.4	0.8 ± 0.1
WEHT:[b]		
0.1 g/kg of bw + *t*-BHP	3.5 ± 0.2	0.9 ± 0.1
0.5 g/kg of bw + *t*-BHP	3.4 ± 0.1	0.8 ± 0.1
1.0 g/kg of bw + *t*-BHP	3.5 ± 0.4	0.8 ± 0.1
1.0 g/kg of bw	3.5 ± 0.2	0.8 ± 0.1

[a] Data are presented as means ± SD from six rats. [b] WEHT was dissolved in 0.9% NaCl solution.

the statistical significance of all parameters were calculated. If necessary, data were tested by two-way ANOVA. p values of <0.05 were assumed to be statistically significant. All data are expressed as the means of three measurements.

RESULTS

Effect of WEHT on the Relative Tissue Weight of Rats.
Table 1 shows the relative kidney and liver tissue weights of rats in each group. There was no difference among the relative kidney and liver weights of *t*-BHP-induced rats with or without gavage of WEHT (0.1−1.0 g/kg of bw) or CA (0.1 g/kg of bw) for 13 consecutive days when compared with controls.

Pathological Histology of the Liver. Figure 1 shows that the treatment with *t*-BHP caused cloudy swelling, pyknosis, cytolysis, and necrosis of hepatocytes in the portal region of rat livers. However, WEHT or CA pretreatment reduced the injury score of leucocyte infiltration as well as ballooning degeneration of the liver lesions and inhibited necrosis of liver cells (**Table 2**). Histological examination showed that the group pretreated with 1.0 g/kg of bw of WEHT exhibited light hepatotoxicity (ballooning degeneration).

Effects of WEHT on *t*-BHP-Induced Hepatic Damage.
Several hepatic enzymes in serum such as AST, ALT, and LDH in liver homogenate were used as the biochemical markers for the early acute hepatic damage. The effects of pretreatment with WEHT on the *t*-BHP-induced elevation of serum AST, ALT, and LDH are shown in **Table 3**. Pretreatment with WEHT (1 g/kg, gavage, 13 days) resulted in no changes in serum AST, ALT, and LDH activities, compared to the control.

When a single dose of *t*-BHP was given to rats by ip injection, after 18 h, elevations of serum AST, ALT, and LDH levels as compared with the control group were seen (**Table 3**). Similar to liver morphology, the acute hepatic damage reaction was significantly (p < 0.05) suppressed in all of the animals pretreated with 0.5 and 1.0 g/kg of bw of WEHT or 0.1 g/kg of bw of CA. WEHT pretreatment prevented the *t*-BHP-induced elevation of AST, ALT, and AST serum levels in a dose-dependent manner. Low does of WEHT (0.1 g/kg) partially prevent the elevation of AST, ALT, and LDH serum levels, and medium or higher doses of WEHT (0.5 or 1.0 g/kg) almost completely prevented hepatic damage. As shown in **Table 3**, pretreatment with CA at a dosage of 0.1 g/kg of bw also provided a significant inhibition (p < 0.05) of the elevated ASL, ALT, and LDH induced by *t*-BHP. The activity exhibited by 1.0 g/kg of extract was similar to that of the CA.

Effects of WEHT on *t*-BHP-Induced Hepatic Lipid Peroxidation. Lipid peroxidation has been recognized as a potential mechanism for cell injury. To evaluate the effect of pretreatment
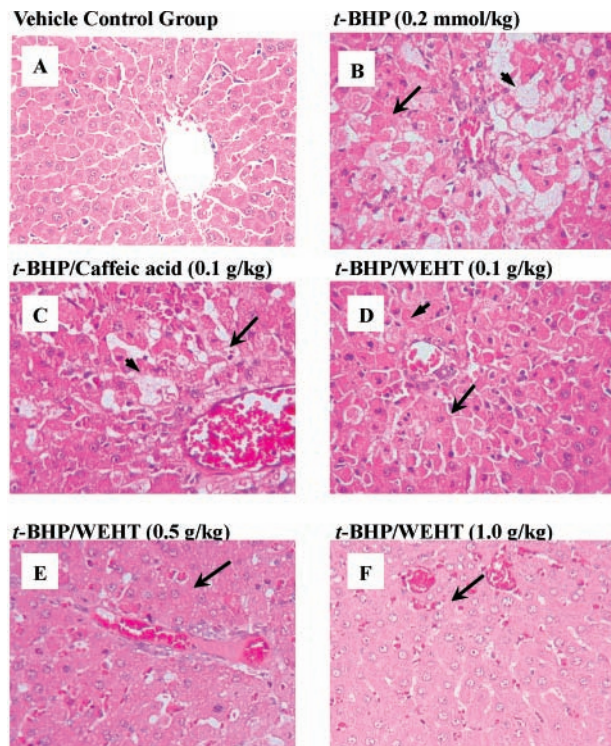

Figure 1. Effect of WEHT or CA on *t*-BHP-induced liver damage in rats: (**A**) vehicle control group ($n = 6$); (**B**) animals treated with *t*-BHP (0.2 mmol/kg) showed severe coagulative necrosis (big arrowhead) and ballooning changes (arrow); (**C**) animals pretreated with 0.1 g/kg CA and then with *t*-BHP showed mild necrosis (big arrowhead) and ballooning changes (arrow); (**D**) animals pretreated with 0.1 g/kg WEHT and then with *t*-BHP showed mild necrosis (big arrowhead) and ballooning changes (arrow); (**E**) animals pretreated with 0.5 g/kg and (**F**) 1.0 g/kg WEHT by gavage, and then with *t*-BHP, showed normal morphology: hematoxylin/eosin staining; magnification ×400. (Figure is reproduced here at 55% of its original size.)

Table 2. Histological Injury Score of Liver under Different Doses of WEHT or CA in Rats Treated with *t*-BHP

	injury score[a]			
	leucocyte infiltration	cell necrosis	ballooning degeneration	bile epithelia hypertrophy
control (0.9% NaCl)	0 ± 0	0 ± 0	0 ± 0	0 ± 0
t-BHP (0.2 mmol/kg)	3.3 ± 0.4	3.9 ± 0.3	3.8 ± 0.4	1.3 ± 0.4
CA: 0.1 g/kg of bw + *t*-BHP	2.2 ± 0.2	2.1 ± 0.4	3.1 ± 0.5	1.4 ± 0.1
WEHT:[b]				
0.1 g/kg of bw + *t*-BHP	2.3 ± 0.4	3.1 ± 0.3	2.8 ± 0.2	1.1 ± 0.3
0.5 g/kg of bw + *t*-BHP	1.2 ± 0.3	1.3 ± 0.2	1.3 ± 0.4	1.1 ± 0.2
1.0 g/kg of bw + *t*-BHP	0 ± 0	1.1 ± 0.2	0 ± 0	1.3 ± 0.2
1.0 g/kg of bw	0 ± 0	0 ± 0	1.2 ± 0.3	0 ± 0

[a] Livers were scored for hepatic injury via light microscopy: 0 = no visible cell damage; 1 = focal hepatocyte damage on <25% of tissue; 2 = focal hepatocyte damage on <25–50% of the tissue; 3 = extensive, but focal, hepatocyte lesion; 4 = global hepatocyte necrosis. Data represent mean ± SD from six rats. [b] WEHT was dissolved in 0.9% NaCl solution.

with WEHT or CA on *t*-BHP-induced liver lipid peroxidation, we monitored the levels of MDA, an indicator of oxidative damage and one of the principal products of lipid peroxidation. When a single dose of *t*-BHP was given to rats by ip injection, it caused an increased formation of MDA in liver (**Figure 2**). WEHT or CA at a dosage of 0.10 g/kg of bw significantly inhibited (p < 0.01) lipid peroxidation in liver homogenate of rat by 57 and 61%, respectively.

4124 *J. Agric. Food Chem.,* Vol. 52, No. 13, 2004

Yen et al.

Table 3. Effect of WEHT or CA on Serum ALT and AST Activities in Rats Treated with *t*-BHP

treatment[a]	AST (μmol/ min/L)	ALT (μmol/ min/L)	LDH (μmol/ min/L)
control (0.9% NaCl)	100 ± 48	34 ± 13	388 ± 358
t-BHP (0.2 mmol/kg)	309 ± 230	65 ± 41	1280 ± 122
CA: 0.1 g/kg of bw + *t*-BHP	118 ± 51[*b]	39 ± 9*	451 ± 605*
WEHT:			
0.1 g/kg of bw + *t*-BHP	238 ± 101*	51 ± 22*	892 ± 440*
0.5 g/kg of bw + *t*-BHP	178 ± 62*	42 ± 11*	692 ± 555*
1.0 g/kg of bw + *t*-BHP	128 ± 38*	31 ± 5*	376 ± 195*
1.0 g/kg of bw	106 ± 40	36 ± 13	340 ± 23

[a] Data are presented as means ± SD from six rats. [b] Asterisks indicate significant difference by comparison with *t*-BHP alone, $p < 0.05$.
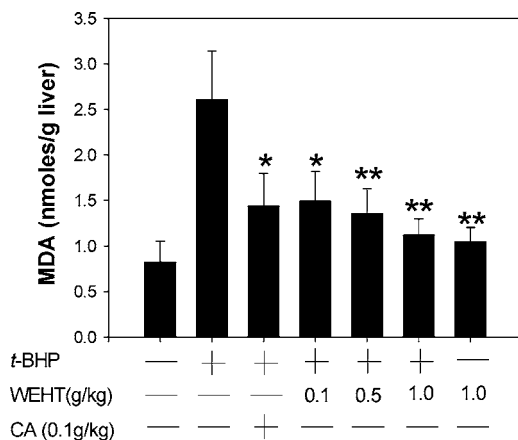

Figure 2. Effect of WEHT or CA on hepatic lipid peroxidation induced by *t*-BHP. Bars represent mean ± SD ($n = 6$). Values are significantly different by comparison with *t*-BHP alone. Asterisks indicate significant difference by comparison with *t*-BHP alone as determined by ANOVA: *, $p < 0.05$; **, $p < 0.01$.
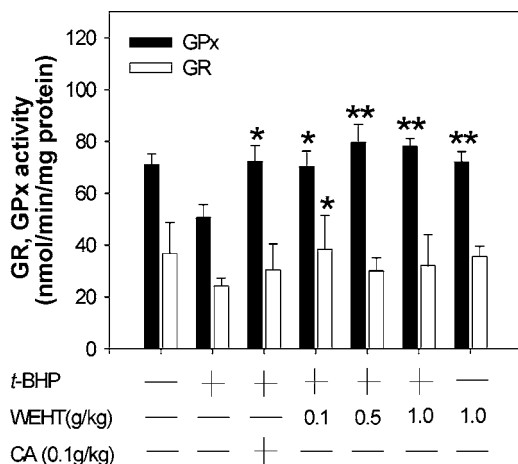

Figure 3. Effect of WEHT or CA on hepatic GSH-related enzymes in *t*-BHP-induced liver damage in rats. GR, GSH reductase; GPx, GSH peroxidase. The data are presented as means ± SD from six rats. Asterisks indicate significant difference by comparison with *t*-BHP alone as determined by ANOVA: *, $p < 0.05$; **, $p < 0.01$. WEHT was dissolved in 0.9% NaCl solution.

Effects of WEHT on Hepatic GSH-Related Enzymes. **Figure 3** shows the effects of WEHT on hepatic GSH-related enzymes in *t*-BHP-induced liver damage of rats. The GSH-related enzyme activities, GPx and GR, in the rat liver from the *t*-BHP-induced damage group elicited 35 and 24% reductions, respectively, compared to the untreated group. Pretreat-

Table 4. Effect of WEHT or CA on the Content of GSH and GST/ GSSG Ratio in Rat Liver That Was Induced by *t*-BHP

treatment[a]	GSH (nmol/mg of prot.ein)	GSSG (nmol/mg of protein)	GSH/GSSG
control (0.9% NaCl)	163 ± 11	17 ± 5	10 ± 2
t-BHP (0.2 mmol/kg)	82 ± 7	40 ± 7	2 ± 0.4
CA: 0.1 g/kg of bw + *t*-BHP	107 ± 7[*b]	33 ± 9	4 ± 1
WEHT:			
0.1 g/kg of bw + *t*-BHP	111 ± 12*	37 ± 8	3 ± 1
0.5 g/kg of bw + *t*-BHP	134 ± 6**	25 ± 5*	5 ± 1*
1.0 g/kg of bw + *t*-BHP	142 ± 7**	24 ± 6*	6 ± 2**
1.0 g/kg of bw	163 ± 10**	18 ± 3*	10 ± 3**

[a] Data are presented as means ± SD from six rats. [b] Asterisks indicate significant difference by comparison with *t*-BHP alone as determined by ANOVA: *, $p < 0.05$; **, $p < 0.01$.

ment with WEHT or CA at a dose of 0.10 g/kg of bw for 13 consecutive days significantly increased the *t*-BHP-induced reduction of GSH-related enzyme activities in rat liver ($p < 0.05$). Liver GPx and GR activities of the untreated group did not show any significant difference ($p > 0.05$) in response to diet supplementation of 1.0 g/kg of bw of WEHT.

Effects of WEHT on GSH Content and GSH/GSSG Ratio. As the oxidative stress of tissue generally involves the GSH system, we therefore measured the level of GSH and the ratio of GSH/GSSG of each group. **Table 4** shows the effect of WEHT or CA on the content of GSH and on the GSH/GSSG ratio in the rat liver that was induced by *t*-BHP. The *t*-BHP-treated group showed a significantly reduced GSH level and GSH/GSSG ratio in the rat liver ($p < 0.01$). Pretreatment with WEHT significantly protected the GSH depletion and also improved the decline in the GSH/GSSG ratio produced by *t*-BHP. Low doses of WETH (0.1 g/kg) partially protected the GSH level and GSH/GSSG ratio, and medium or higher doses of WEHT (0.5 or 1.0 g/kg) almost completely prevented the elevation of GSH level and GSH/GSSG ratio in the rat liver. As shown in **Table 4**, pretreatment with CA at a dosage of 0.1 g/kg of bw also significantly protected ($p < 0.05$) the GSH depletion produced by *t*-BHP.

Effect of WEHT on *t*-BHP-Induced Oxidative Damage in Rat Liver. To examine the *t*-BHP-induced oxidative damage, the 8-OHdG level was determined following ip injection with a single dose of *t*-BHP (0.2 mmol/kg). In the *t*-BHP-administered group, the 8-OHdG level in the liver DNA significantly ($p < 0.05$) increased as compared to that of the control (**Figure 4**). WEHT or CA at dosage of 0.10 g/kg of bw significantly inhibited *t*-BHP-induced oxidative DNA damage in rat liver ($p < 0.05$).

DISCUSSION

Diet appears to play an important role in human health and in the development of certain diseases, especially cancer (*4*). The frequent consumption of fresh fruits and vegetables is associated with a low cancer incidence (*26*). Recently, much attention has focused on the protective function of naturally occurring antioxidants in biological systems and on the mechanism of their actions. Phenolic compounds, which are widely distributed in plants, have been considered to play an important role as dietary antioxidants for the prevention of oxidative damage in living system (*27*). In our previous study, it was shown that the phenolic acids could be important antioxidant components in Hsian-tsao, among which CA with the highest antioxidant activity and the greatest content is most important
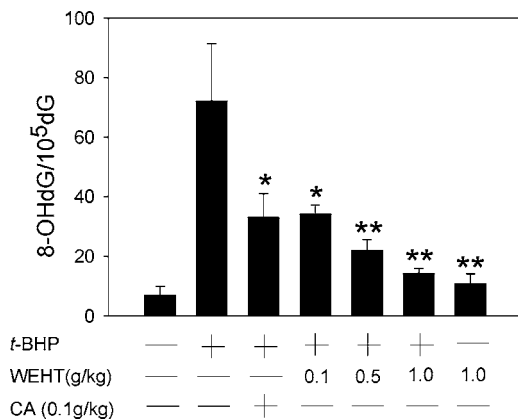
Mesona procumbens against Hepatic Damage

J. Agric. Food Chem., Vol. 52, No. 13, 2004   **4125**

Figure 4. Effects of WEHT or CA on DNA damage in rat liver induced by *t*-BHP (*n* = 6). Asterisks above column indicate significant difference from *t*-BHP alone (*, $p < 0.05$; **, $p < 0.01$).

(*10*). To clarify the biological and pharmacological action of Hsian-tsao and its active compound (caffeic acid), in vivo hepatoprotective experiments were carried out. According to the reports of Wang et al. (*28*) and our preliminary test, we used 13 days for pretreatment. Our experimental results show that pretreatment with WEHT (0.5 g/kg of bw) or CA (0.1 g/kg of bw) significantly (*p* < 0.05) protected rats from hepatic damage and liver inflammation caused by *t*-BHP, as indicated by the reduced serum leakage of AST, ALT, and LDH and decreased formation of MDA (**Table 3** and **Figure 2**), pyknosis, cytolysis, necrosis, cell swelling, and DNA damage in the liver (**Figures 1** and **4** and **Table 2**). AST, ALT, and LDH are known to be general indices of hepatic cytotoxicity. The rise in the serum levels of AST, ALT, and LDH has been attributed to the damaged structural integrity of the liver, because these are cytoplasmic in location and are released into circulation after cellular damage (*29*). MDA, on the other hand, is a major oxidative degradation product of membrane unsaturated fatty acid and has been shown to be biologically active with hepatotoxic and genotoxic properties (*30*). In the present study, *t*-BHP was shown to enhance hepatic damage and lipid peroxidation in the animal system (**Figure 2**). This could be due to the cytochrome P450-mediated metabolism of the organic hydroperoxide to active alkoxyl radicals that, then, initiated lipid peroxidation and led to liver damage. The results of the present study demonstrate that pretreatment of rat with WEHT or CA effectively protected the rats against *t*-BHP-induced hepatic damage, as evidenced by decreased serum AST, ALT, and LDH activities and hepatic lipid peroxidation (**Table 3** and **Figure 2**). Moreover, these protective effects were found to be dose-dependent, especially, WEHT pretreatment at doses form 0.1 to 1.0 g/kg was found to significantly decrease *t*-BHP-induced hepatic damage. This phenomenon was also confirmed by histological observation (**Figure 1**). It is now generally accepted that the rat hepatocytes metabolized *t*-BHP by two distinct pathways. One pathway involves the cytochrome P-450 and leads to the formation of toxic peroxyl and alkoxy radical (*11*). The second is a detoxification reaction that involves glutathione peroxidase, producing *tert*-butyl alcohol and oxidative glutathione (*31*). These metabolic pathways could increase cellular free radicals, which may attack phospholipids, proteins, and nucleic acid. Thus, antioxidant activity and the inhibition of free radical generation are important in terms of protecting the liver from *t*-BHP-induced damage (*7*).

The physiological role of GSH is as an essential intracellular reducing agent for the maintenance of thiol groups on intracellular protein and antioxidant molecules. It was well estab-

lished that GSH, the most important biomolecule protecting against chemically induced cytotoxicity, can participate in the elimination of reactive intermediates by conjugation and hydroperoxide reduction. The ratio of GSH to GSSG would be expected to be a more sensitive marker of oxidative stress, because small increases in GSSG and decreases in GSH can appear to be more amplified by examining the ratio than by measuring either one separately (*32*). In addition, the GSH antioxidant system plays a fundamental role in cellular defense against reactive free radicals and other oxidant species. This system consists of GSH and an array of functionally related enzymes, of which GR is responsible for the regeneration of GSH, whereas GPx worked together with GSH in the decomposition of hydrogen peroxide or other organic hydroperoxides (*33*). Thus, the levels of GSH and activities of the GPx and GR were used to monitor the peroxidative balance (*34*). Furthermore, induction of the hepatic GSH antioxidant system by chemopreventive agents has been reported in several studies (*35*).

Impaired activity of GPx and GR with increased lipid peroxidation and a marked decrease in the GSH/GSSG ratio have been observed in the liver of *t*-BHP-intoxicated rats (**Figure 3** and **Table 4**). Enhanced lipid peroxidation in rat liver reflects an excessive generation of free radicals exacerbated by a deficiency of hepatic antioxidant and detoxification systems. In contrast, reduced lipid peroxidation is associated with a significantly greater increase of the GSH-related enzymes and with a concomitant increase in the GSH/GSSG ratio in rats with oral pretreatment of WEHT or CA (**Figure 3** and **Table 4**). These results show that the protection afforded by WEHT or CA against *t*-BHP-induced hepatotoxicity might be due to regulation of hepatic GSH redox balance.

Currently, there is increasing evidence that oxidative damage has been linked to chemical carcinogenesis (*36*). Oxidative DNA damage products such as 8-OHdG caused misincorporation during replication, which subsequently led to G → T transversion (*37*). At the same time, the generation of oxidative damaging ROS has been widely accepted to have promotion effects (*38*). Therefore, 8-OHdG was a key marker for the study of DNA damage caused by reactive oxygen species (*39*). As in the results shown in **Figure 4**, WEHT or CA had a protective effect on *t*-BHP, which induced DNA damage in rat liver. Many researchers indicated that the formation of 8-OHdG was induced by various environmental factors, such as ionizing radiation, UV light, or Fenton reaction, indicating that the formation of 8-OHdG occurred through the reaction of DNA with hydroxyl radicals and singlet oxygen (*40*). Furthermore, antioxidants and vitamins such as β-carotene and vitamin C, as well as vegetables such as Brussels sprouts, are known to reduce the level of 8-OHdG in animal studies (*41*). In the present study, it was shown that *t*-BHP induced oxidative damage in the liver and that pretreatment of WEHT or CA inhibited this harmful effect (**Figure 4**). The inhibitory effect of WEHT or CA on *t*-BHP-induced hepatic damage may be partially associated with the blocking of oxidative stress-induced 8-OHdG formation. Hung and Yen (*10*) reported that WEHT inhibited the peroxidation of linoleic acid and had antioxidant activity equal to that of Trolox and BHA. In addition, WEHT also had a scavenging activity on free radicals and ROS, such as hydroxyl radicals or peroxyl/hydroperoxy radicals. Thus, the antioxidant activity and scavenging effect of WEHT might be mainly related to its inhibitory effect on hepatic damage in rat induced by *t*-BHP. CA is the major representative of hydroxycinnamic acid that is found naturally in a wide variety of foods such as vegetables,

4126 *J. Agric. Food Chem.,* Vol. 52, No. 13, 2004

Yen et al.

fruits, tea, coffee, and wine (*42*), with high concentrations in certain foods and beverage. For instance, red wine may contain 25 mg of CA/L (*43*). CA has been reported to have anti-inflammatory, antimutagenic, and anticarcinogenic activities (*44*). Furthermore, CA at an oral dose of 200 mg/kg exhibited a significant hepatoprotective effect on bromobenzene-induced hepatotoxicity in mice (*45*). Therefore, WEHT contains many important antioxidant components, of which CA with the highest antioxidant activity and the greatest content is most important (*10*). The CA content in extracts isolated form Hsian-tsao (*M. procumbens* Hemsl.) was 17.17 mg/g of lyophilized powder. Phenolic compounds have a variety of biological effects in numerous mammalian cell systems in vitro as well as in vivo. The CA in WEHT might play an important role in decreasing *t*-BHP-induced hepatic damage in rat. The effect of antioxidants in decreasing oxidative damage is believed to contribute to the low cancer incidence (*46*). Therefore, we suggest that daily consumption of WEHT might be effective in lowering possible oxidative damage in living systems.

In conclusion, the WEHT showed efficient protective action against *t*-BHP-induced hepatic and oxidative damage. It was due to the possibly different mechanisms associated with different antioxidants, especially CA, existing in the crude extracts. The exact mechanisms and constituents need further investigation.

ABBREVIATIONS USED

WEHT, water extract of Hsain-tsao; *t*-BHP, *tert*-butyl hydroperoxide; MDA, malondialdehyde; GSH, (reduced) glutathione; GSSG, glutathione disulfide; GR, glutathione reductase: GPx, glutathione peroxidase; CA, caffeic acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; bw, body weight.

ACKNOWLEDGMENT

LITERATURE CITED

(1) Esposito, F.; Ammendola, R.; Faraonio, R.; Russo, T.; Cimino, F. Redox control of signal transduction, gene expression and cellular senescence. *Neurochem. Res.* **2004**, *29*, 617−628.

(2) Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*; Oxford University Press: New York, 1998; pp 27−30.

(3) Halliwell, B. Antioxidants in human health and disease. *Annu. Rev. Nutr.* **1997**, *16*, 33−50.

(4) Ames, B. N. Dietary carcinogens and anticarcinogens: oxygen radicals and degenerative disease. *Science* **1983**, *221*, 1256−1264.

(5) Sheu, S. Y.; Liu, C.; Chiang, H. C. The hypoglycemic principle of *Mesona procumbens* and *Orthosiphon stamineus. Taiwan K'o Hsueh* **1984**, *38*, 26−31 (in Chinese).

(6) Liu, J. Pharmacology of oleanolic acid and ursolic acid. *J. Ethnopharmacol.* **1995**, *49*, 57−68.

(7) Yen, G. C.; Hung, C. Y. Effects of alkaline and heat treatment on antioxidative activity and total phenolic of extracts from Hsian-tsao (*Mesona procumbens* Hemsl.). *Food Res. Int.* **2001**, *33*, 487−492.

(8) Yen, G. C.; Hung, Y. L.; Hsieh, C. L. Protective effect of extracts of *Mesona procumbens* Hemsl. on DNA damage in human lymphocytes exposed to hydrogen peroxide and UV irradiation. *Food Chem. Toxicol.* **2000**, *38*, 747−754.

(9) Yen, G. C.; Dah, P. D.; Hung, Y. L. Contribution of major components to the antimutagenic effect of Hsiao-tsao (*Mesona procumbens* Hemsl.). *J. Agric. Food Chem.* **2001**, *49*, 5000−5004.

(10) Hung, C. Y.; Yen, G. C. Antioxidant activity of phenolic compounds isolated from *Mesona prcumbens* Hemsl. *J. Agric. Food Chem.* **2002***, 59*, 2993−2997.

(11) Rush, G. F.; Gorski, J. R.; Ripple, M. G.; Sowinski, J.; Bugelski, F.; Hewitt, W. R. Organic hydroperoxide-induced lipid peroxidation and cell death in isolated hepatocytes. *Toxicol. Appl. Pharmacol.* **1985**, *78*, 473−483.

(12) Thornalley, P.; Trotta, R. J.; Stern, A. Free radical involvement in the oxidative phenomena induced by *tert*-butyl hydroperoxide in erythrocytes. *Biochim. Biophys. Acta* **1983**, *759*, 16−22.

(13) Davies, M. J. Detection of peroxyl and alkoxyl radicals produced by reaction of hydroperoxides with rat liver microsomal fraction. *Biochem. J.* **1989**, *257*, 603−606.

(14) Cerutti, P. A. Proxidant states and tumor promotion. *Science* **1985**, *227*, 375−381.

(15) Breimer, L. H. Molecular mechanism of oxygen radical carcinogenesis and mutagenesis: the role of DNA base damage. *Mol. Carcinog.* **1990**, *3*, 188−197.

(16) Reitman, S.; Frankel, S. A colorimetric method for the determination of serum GOT and GPT. *Am. J. Clin. Pathol.* **1957**, *28*, 57−60.

(17) Yagi, K. Lipid peroxides and human diseases. *Chem. Phys. Lipids* **1987**, *45*, 337−351.

(18) French, S. W.; Miyamoto, K.; Ohta, Y.; Geoffrion, Y. Pathogenesis of experimental alcoholic liver disease in the rat. *Methods Achiev. Exp. Pathol.* **2000**, *13*, 181−207.

(19) Gray, P. *Handbook of Basic Microtechnique*, 3rd ed.; McGraw-Hill: New York, 1964; pp 85−145,

(20) Mohandas, J.; Marshall, J. J.; Duggin, G. G.; Horvath, J. S.; Tiller, D. J. Low activities of glutathione-related enzymes as factors in the genesis of urinary bladder cancer. *Cancer Res.* **1984**, *44*, 5086−5091.

(21) Bellomo, G.; Mirabelli, F.; Dimonte, D.; Richelmi, P.; Thor, H.; Orrenius, C.; Orrenius, S. Formation and reduction of glutathione-mixed disulfides during oxidative stress. *Biochem. Pharmcol.* **1987**, *36*, 1313−1320.

(22) Reed, D. J.; Babson, J. R.; Beatty, P. W.; Brodie, A. E.; Ellis, W. W.; Potter, D. W. High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. *Anal. Biochem.* **1980**, *106*, 55−62.

(23) Adachi, S.; Zeisig, M.; Moller, L. Improvements in the analytical method for 8-hydroxydeoxyguanosine in nuclear DNA. *Carcinogenesis* **1995**, *16*, 279−288.

(24) Frenkel, K. Methods used for analysis of "environment" damaged nucleic acids. *J. Chromatogr.* **1993**, *68*, 289−314.

(25) Liu, T. Y.; Chen, C. L.; Chi, C. W. Oxidative damage to DNA induced by areca nut extract. *Mutat. Res.* **1996**, *367*, 25−31.

(26) Shibata, A.; Paganini-Hill, A.; Ross, R. K.; Henderson, B. E. Intake of vegetables, fruits, β-carotene, vitamin C and vitamin supplements and cancer incidence among the elderly: a prospective study. *Br. J. Cancer* **1992**, *66,* 673−679.

(27) Hertag, M. G. L.; Feskens, E. J. M. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Stduy. *Lancet* **1993**, *342*, 1007−1011.

(28) Wang, C. J.; Wang, J. M.; Lin, W. L.; Chu, C. Y.; Chou, F. P.; Tseng, T. H. Protective effect of Hibiscus anthocyanins against *tert*-butyl hydroperoxide-induced hepatic toxicity in rats. *Food Chem. Toxicol.* **2000**, *38*, 411−416.

(29) Recknagel, R. O.; Glende, E. A., Jr.; Dolak, J. A.; Waller, R. L. Mechanisms of carbon tetrachloride toxicity. *Pharm. Ther.* **1989**, *43*, 139−145.

(30) Draper, H. H.; Hadley, M. A review of recent studies on the metabolism of exogenous and endogenous malondialdehyde. *Xenobiotica* **1990**, *20*, 901−907.

Mesona procumbens against Hepatic Damage

J. Agric. Food Chem., Vol. 52, No. 13, 2004   **4127**

(31) Borrello, S.; Palombini, G.; Galeotti, T.; van Rossum, G. D. Effect of *tert*-butyl hydroperoxide on metabolism of liver and hepatoma. *Ann. N. Y. Acad. Sci.* **1988**, *551*, 144−146.

(32) Kadiiska, M. B.; Gladen, B. C.; Baird, D. D.; Dikalova, A. E.; Sohal, R. S.; Hatch, G. E.; Jones, D. P.; Mason, R. P.; Barrett, J. C. Biomarker of oxidative stress study: are plasma antioxidant markers of CCl_4 poisoning? *Free Radical Biol. Med.* **2000**, *28*, 838−845.

(33) Mak, D. H.; Ip, S. P.; Li, P. C.; Poon, M. K.; Ko, K. M. Alterations in tissue glutathione antioxidant system in Streptozotocin-induced diabetic rats. *Mol. Cell. Biochem.* **1996**, *162*, 153−158.

(34) Velmurugan, B.; Bhuvaneswari, V.; Balasenthil, S.; Nagini, S. Lycopene, an antioxidant carotenoid modulates glutathione dependent hepatic biotransformation enzymes during experimental gastric carcinogenesis. *Nutr. Res.* **2001**, *21*, 1117−1124.

(35) Arivazhagan, S.; Balasenthil, S.; Nagini, S. Garlic and neem leaf extracts ehhance hepatic glutathione and glutathione dependent enzymes during *N*-methyl-*N*-nitrosoguanidine (MNNG)-induced gastric carcinogenesis. *Phytother. Res.* **2000**, *14*, 291−293.

(36) Cerutti, P. A. Oxy-radicals and cancer. *Lancet* **1994**, *344*, 862−863.

(37) Cheng, K. C.; Cahill, D. S.; Kasai, H. Nishimura, S.; Loeb, L. A. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G−T and A−C substitutions. *J. Biol. Chem.* **1992**, *267*, 166−172.

(38) Spector, A. Review: Oxidative stress and disease. *J. Ocul. Pharmacol. Ther.* **2000**, *16*, 193−201.

(39) Kasai, H. Analysis of a form of oxidative DNA damage, 8-hydroxy-2′-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. *Mutat. Res.*. **1997**, *387*, 147−163.

(40) Floyd, R. A. The role of 8-hydroxydeoxyguanososine in carcinogenesis. *Carcinogenesis* **1990**, *11*, 1447−1450.

(41) Verhagen, H.; Poulsen, H. E.; Loft, G.; van Poppel, G.; Willems, M. I.; van Bladeren, P. J. Reduction of oxidative DNA damage in human by Brussels sprouts. *Carcinogenesis* **1995**, *16*, 969−970.

(42) Nardini, M.; D'Aquino, M.; Tomassi, G.; Gentili, V.; Difelice, M.; Saccini, C. Inhibition of human low-density lipoprotein oxidation by caffeic acid and other hydrocinnamic acid derivatives. *Free Radical Biol. Med.* **1995**, *19*, 541−552.

(43) Moridani, M. Y.; Scobie, H.; Jamshizadeh, A.; Salehi, P.; O'Brien, P. J. Caffeic acid, chlorogeic acid and dihydrocaffeic acid metabolism: glutathione conjugate formation. *Drug Metab. Dispos.* **2001**, *29*, 1432−1439.

(44) Kono, Y.; Kobayashi, K.; Tagawa, S.; Adachi, K.; Ueda, A.; Sawa, Y.; Shibata, H. Antioxidant activity of polyphenolics in diets. Rate constants of reactions of chlorogenic acid and caffeic acid with reactive species of oxygen and nitrogen. *Biochim. Biophys. Acta* **1997**, *1335*, 335−342.

(45) Paya, M.; Ferrandiz, M. L.; Sanz, M. J.; Alcaraz, M. J. Effects of phenolc compounds on bromobenzene-mediated hepatotoxicity in mice. *Xenobiotica* **1993**, *23*, 327−333.

(46) Rice-Evans, C. Implications of the mechanism of action of tea polyphenols as antioxidants in vitro for chemoprevention in human. *Proc. Soc. Exp. Biol. Med.* **1999**, *220*, 262−266.