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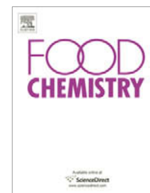

Cytoprotective effects of pu-erh tea on hepatotoxicity *in vitro* and *in vivo* induced by tert-butyl-hydroperoxide

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ABSTRACT

The *in vitro* and *in vivo* protective effects of water extract of pu-erh tea (WEPT) on tert-butyl-hydroperoxide (t-BHP)-induced oxidative damage in hepatocytes of HepG2 cells and in rat livers were investigated. After treatment with 200 μg/ml of samples, the survival rate of HepG2 cells induced by t-BHP increased. WEPT concentration-dependently inhibited reactive oxygen species (ROS) generation in HepG2 cells in response to the oxidative challenge induced by t-BHP. Administration of WEPT (0.2, 0.5 and 1.0 g/kg of body weigh) to rats for 56 consecutive days before a single dose of t-BHP (0.5 mmol/kg, i.p.) exhibited a significant ($p < 0.01$) protective effect by lowering serum levels of glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT), as well as reducing the formation of malondialdehyde. Taken together, these results demonstrate that WEPT is able to protect against hepatic damage *in vitro* and *in vivo*, suggesting that the drinking of pu-erh tea may protect liver tissue from oxidative damage.

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1. Introduction

Oxidative stress is recognized to be a factor in liver disease and carcinogenicity (Pryor, 1986). Liver injury occurs as a result of exposure to the free radicals derived from environmental toxicants, chemicals and drugs (Leal, Begona Ruiz-Larrea, Martinez, & Lacort, 1998). The liver plays a crucial role in metabolic function. Thus, hepatic injury is concerned with the disorder of metabolic function (Bhandarkar & Khan, 2004). Therefore, studies investigating scavenging free radicals as well as reducing oxidative stress, and thereby protecting liver from damage, have attracted much attention.

Tea (*Camellia Sinensis*) is a popular drink due to its pleasant taste and is now the second most widely consumed beverage in the world (Kuroda, 1990). In particular, tea has been used as a crude medicine for 4000 years (Kuroda, 1990). According to extensive *in vitro* and *in vivo* experiments and epidemiological evidence, tea extracts have displayed many biological actions, such as antifungal (Okubo, Toda, Hara, & Shimamura, 1991), antiviral (Green, 1949), antioxidant (Matsuzaki & Hara, 1985), antimutagenic (Okuda, Mori, & Jayatsu, 1984), and antitumor (Conney, 1982) activities. According to period of fermentation, teas are classified as green tea (fermentation-free), oolong tea (mild fermentation) and black tea (full fermentation). However, like black tea, pu-erh tea is prepared by full fermentation, but it is prepared for a longer period. *Aspergillus niger* is found in pu-erh tea during fermentation. It is believed that the longer the preservation period, the better are the taste and quality (Sano et al., 1986). Although the biological effects of tea extracts and tea-derived products, such as green tea, oolong tea and black tea, have been demonstrated, data regarding the biological effect of pu-erh tea are limited in the literature. Sano et al. (1986) noted that the levels of plasma cholesterol ester and triglyceride in the plasma of rats were significantly reduced by administration of pu-erh tea. In our previous studies, pu-erh tea has been shown to display remarkable inhibition of lipid and non-lipid oxidation, similar to that of other tea extracts (Duh, Yen, Yen, Wang, & Chang, 2004). We also found that pu-erh tea directly scavenged nitric oxide (NO) radicals (Duh et al., 2004), and the regulating mechanism of pu-erh tea on NO generation has been clearly elucidated (Wang, Yu, Chang, Yen, & Duh, 2008). In addition, we reported that pu-erh tea inhibited LDL oxidation and positively modulated the glutathione (GSH) and antioxidant enzyme systems in 3T3 cells (Wang et al., 2008). We noted that pu-erh tea showed antimutagenic action against aflatoxin B1 (AFB_1) and 4-nitroquinoline-N-oxide (NQNO), as well as antimicrobial effects against *Staphylococcus aureus* and *Bacillus subtilis* (Wu et al., 2007). Apart from these observations, little information is available about the biological action of pu-erh tea. Although pu-erh tea has demonstrated the potential biological effects mentioned above, whether pu-erh tea has protective effects against hepatotoxicity has not been elucidated. Thus, the present study aimed to investigate, both *in vitro* and *in vivo*, the protective effect of pu-erh tea on hepatotoxicity induced by oxidative stress.

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2. Materials and methods

2.1. Samples

Pu-erh tea and green tea were purchased from a local market in Tainan, Taiwan, Republic of China. Each tea (300 g) was extracted twice with boiling water (3000 ml) for 5 min. Then, the filtrate was freeze-dried and weighed. These final freeze-dried samples (60 g and 64 g for pu-erh tea and green tea, respectively) were designated water extracts of pu-erh tea (WEPT) and green tea (WEGT), respectively.

2.2. Measurement of HepG2 cells viability

HepG2 cells (ATCC number: CRL-11997) were purchased from Bioresources Collection and Research Center (Shin-chu, Taiwan) and cultured in minimum essential medium (MEM) containing 10% fetal bovine serum, 2 mM glutamine, and maintained in humidified 5% CO_2/95% air at 37 °C. After cells were cultured with samples, in the presence of 2 mM t-BHP or not, for 2 h, cell viability was determined by colorimetric measurement of the reduction product of (3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). After the original medium was removed, MTT (final 0.5 mg/ml) were added to each well. After 1 h of incubation, the reaction was terminated and the plates were incubated for 30 min to solubilize the formazan dye by addition of dimethyl sulfoxide. The optical density of each well was measured at 570 nm.

2.3. Measurement of intracellular reactive oxygen species

Intracellular reactive oxygen species were determined by using the 2′,7′-dichlorofluorescin diacetate (H_2DCFDA) fluorescence probe. H_2DCFDA is nonfluorescent until removal of the acetate groups by intracellular esterases and reacts with reactive oxygen species to produce the fluorescent product, dichlorofluorescein (DCF) within the cells. Prior to tert-butyl-hydroperoxide (t-BHP) stimulation, cells were cultured with H_2DCFDA (10 μM). After 20 min of incubation, various concentrations of samples were added to cells for 30 min, and then incubated with t-BHP (2 mM) for 30 min. The control wells, containing dye, samples and t-BHP, did not produce any fluorescent signal. After incubation, reactive oxygen species produced from cells was determined using a Bio-Tek FLx800 microplate fluorescence reader with excitation and emission wavelengths of 485 and 535 nm, respectively.

2.4. Animals

This study was conducted in conformity with the policies and procedure details in the ''Guide for the Care and Use of Laboratory Animals'' (NIH Publication No. 86–23 1985). Fifty-five Wistar male rats (100–105 g/rat) were purchased from BioLASCO Taiwan Co., Ltd., Taipei, Taiwan and divided into eleven groups. Rats were housed in an environmentally controlled room (temperature 25 ± 1 °C; 12 h light/12 h dark cycle) and were given free access to a standard commercial chow diet. Then WEPT (0.2, 0.5, 1 g/kg weight), WEGT (0.2, 0.5, 1 g/kg weight), gallic acid (GA) (0.02, 0.2 g/kg weight), and silymarin (0.5 mmol/kg weight), respectively, were fed daily for 56 days. Daily feed intake and weekly body weight gains of animals were routinely recorded throughout the experimental period. No significant differences were found between the body weights of control and induced rats at the end of the experimental period (data not shown). On day 56, t-BHP (0.5 mmol/kg weight) in PBS was injected (i.p.) to induced groups rats and, 18 h later, all rats were sacrificed under anesthesia. The blood samples of rats were collected for serum total antioxidant activity, GOT and GPT assays, respectively, and livers were excised from the animals to analyze malondialdehyde (MDA) formation and antioxidant enzyme activity.

2.5. The trolox equivalent antioxidant capacity of serum

This method is based on the capacity of serum to scavenge the 2, 2′-azinobis 3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{·+}$) radical cation compared with trolox in a concentration-dependent response. The $ABTS^{·+}$ radical-scavenging activity was measured as previously described (Arnao, Cano, & Acosta, 2001). The $ABTS^{·+}$ radical was generated by reacting 1 mM ABTS, 0.5 mM hydrogen peroxide and 10 units/ml of horseradish peroxidase in the dark at 30 °C for 2 h. After 1 ml of $ABTS^{·+}$ was added to 0.25 ml of rat serum or trolox standards, the absorbance at 734 nm was recorded after 10 min. The radical-scavenging capacity was plotted as a function of concentration and the trolox equivalent antioxidant capacity (TEAC) was calculated against a trolox calibration curve.

2.6. Hepatotoxicity assessment

Early acute hepatic damages were determined by detecting serum GOT and GPT activities. Serum activities of GOT and GPT were determined by using GOT and GPT kits (RANDOX laboratories Ltd., Crumlin, UK) according to the manufacturer's instructions.

2.7. Liver lipid peroxidation and antioxidant enzyme activity determination

The livers were weighed, minced into small pieces, and rinsed twice with ice-cold homogenization buffer (250 mM sucrose, 50 mM Tris–HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA) at 4 °C. The livers were homogenized in five volumes of ice-cold homogenization buffer with a motor-driven Teflon pestle. The homogenates were centrifuged at 1000*g* for 10 min. The pellets were discarded, and supernatants were centrifuged at 12,000*g* for 30 min. The final supernatant protein contents were determined by using the bicinchoninic acid protein assay reagents (Pierce). Lipid peroxidation products were estimated by measuring the concentration of thiobarbituric acid-reactive substances (TBARS) in fluorescence at 530 nm ex/552 nm em.

Further, the antioxidant enzyme activities of liver homogenate supernatants were determined as previously described. Hepatic glutathione peroxidase (GPx) and glutathione reductase (GRd) activities were measured by following the decrease in the absorbance due to oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) (Wang et al., 2008). Briefly, a reaction mixture containing 1 mM GSH, 1 unit/ml of GRd, 1 mM NaN_3, 1 mM EDTA, 0.2 mM NADPH and 0.1 ml of liver supernatants was mixed with 0.1 ml of 2.5 mM hydrogen peroxide (H_2O_2) for GPx activity determination. To another mixture containing 1 mM $MgCl_2$, 1 mM oxidized glutathione (GSSG), 0.2 mM NADPH, was added 0.1 ml of liver supernatants for GRd activity determination. The decreased absorbance at 340 nm was measured for 3 min. The superoxide dismutase (SOD) activity was determined using the xanthine–xanthine oxidase-iodophenyl nitrophenyl phenyltetrazolium chloride (INT) system, as previously described (Durackova & Labuda, 1995). The reaction mixture containing 0.05 mM xanthine, 0.025 mM INT, 1 mM EDTA, and 0.1 ml of liver supernatants was mixed with 0.1 ml xanthine oxidase (80 unit/ml). The change in absorbance at 510 nm in 3 min was monitored. The activity of catalase (CAT) was measured as described previously (Armstrong & Browne, 1994). Liver supernatants were reacted with 20 mM H_2O_2 in 50 mM potassium phosphate buffer, pH 7.0. The change in absorbance was monitored at 240 nm at 30 and 120 s.

2.8. Statistical analysis

Statistical analysis involved use of the Statistical Analysis System software package. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple range tests at a level of $p < 0.05$.

3. Results

In our previous study (Wang et al., 2008), HPLC analysis showed that gallic acid (GA), epicatechin (EC) and caffeine, which are polyphenols with bioactive action, were present in WEPT. In addition, these three bioactive compounds are also present in WEGT (Zuo, Chen, & Deng, 2002). Therefore, GA, EC and caffeine were used as reference compounds for the following tests.

The HepG2 cells were treated with samples for 2 h, as indicated. The cell viability, which was measured by MTT assay, in the presence of WEPT, WEGT, caffeine, EC and GA in the range of 0–1000 μg/ml, was ⩾95%, indicating that WEPT, WEGT, GA, EC and caffeine showed no cytotoxicity to $HepG_2$ cells. Therefore, concentrations below 1000 μg/ml were used for the following experiments. To evaluate whether WEPT protects $HepG_2$ cells from oxidative stress induced by t-BHP, the direct cytotoxic effect of t-BHP on $HepG_2$ cells in the presence and absence of WEPT, WEGT or GA, EC and caffeine was determined. The results are shown in Fig. 1. The cell viability of HepG2 induced by t-BHP (2 mM) in the absence of WEPT, WEGT, GA, EC and caffeine, was 50%, indicating that t-BHP showed remarkable cytotoxicity on $HepG_2$ cells. However, treatment of $HepG_2$ cells with different concentrations of WEPT, WEGT, GA or EC protected cells against t-BHP-induced cytotoxicity in a dose-dependent manner. Nevertheless, caffeine showed no protective effect on HepG2 cells against t-BHP-induced cytotoxicity. WEPT, WEGT, EC and GA at 1000 μg/ml, significantly increased cell viability up to 78%, 102%, 100% and 97%, respectively, from 50%, for t-BHP-treated cells. Obviously, WEPT, WEGT, GA, and EC exhibited comparable protection against t-BHP-induced cytotoxicity.

To investigate the effects of WEPT, WEGT, EC and GA on intracellular ROS generation, $HepG_2$ cells were pretreated with WEPT, WEGT, EC or GA in the concentration range 0–1000 μg/ml for 30 min prior to the addition of t-BHP (2 mM) for 30 min. The levels of intracellular ROS in $HepG_2$ cells induced by t-BHP were determined using a fluorescent probe, H_2DCFDA. The results are shown in Fig. 2. The level of intracellular ROS in HepG2 cells treated with t-BHP alone was 294% compared to the control (100%) treated without t-BHP. The HepG2 cells treated with WEPT, WEGT, EC and GA at 1000 μg/ml showed 185%, 168%, 60% and 100%, respectively, of intracellular ROS production compared to control (100%). The ROS generation in $HepG_2$ cells treated with WEPT, WEGT, EC and GA significantly decreased in a dose-dependent manner. t-BHP is known to generate ROS and induce oxidative stress in the liver (Wang et al., 2000). Although the suppression of ROS by WEPT was inferior to those of WEGT, EC and GA, WEPT displayed a potent suppression of ROS and appear to suppress toxicity induced by t-BHP.

According to Figs. 1 and 2, WEPT was able to decrease the cytotoxicity and suppress the ROS formation in HepG2 cells induced by t-BHP. Thus, this antioxidant bioactivity of WEPT was further evaluated by finding whether WEPT protected hepatotoxicity in rats induced by t-BHP. In addition, among the reference compounds, although EC and GA are the main constituents in WEPT (Wang et al., 2008), there was a trace of EC in WEGT (Duh et al., 2004). Therefore, GA was used as a reference compound in the following *in vivo* experiments.

Table 1 shows the effect of WEPT on total antioxidant activity of serum in rats induced by t-BHP. The total antioxidant activity of serum in rats induced by t-BHP was significantly decreased compared to normal control ($p < 0.05$). Total antioxidant activity in serums of t-BHP-induced rats administered with 0.5 and 1.0 g/kg of WEPT and 0.02 g/kg GA, respectively, was significantly increased compared to the t-BHP-induced group ($p < 0.05$). In contrast, no significant differences were found between the rats administered with WEGT in the concentration range 0.2–1.0 g/kg as well as 0.2 g/kg GA and the t-BHP-induced group ($p > 0.05$).

The leakage of hepatic enzymes, such as GOT and GPT, in the serum indirectly reflects the failure of liver function due to hepatotoxicity (Yen, Wu, Lin, & Lin, 2007). Thus, the levels of these enzymes are used as the index of hepatic damage. The effects of pretreatment of WEPT, WEGT and GA on the t-BHP-induced elevation of GOT and GPT are shown in Table 2. t-BHP intoxication in normal rats elevated the levels of GOT and GPT as compared to the normal control. Pretreatment with WEPT (0.2, 0.5 and 1.0 g/kg),

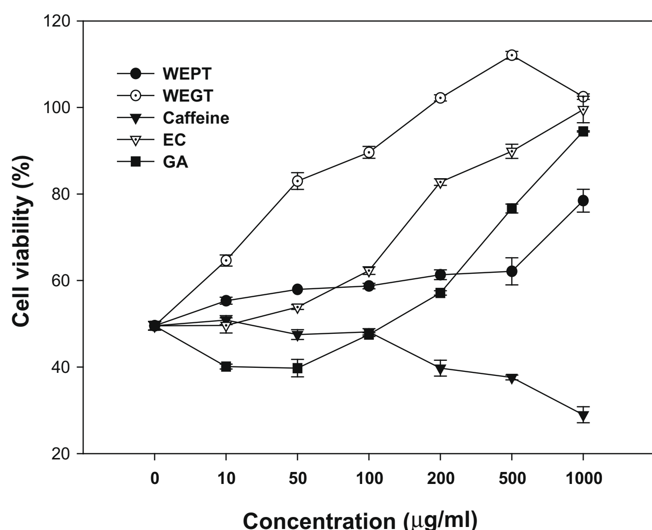

Fig. 1. Effect of WEPT on viability of HepG2 cells induced by t-BHP. WEPT, water extract of pu-erh tea; WEGT, water extract of green tea; EC, epicatechin; GA, gallic acid.

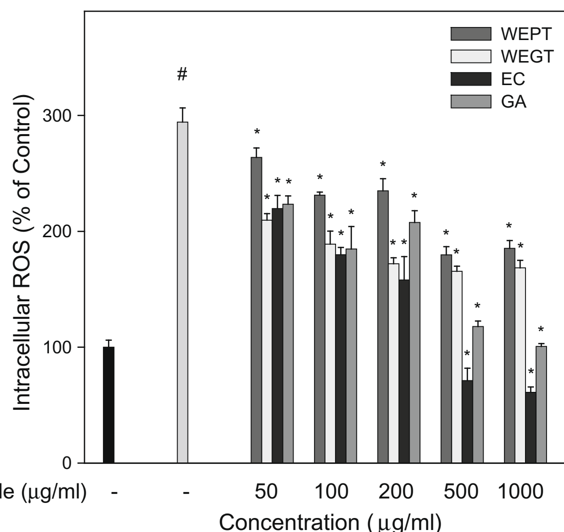

Fig. 2. Effect of WEPT on ROS generation in HepG2 cells induced by t-BHP. WEPT, water extract of pu-erh tea; WEGT, water extract of green tea; EC, epicatechin; GA, gallic acid. #, $p < 0.05$, compared with control; *, $p < 0.05$, compared to treatment with t-BHP.

Table 1
Effect of WEPT on total antioxidant activity of serum in rats induced by t-BHP.

Treatment	TEAC (μg/0.02 ml serum)
Control	3.07 ± 0.11
t-BHP 0.5 mmol/kg	2.79 ± 0.12[a]
Silymarin 0.2 g/kg + t-BHP	3.25 ± 0.4
WEPT 0.2 g/kg + t-BHP	3.26 ± 0.18
WEPT 0.5 g/kg + t-BHP	3.38 ± 0.47[b]
WEPT 1.0 g/kg + t-BHP	3.8 ± 0.34[b]
WEGT 0.2 g/kg + t-BHP	2.96 ± 0.16
WEGT 0.5 g/kg + t-BHP	3.03 ± 0.09
WEGT 1.0 g/kg + t-BHP	3.11 ± 0.19
GA 0.02 g/kg + t-BHP	3.83 ± 0.32[b]
GA 0.2 g/kg + t-BHP	3.25 ± 0.10

WEPT, water extract of pu-erh tea; WEGT, water extract of green tea; GA, gallic acid.
[a] $p < 0.05$, compared with control.
[b] $p < 0.05$, compared to treatment with t-BHP.

WEGT (0.5 and 1.0 g/kg), and GA (0.02 and 0.2 g/kg) significantly decreased the elevation of GOT ($p < 0.05$). In addition, WEPT at high doses of 1.0 g/kg, WEGT at 0.5 and 1.0 g/kg and GA at 0.02 and 0.2 g/kg significantly reduced the elevation of GPT. These results indicate that administration of WEPT, WEGT or GA resulted in a significantly reduced the t-BHP-induced hepatic damage.

In order to further evaluate the possible mechanism involved in the protective effect of WEPT on hepatotoxicity, MDA formation in t-BHP-induced liver damage in rats was further evaluated. As seen in Fig. 3, t-BHP-treated rats administered with 0.2, 0.5, or 1.0 g/kg WEPT significantly reduced the amount of MDA by 54.7%, 57.6% and 89.1% in liver homogenate, respectively, as compared with the t-BHP-intoxicated group ($p < 0.05$), indicating that WEPT, WEGT and GA may prevent the oxidative damage in the liver of rats induced by t-BHP.

Since the oxidative stress derived from t-BHP-induced hepatotoxicity occurred in rats, the levels of liver antioxidant enzymes SOD, GPx, GRd and CAT were determined. Table 3 shows the effect of WEPT on hepatic antioxidant enzymes in t-BHP-induced liver damage of rats. WEPT and WEGT, at doses of 0.2, 0.5 and 1.0 g/kg, did not raise SOD, GPx and GRd activities as compared to the rats treated with t-BHP. As for CAT activity, WEPT at doses of 0.2, 0.5 g/kg did not raise CAT activity except when a high dosage of 1.0 g/kg was used ($p < 0.05$).

4. Discussion

t-BHP is a well-known oxidant which is often used as a model compound to induce oxidative stress during *in vitro* and *in vivo* studies. In the present study, t-BHP dose-dependently decreased the viability of HepG2 in the model system used (data not shown).

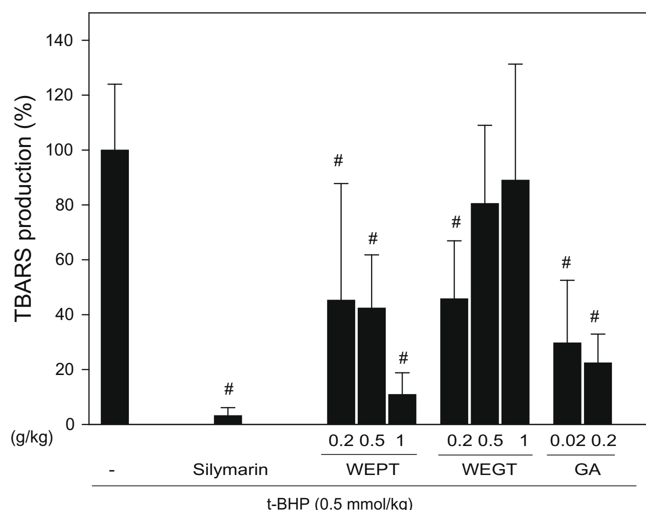

Fig. 3. Effect of WEPT on TBARS production of liver in rats induced by t-BHP. WEPT, water extract of pu-erh tea; WEGT, water extract of green tea; GA, gallic acid. #, $p < 0.05$, compared with t-BHP group.

t-BHP at 2 mM showed 50% inhibition of HepG2 cell viability. Treatment with non-cytotoxic concentrations (in the range of 0–1000 μg/ml) of WEPT increased cell survival after t-BHP exposure, indicating that WEPT, in the range of concentrations tested, may protect HepG2 cells from t-BHP-induced cytotoxicity. In addition, t-BHP significantly enhanced the ROS generation in HepG2 cells (Fig. 2). The increase in ROS generation may be instrumental in inducing cell injury, and consequently, may decrease the cell viability (Fig. 1). Wang et al. (2000) noted that t-BHP can be metabolized into free radical form intermediated by cytochrome P450, and subsequently can initiate lipid peroxidation and affect cell membrane integrity, leading to cell damage. The present results show that WEPT inhibited t-BHP-induced hepatocyte ROS generation, as well as increasing viability of HepG2 cells induced by t-BHP. This observation suggests that WEPT reduced t-BHP-induced cell killing associated with its effects on the suppression of ROS generation. In other words, the addition of WEPT decreases ROS levels, and exerts its protective effects against t-BHP cytotoxicity through the scavenging of free radicals (Park et al., 2003). Thus, the antioxidant bioactivity of WEPT on hepatocyte, as an efficient inhibitor of ROS generation as well as a good protector against hepatic damage, is demonstrated.

Until now, few studies regarding the biological activity of WEPT have been reported. Based on the data mentioned above, WEPT showed biological action in HepG2 cells induced by t-BHP

Table 2
Effects of WEPT on serum biochemical marker in rats induced by t-BHP.

Treatment	GOT (U/l)	GPT (U/l)	BUN (mg/dl)	CREA (mg/dl)
Control	365 ± 30[d]	59 ± 4[c]	24 ± 1[a]	0.8 ± 0.06[a,b,c]
t-BHP 0.5 mmol/kg	1829 ± 265[a]	353 ± 121[a]	24 ± 10[a,b]	0.8 ± 0.15[a,b,c]
Silymarin 0.2 g/kg + t-BHP	1353 ± 238[b]	257 ± 97[a,b]	19 ± 5[a,b,c]	0.9 ± 0.21[a,b,c]
WEPT 0.2 g/kg + t-BHP	1060 ± 303[b,c]	247 ± 111[a,b]	20 ± 3[a,b,c]	1.0 ± 0.12[a]
WEPT 0.5 g/kg + t-BHP	1312 ± 53[b,c]	231 ± 24[a,b]	16 ± 1[b,c]	0.8 ± 0.06[a,b,c]
WEPT 1.0 g/kg + t-BHP	1271 ± 204[b,c]	216 ± 30[b]	20 ± 3[a,b,c]	0.9 ± 0.06[a,b]
WEGT 0.2 g/kg + t-BHP	1669 ± 152[a]	243 ± 58[a,b]	15 ± 1[c]	0.8 ± 0.00[a,b,c]
WEGT 0.5 g/kg + t-BHP	1223 ± 127[b,c]	194 ± 20[b]	16 ± 3[bc]	0.8 ± 0.06[b,c]
WEGT 1.0 g/kg + t-BHP	1130 ± 130[b,c]	180 ± 61[b]	18 ± 4[a,b,c]	0.7 ± 0.06[c]
GA 0.02 g/kg + t-BHP	959 ± 182[c]	152 ± 35[b,c]	21 ± 4[a,b,c]	0.9 ± 0.06[a,b,c]
GA 0.2 g/kg + t-BHP	1115 ± 160[b,c]	201 ± 35[b]	19 ± 3[a,b,c]	0.8 ± 0.00[a,b,c]

WEPT, water extract of pu-erh tea; WEGT, water extract of green tea; GA, gallic acid; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase; BUN, blood urine nitrogen; CREA, creatinine. Values with different superscripts in a column are significantly different ($p < 0.05$).

Table 3
Effects of WEPT on hepatic antioxidant enzyme activities in rats induced by t-BHP.

Treatment	SOD (U/g protein)	GPx (U/g protein)	GRd (U/g protein)	CAT (UM/g protein)
Control	$1284 \pm 167^{a,b}$	2032 ± 383^{b}	152 ± 56^{a}	$10.1 \pm 1.8^{c,d,e}$
t-BHP 0.5 mmol/kg	$1256 \pm 295^{a,b}$	$2151 \pm 275^{a,b}$	$106 \pm 10^{a,b,c}$	7.1 ± 3.2^{de}
Silymarin 0.2 g/kg + t-BHP	$1180 \pm 76^{a,b}$	1987 ± 412^{b}	$104 \pm 11^{a,b,c}$	$7.7 \pm 1.9^{d,e}$
WEPT 0.2 g/kg + t-BHP	$1305 \pm 365^{a,b}$	1546 ± 224^{b}	$100 \pm 11^{a,b,c}$	$10.9 \pm 2.7^{c,d,e}$
WEPT 0.5 g/kg + t-BHP	$1302 \pm 161^{a,b}$	1341 ± 343^{b}	$129 \pm 48^{a,b,c}$	$12.6 \pm 5.4^{b,c,d}$
WEPT 1.0 g/kg + t-BHP	1601 ± 407^{a}	3091 ± 694^{b}	$145 \pm 5^{a,b}$	$18.4 \pm 5.4^{a,b}$
WEGT 0.2 g/kg + t-BHP	1578 ± 288^{a}	1837 ± 1567^{b}	$141 \pm 48^{a,b,c}$	20.8 ± 3.8^{a}
WEGT 0.5 g/kg + t-BHP	$1427 \pm 235^{a,b}$	1513 ± 138^{b}	$114 \pm 28^{a,b,c}$	$15.1 \pm 3.7^{a,b,c}$
WEGT 1.0 g/kg + t-BHP	$1034 \pm 253^{b,c}$	1377 ± 230^{b}	$103 \pm 3^{a,b,c}$	$11.2 \pm 5.3^{c,d,e}$
GA 0.02 g/kg + t-BHP	706 ± 80^{c}	1818 ± 382^{b}	$91 \pm 6^{b,c}$	$6.0 \pm 0.6^{d,e}$
GA 0.2 g/kg + t-BHP	659 ± 80^{c}	1591 ± 166^{b}	87 ± 11^{c}	5.7 ± 0.8^{e}

WEPT, water extract of pu-erh tea; WEGT, water extract of green tea; GA, gallic acid. SOD, superoxide dismutase; GPx, glutathione peroxidase; GRd, glutathione reductase; CAT, catalase. Values with different superscripts in a column are significantly different ($p < 0.05$).

in vitro. Thus, WEPT was expected to display biological activity against liver damage in rats. To confirm this, an *in vivo* hepatoprotective model system was studied.

Polyphenolic compounds play an important role in the protection against oxidative damage. The health effects of polyphenolic compounds depend on the amount consumed and on their bioavailability (Manach, Scalbert, Morand, Remesy, & Jimenez, 2004). The present study showed that rats administered with WEPT significantly increased their levels of total polyphenolic compounds in serum compared to the control groups ($p < 0.05$) (data not shown). Previous studies (Scalbert & Williamson, 2000) noted that the consumption of 300 ml of red wine, containing ~500 mg of polyphenols, induced an increase of the plasma antioxidant activity. Apparently, an increase in the serum polyphenol concentration may partially explain why the total antioxidant activity in the serum of t-BHP-induced rats administered WEPT was significantly increased compared to the t-BHP-induced group ($p < 0.05$).

Although there were no significant differences in relative organ weights of liver and kidney between rats treated with t-BHP in the presence of WEPT (data not shown), there were significant differences ($p < 0.05$) in GOT and GPT levels between rats treated with t-BHP alone and those treated with t-BHP in the presence of WEPT. Apparently, in the present study, t-BHP exerted its cytotoxicity in both cell cultures and animal model systems. These may be a result of cytochrome P450-mediated metabolism of the hydroperoxide to active alkoxyl radicals, which consequently leads to initiated lipid peroxidation and causes liver damage (Wang et al., 2000). According to Table 1, WEPT was able to attenuate the elevation of GOT and GPT levels and protect the hepatocyte and rat liver from the oxidative stress caused by t-BHP. In addition, t-BHP can be converted into active peroxyl and alkoxyl radicals by cytochrome P450 enzymes, and can then initiate lipid peroxidation that leads to liver injury (Lima, Fernandes-Ferreira, & Pereira-Wilson, 2006). On the other hand, in our previous studies (Duh et al., 2004; Wang et al., 2008), WEPT containing bioactive components, such as ascorbic acid, caffeine, GA and EC, exhibited a significant metal-binding activity, reducing ability and scavenging effect for free radicals, which may be responsible for the inhibitory effect on lipid peroxidation. Our *in vivo* studies showed that the MDA formation due to lipid peroxidation in the livers of rats induced by t-BHP was attenuated by the addition of WEPT (Fig. 3). In addition, the hepatic injury of rats induced by t-BHP was inhibited by WEPT (Table 1). Taken together, we hypothesize that there is a good correlation between hepatoprotective effects and the inhibitory action of lipid peroxidation. Moreover, WEPT enhanced CAT activity and thereby decreased the amounts of hydroperoxide against t-BHP-induced hepatoxicity in rats. These findings indicate that the reduction of oxidative stress plays a crucial role in the mechanism of the hepatoprotective effects (Yen et al., 2007).

Epigallocatechin gallate (EGCG) and epigallocatechin (EGC) are the main constituents in the WEGT but were not detected in WEPT (Duh et al., 2004). On the contrary, EGCG and EGC are not detected in WEPT but, instead, EC is present in WEPT. According to our previous study (Wang et al., 2008), the amount of GA in WEPT was higher than in WEGT due to the higher degree of fermentation in pu-erh tea. Instead, the amounts of ascorbic acid, flavonoids and polyphenolic compounds in WEPT were less than those in WEGT. One study (Galati, Lin, Sultan & O'Brien, 2006) noted that administration of a single dose of the tea's phenolic acids, GA (200–800 mg/kg) and propyl gallate (100–200 mg/kg), as well as EGCG (100–300 mg/kg), resulted in a dose-dependent increased liver injury, *in vivo*, in male CD-1 mice. EC was also found to have cytotoxicity (Galati, Lin, Sultan, & O'Brien, 2006). However, as shown in Fig. 1, GA and EC, in the range 0–1000 μg/ml, were not found to be toxic to HepG2 cells. In addition, recent studies have clearly demonstrated that ascorbic acid (Ahn, Yun, & Oh, 2006), GA (Park, Han, Park, Choi, & Choi, 2005), EC and EGCG (Raza & John, 2007) protected the liver from various types of toxicity. Thus, WEGT significantly lowered the serum levels of GOT and GPT, as well as reducing the oxidative stress of the liver, which may be associated with the presence of ascorbic acid, GA, EC and other catechins. Due to the fermentation and preservation over a long period, the contents of polyphenolics, EC and ascorbic acid in WEPT were less than those in WEGT (Duh et al., 2004). However, WEPT still showed hepatoprotective action for both *in vitro* and *in vivo* model systems, suggesting that this hepatoprotective action of WEPT may not be mainly attributed to the contents of ascorbic acid, EC, GA and polyphenolics. In addition, some active compounds may be formed during a long period of fermentation (Unno, Sugimoto, & Kakuda, 2000). We speculate that some active compounds formed during fermentation may complement the ascorbic acid, EC, GA and polyphenolics, which would account for the hepatoprotective activity of WEPT.

In conclusion, the results of this investigation demonstrate that the decrease of oxidative damage by WEPT contributes to hepatoprotective effects against t-BHP-induced hepatotoxicity for both *in vitro* and *in vivo* model systems. The inhibition of ROS generation and reduction of MDA formation are likely to be major factors in the mechanism of hepatoprotective effects of WEPT. This hepatoprotective activity of WEPT may be due to bioactive compounds present in WEPT in conjunction with its antioxidant activity. In addition, because WEPT has a potential hepatoprotective effect, this study provides positive indications that the daily intake of pu-erh tea may be beneficial to human health and may lessen liver damage induced by environmental and dietary toxicants.

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