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Effects of Pu-erh Tea on Oxidative Damage and Nitric Oxide Scavenging

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The effects of pu-erh tea, which is prepared by fermentation of tea, on oxidative damage and nitric oxide scavenging, compared with various other brands of tea were investigated. The total antioxidant activity was determined using the Trolox equivalent antioxidant capacity (TEAC) assay. The results showed that TEAC values of the 200 μg/mL water extracts of pu-erh tea (WEPT), green tea, oolong tea, and black tea were 86.3, 85.3, 87.4, and 80.3 (μg/mL), respectively, indicating that WEPT showed a significant antioxidant activity. WEPT, like green tea extract, oolong tea extract, and black tea extract, exhibited a remarkable protective effect in lipid (liposome) and nonlipid (protein and deoxyribose) model systems, implying that it is an inhibitor of lipid and nonlipid oxidative damage. It also exhibited metal-binding ability, reducing power, and scavenging effect for free radicals. Moreover, WEPT showed a decreasing effect on nitric oxide production of lipopolysaccharide-induced RAW 264.7 macrophages. In addition, the results revealed that epicatechin (EC), flavonoid, ascorbic acid, and polyphenolic compounds are present in WEPT, which may partially account for the protective effect on oxidative damage. Thus, WEPT may have potential as an antioxidant and as a nitric oxide scavenging agent.

KEYWORDS: Pu-erh tea; antioxidant; total catechin; flavonoid; oxidative damage; nitric oxide; free radical scavenging

INTRODUCTION

Free-radical-initiated autoxidation of cellular membrane lipids can lead to cellular necrosis and a variety of pathological conditions such as cancer and even aging (*1*). Hence, a considerable number of investigations have been focused on the prevention of oxidative damage initiated by free radicals. Epidemiological data indicated that >80% of cancers are connected to lifestyle, of which at least one-third is diet-related (*2*). In addition, diets rich in fruits and vegetables are associated with a lower risk of several degenerative diseases (*3*). These observations imply that some phytochemicals, such as antioxidants, and other bioactive compounds are present in these foods. Therefore, investigations of natural antioxidants and bioactive compounds for food preservation and certain human diseases have received much attention.

Pu-erh tea, produced mainly in the Yunnar province of China, is consumed in a large amount by the Chinese people. Pu-erh tea is prepared by fermentation, like black tea, but it is fermented for a long time. The microorganism *Aspergillus niger* is often found in pu-erh tea. As for the quality and taste of pu-erh tea, it is believed that the longer the preservation period, the better the quality and taste. These characteristics of pu-erh tea differ greatly from those of green tea, which is nonfermented and consumed as fresh as possible (*4*).

Tea is one of the most popular drinks due to its pleasant taste and its use as a drug with medical effect. On the basis of extensive animal experiments and some epidemiological data, tea has been attributed the properties of being anti-inflammatory, antioxidative, and anticarcinogenic (*5*). The literature is replete with reports of antioxidant activity and other biological effects in tea and tea-derived products. However, there are considerably fewer reports on the antioxidant activity of pu-erh tea. Sano et al. (*4*) noted that the levels of plasma cholesterol ester and triglyceride in the plasma of rats given pu-erh tea were significantly reduced. Apart from this observation, no reports of pu-erh tea on the antioxidant activity have been reported so far. Regarding the inhibition of nitric oxide (NO) production, a number of polyphenolic phytochemicals have been found to inhibit NO production and iNOS gene expression (*6*). With respect to NO-inhibitory production in tea, green tea extract

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and epigallocatechin gallate (EGCG) showed direct scavenging activity against NO (*5*, *7*). Black tea also has been demonstrated as a powerful chemopreventor of nitrogen species (*8*). Although pu-erh tea is prepared from tea, the inhibitory effects of pu-erh tea on NO production have not been clearly elucidated. Therefore, the aims of the present study were to investigate the antioxidant activity of pu-erh tea and its nitric oxide scavenging activity.

MATERIALS AND METHODS

Materials. Pu-erh tea, green tea, oolong tea, and black tea were purchased from a local market in Tainan, Taiwan, Republic of China. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Fluka Chemie AG (Buchs, Switzerland). 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and ferrozine were obtained from Sigma Chemical Co. (St. Louis, MO). Trolox was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other reagents were of analytical grade.

Extracts. Each tea (50 g) was extracted with boiling water (500 mL) for 5 min, and the filtrate was freeze-dried.

Measurement of Antioxidant Ability by TEAC Method. The total antioxidant activity of the water extract of pu-erh tea (WEPT) was measured using the Trolox equivalent antioxidant capacity (TEAC) assay as described by Miller et al. (*9*) with minor modification. The TEAC value is based on the ability of the antioxidant to scavenge the blue-green ′$ABTS^{\bullet+}$ radical cation relative to the $ABTS^{\bullet+}$ scavenging ability of the water-soluble vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). $ABTS^{\bullet+}$ is generated by the interaction of ABTS (100 μM), H_2O_2 (50 μM), and peroxidase (4.4 units/mL). To measure the antioxidant activity, 0.25 mL of extracts was mixed well with an equal volume of ABTS, H_2O_2, peroxidase, and 1.5 mL of deionized water. Absorbance was measured at 734 nm after the interaction of sample solution for 10 min. The decrease in absorption at 734 nm after the addition of reactant was used to calculate the TEAC value. A dose−response curve was plotted for Trolox, and antioxidant ability was expressed as the TEAC. The higher the TEAC value of a sample, the stronger the antioxidant activity.

Determination of Antioxidant Effect on Liposome Oxidation. Lecithin (580 mg) was sonicated in an ultrasonic cleaner (Branson 8210, Branson Ultrasonic Corp., Danbury, CT) in 58 mL of 10 mM phosphate buffer (pH 7.4) for 2 h. The sonicated solution (10 mg of lecithin/mL), $FeCl_3$, ascorbic acid, and extracts (0.2 mL, 0.500 mg/mL) were mixed to produce a final concentration of 100 μM $FeCl_3$, and 100 μM ascorbic acid. The mixture was incubated for 1 h at 37 °C according to the thiobarbituric acid (TBA) method (*10*). The absorbance of the sample was read at 532 nm against a blank, which contained all reagents except lecithin.

Determination of Effect on Protein Oxidation. The effects of WEPT on protein oxidation were determined according to the method of Lenz et al. (*11*). The reaction mixture (1.2 mL), containing sample (0−500 mg/mL), phosphate buffer (20 mM, pH 7.4), bovine serum albumin (20 mg/mL), $FeCl_3$ (400 μM), H_2O_2 (3.0 mM), and ascorbic acid (400 μM), was incubated for 1 h at 37 °C, and 1 mL of 20 mM dinitophenyl hydrazine (DNPH) in 2 M HCl was added to the reaction mixture. One milliliter of cold trichloroacetic acid (TCA) (20%, w/v) was added to the mixture and centrifuged at 650*g* for 10 min. The protein was washed three times with 2 mL of ethanol/ethyl acetate (1:1, v/v) and dissolved in 2 mL of 6 M guanidine−HCl (pH 6.5). The absorbance of the sample was read at 370 mM. Triplicate samples were run for each set.

Determination of Effects on Oxidation of Deoxyribose. The determination was carried out as described by Halliwell et al. (*12*). The reaction mixture (1.4 mL), which contained extracts (0.2 mL, 0−500 mg/mL), deoxyribose (6 mM), H_2O_2 (3 mM), KH_2PO_4−K_2HPO_4 buffer (20 mM, pH 7.4), $FeCl_3$ (400 μM), ethylenediaminetetraacetic acid (EDTA, 400 μM), and ascorbic acid (400 μM), was incubated at 37 °C for 1 h. The extent of deoxyribose degradation was tested by using the TBA method. One milliliter of 1% TBA and 1 mL of 2.8% TCA were added to the mixture, which was then heated in a water bath at 90 °C for 20 min. The absorbance of the mixture was read spectrophotometrically at 532 nm. All analyses were run in three replicates and averaged.

Table 1. Total Antioxidant Activity of Water Extracts of Pu-erh Tea (WEPT)

sample (μg/mL)	Trolox equiv antioxidant capacity (TEAC,[a] μg/mL)
WEPT	
50	68.3 ± 2.47
100	77.1 ± 2.13
200	86.3 ± 0.11
500	84.5 ± 0.17
green tea	
50	59.5 ± 1.07
100	66.6 ± 0.06
200	85.3 ± 1.29
500	87.0 ± 0.06
oolong tea	
50	55.5 ± 3.48
100	63.1 ± 0.28
200	87.4 ± 0.00
500	87.2 ± 0.00
black tea	
50	50.1 ± 2.42
100	59.0 ± 0.22
200	80.3 ± 0.45
500	86.3 ± 0.06

[a] TEAC is the μg/mL concentration of a Trolox solution having the antioxidant capacity equivalent to a μg/mL solution of the sample under investigation.

Reducing Power. The reducing power of the extracts was determined according to the method of Oyaizu (*13*). Extracts (0−500 mg/mL) in phosphate buffer (2.5 mL, 0.2M, pH 6.6) were added to potassium ferricyanide (2.5 mL, 10 mg/mL), and the mixture was incubated at 50 °C for 20 min. TCA (2.5 mL, 100 mg/mL) was added to the mixture, which was then centrifuged at 650*g* for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 1.0 mg/mL), and then the absorbance was read spectrophotometrically at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing power.

Determination of Effect on DPPH Radical. The effect of extracts on the DPPH radical was estimated according to the method of Hatano et al. (*14*). The extracts (0−500 mg/mL) were added to a methanolic solution (0.5 mL) of DPPH radical (final concentration of DPPH was 0.2 mmol/L). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min; the absorbance of the resulting solution was then measured spectrophotometrically at 517 nm. The tests were run in duplicate, and analyses of all samples were run in triplicate and averaged.

Measurement of Chelating Activity on Metal Ions. The chelating activity of sample on Fe^{-2} was measured according to the method of Carter (*15*). Briefly, extracts (0−500 mg/mL) were incubated with 0.05 mL of $FeCl_2 \cdot 4H_2O$ (2.0 mM). The reaction was initiated by the addition of 0.2 mL of ferrozine (5.0 mM) and finally quantified to 0.8 mL with methanol. After the mixture had reached equilibrium (10 min), the absorbance at 562 nm was read. Triplicate samples were run for each set and averaged.

Determination of Total Phenolic Compounds. The concentration of phenolic compounds was measured according to the method of Taga et al. (*16*) and calculated using gallic acid as standard. A sample (0.1 mL) was added to 2.0 mL of 0.02 g/mL Na_2CO_3. After 2 min, 0.5 mL/mL Folin−Ciocalteu reagent (100 μL) was added to the mixture, which was then left for 30 min. Absorbance was measured at 750 nm using a spectrophotometer.

Determination of Ascorbic Acid Content. Determination of ascorbic acid content was performed according to the method of Klein and Perry (*16*). Extracts of 0.05 g were extracted with 10 mL of 1% metaphosphoric acid. After being filtered, the filtrate (1 mL) was added to 9 mL of 2,6-dichloroindophenol (DIP), and the absorbance at 515 nm was read with a Hitach model 2000 spectrophotometer.

Determination of Flavonoid Content. The spectrophotometer assay for the quantitative determination of flavonoid content was carried out as described by Hairi et al. (*18*). Briefly, the extract (1 mL, 1 mg/mL) was diluted twice with distilled water, and absorption was measured spectrophotometrically at 404 nm after the addition of 100 μL of 1% 2-aminoethyl diphenylborate solution. Extract absorption was compared to that of a standard rutin curve.

High-Performance Liquid Chromatography (HPLC) Analysis. Epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG) in tea extract were analyzed by HPLC, performed with a Hitachi liquid chromatograph (Hitachi Ltd., Tokyo, Japan) consisting of a model L-7100 pump and a model L-7420 UV−vis detector set at 280 nm. A Hypersil BDS RP-18 reversed-phase column (5 μm, 250 mm × 4.6 mm, i.d.), protected with a guard column RP-18 (5 μm, 10 mm × 4 mm), was used for analysis. The volume injected was 20 μL. The gradient elution program was modified from that of Lamuela-Raventos and Waterhouse (*19*). The solvents were (A) 50 mM ammonium dihydrogen phosphate, pH 2.6; (B) 0.2 mM orthophosphoric acid, pH 1.5; and (C) 20% solvent A in 80% acetonitrile. The solvent gradient elution program was as follows:

	solvent (%)		
time (min)	A	B	C
0	100	0	0
5	96	0	4
10	92	0	8
10.1	0	92	8
20	0	80	20
30	0	70	30
60	0	50	50
75	0	20	80
80	100	0	0

Cell Culture. RAW 264.7 cells, an Abelson virus-transformed murinemacrophage cell line (American Type Culture Collection), were cultured in RPMI-1640 medium containing 50 mL/L heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 1 mM pyruvate and were maintained in humidified 5% CO_2/95% air at 37 ± 0.5 °C. For cell stimulation, cells were grown in a six-well plate (1×10^6 cells/well) in 2 mL of growth medium for 18 h to allow the cell number to approximately double. Then, the growth medium was replaced and the indicated stimulants were added.

MTT Assays. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was performed as previously described. Briefly, cells were dispensed into 96-well plates and drugs were added at appropriate concentrations for 24 h. Then, MTT (5 mg/mL stock solution) was added to each well. After 1 h, the reaction was terminated and the plates were incubated for 30 min to solubilize the formazan dye by addition of DMSO. The optical density (OD) of each well was measured with an Anthos 2010 microplate reader at 570 nm.

Scavenging NO Production in LPS-Activated RAW 264.7 Cells. Cells were seeded in 96 wells (2×10^4 cells/well), cultured for 2 days, and then incubated with or without LPS (1.0 μg/mL) in the absence or presence of WEPT, individually, at various concentrations for 20 h. The nitrite concentration in the supernatant was assessed, on the basis of the Griess reaction, and determined through comparison with a sodium nitrite standard curve (*20*).

RESULTS AND DISCUSSION

The antioxidant activity of various tea extracts was evaluated by means of TEAC assay, and the results are shown in **Table 1**. The antioxidant activity of WEPT increased with increasing concentration up to 200 μg/mL, and then no significant differences ($P > 0.05$) were shown in the antioxidant activity with concentration from 200 to 500 μg/mL. Similar results were obtained with the other extracts. As can be seen in **Table 1**, even the addition of 50 μg/mL of WEPT in the solution of sample was able to reduce the formation of peroxides. Apparently, the process of pu-erh tea preparation is different from that of other tea; however, the antioxidant activity of pu-erh tea is similar to that of other tea.

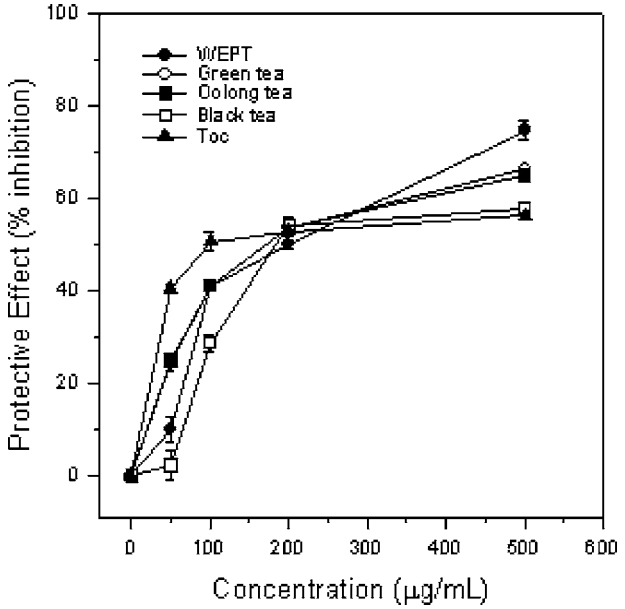

Figure 1. Effect of water extracts of pu-erh tea (WEPT) on liposome peroxidation. Toc, tocopherol.

To determine the protective effect of WEPT on lipid and nonlipid oxidation, the formation of liposome, protein, and deoxyribose oxidation was measured. **Figure 1** shows the effect of WEPT on liposome peroxidation compared to various tea extracts and tocopherol (Toc). WEPT in the range of 0−500 μg/mL shows 10.0−74.7% inhibition of peroxidation compared with the control, indicating that the observed inhibition was concentration dependent. There were no significant differences ($P > 0.05$) between 200 μg/mL of WEPT and 200 μg/mL of other tea extracts and Toc. However, of the five samples, 500 μg/mL WEPT showed the highest inhibitory effect. In this model system, liposome peroxidation was assessed by the amount of malonaldehyde (MDA) produced. MDA is very reactive due to its bifunctional aldehyde. Of special note is that it has been found to take part in the cross-linking reaction with DNA and proteins (*21*). As shown in **Figure 1**, WEPT significantly lowered MDA when compared with the control. This result implies that WEPT may protect against damage to cell membrane because it reduces the formation of MDA.

The effect of WEPT on protein oxidation, induced by $FeCl_2$, H_2O, and ascorbic acid, is plotted in **Figure 2**. Various tea extracts in the range of 0−500 μg/mL showed that the protective effect on protein oxidation was concentration dependent. Especially, in the presence of 200 μg/mL WEPT, protection of protein oxidation was 87.9%, which was the highest protection and statistically significantly different ($P < 0.05$) among the protective effect of other tea extracts and Toc. However, in the 500 μg/mL of sample used, there was no statistical significance ($P > 0.05$) among the protective effects of various tea extracts. As for other tea extracts in the range of 0−500 μg/mL, the protective effects on protein oxidation were 29.3−84.9, 53.9−96.7, and 52.0−94.8% for green tea extract, oolong tea extract, and black tea extract, respectively. This result agrees with the findings of Chen (*22*), who reported that various tea extracts (green tea, oolong tea, paucha tea extract, and black tea extract) showed remarkable inhibitory effects on protein oxidation. Stadtman (*23*) noted that amino acid residues are oxidized to

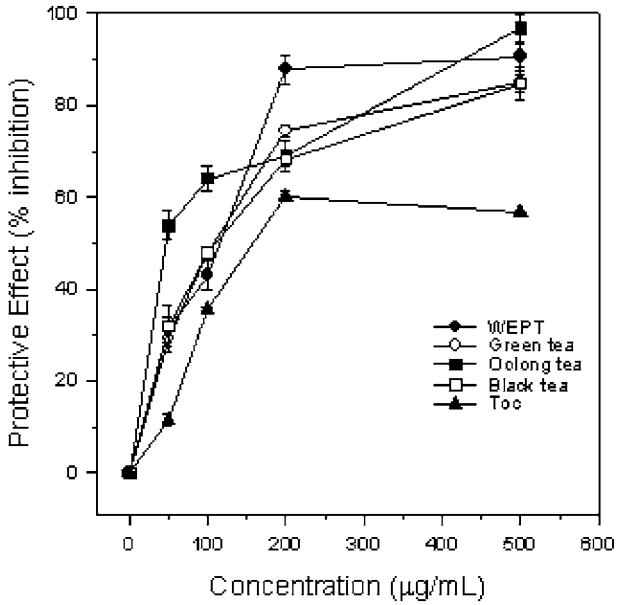

Figure 2. Effect of water extracts of pu-erh tea (WEPT) on albumin oxidative damage. Toc, tocopherol.

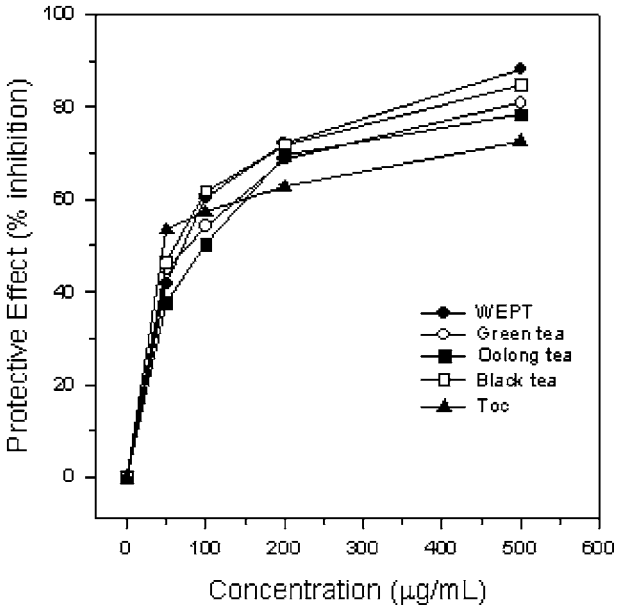

Figure 3. Effect of water extracts of pu-erh tea (WEPT) on deoxyribose oxidative damage. Toc, tocopherol.

carbonyl derivatives, and the amounts of protein carbonyl in patients with premature aging diseases are about the same as those found in fibroblasts of 80-year-old normal individuals. In addition, oxidized protein in old individuals could amount to 20−30% of the total cellular protein. Accordingly, protein oxidation contributes to the pool of damaged enzymes, which increases in size during aging and in various pathological states (*24*). From **Figure 2**, it can be seen that WEPT and other tea extracts have marked inhibitory effect on protein oxidation, indicating that constant consumption of WEPT and other tea could stimulate the protection of protein oxidation and thereby decrease the accumulation of oxidized proteins.

The effect of WEPT and other tea extracts on deoxyribose damage induced by Fe^{3+}/H_2O_2, measured by the thiobarbituric acid method, is plotted in **Figure 3**. The inhibitory effect on deoxyribose damage by WEPT and other tea extracts was found to be concentration dependent. These was no significant difference ($P > 0.05$) between 200 μg/mL of WEPT and other

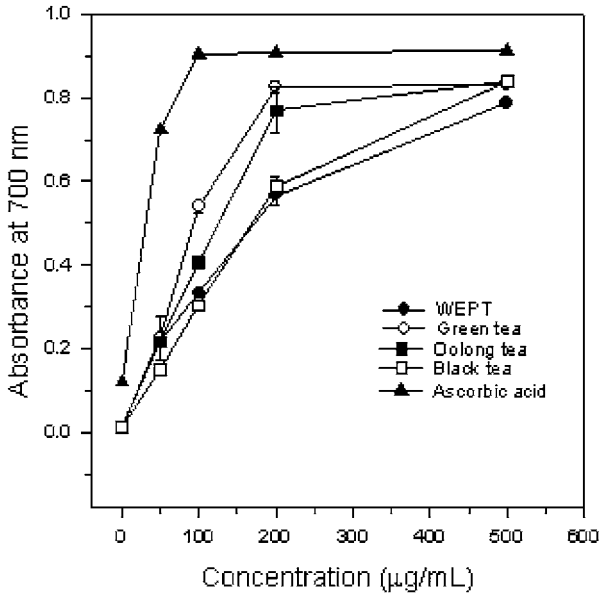

Figure 4. Reducing ability of water extracts of pu-erh tea (WEPT).

tea extracts (200 μg/mL). WEPT (200 μg/mL) exhibited a more significant ($P < 0.05$) inhibitory effect on deoxyribose damage than 200 μg/mL Toc. Obviously, WEPT and other tea extracts exhibited comparable activities in the inhibition of deoxyribose oxidative damage. Antosiewicz et al. (*24*) noted that proteins have a different oxidation pattern from that of lipid; however, according to **Figures 1−3**, WEPT, like other tea extracts, exhibited a protective effect not only on the lipid model system but also on the nonlipid model system.

Like the reducing ability of green tea extract, oolong tea extract and black tea extract, which showed remarkable reducing abilities (*25*), WEPT also exhibited reducing ability, and it is increased upon increasing the concentration of WEPT (**Figure 4**). Although WEPT and other tea extracts showed marked reducing ability, the reducing ability of those were lower than that of ascorbic acid, which is a strong reducing agent. Yen and Chen (*25*) reported that green tea, paucha tea, oolong tea, and black tea exhibited reducing power, and the greatest reducing power was observed in oolong tea relative to other teas. In this model system, at 200 μg/mL the difference was not very significant ($P > 0.05$) between 200 μg/mL WEPT and black tea extract, but significantly ($P < 0.05$) lower than green tea extract, oolong tea extract, and ascorbic acid. Tanaka et al. (*26*) noted that the antioxidant activity is concomitant with the development of reducing power. Yildirim et al. (*27*) reported that the reducing power of a compound is related to the electron-transfer ability of that compound; thus, the reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Therefore, WEPT acts as an electron donor and can react with free radicals to convert them to more stable products and terminate the chain reaction.

Free radicals are reported to be related to various physiological and pathological events, such as inflammation, immunization, aging, mutagenicity, and carcinogenicity (*28*). Green tea, oolong tea, and black tea exhibited significant scavenging effects on free radicals (*25*). In this paper, the percent DPPH scavenging activities of WEPT and other tea extracts were in a manner dependent on concentration (**Figure 5**). There were no significant differences ($P > 0.05$) between 200 μg/mL of WEPT and 200 μg/mL of other tea extracts. Wang et al. (*29*) assessed the perlyene free radical scavenging potential of green tea polyphenols; EGCG showed the strongest scavenging effect among the

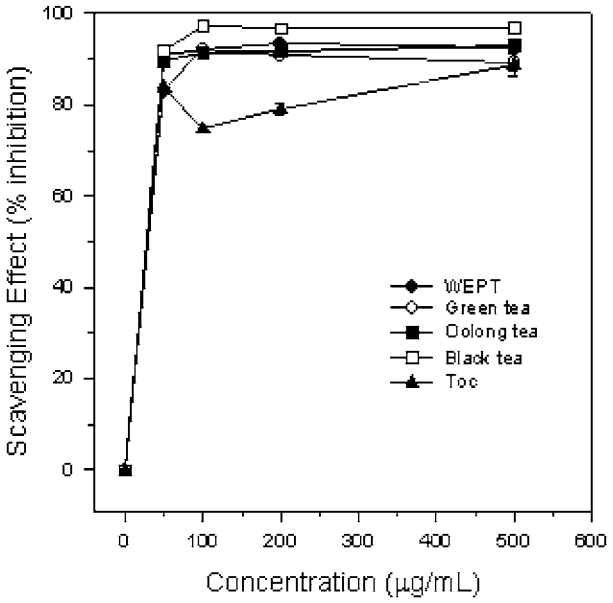

Figure 5. Scavenging effect of water extracts of pu-erh tea (WEPT) on free radical Toc, tocopherol.

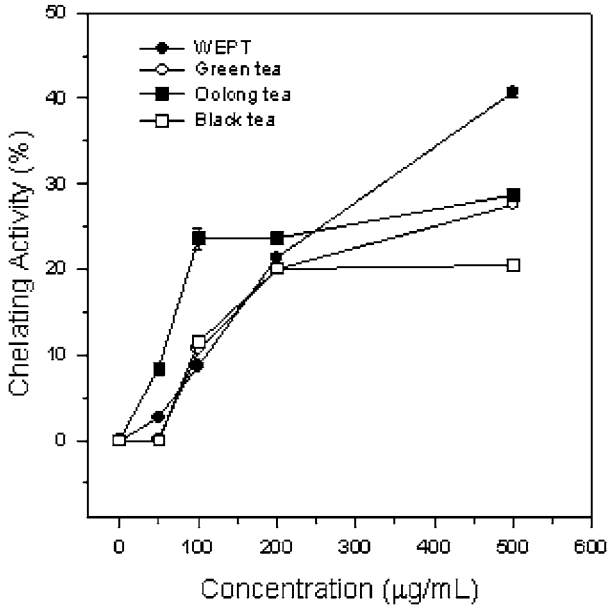

Figure 6. Chelating effect of water extracts of pu-erh tea (WEPT) on ferrous ions.

four catechins (EC, ECG, EGC, and EGCG). These results revealed that WEPT and other tea extracts showed remarkable antiradical activity and are free radical inhibitors. Consequently, WEPT acts as hydrogen donors and as primary antioxidants by reacting with the free radical. This may be responsible for the main cause of suppression of autoxidation, in both lipid and nonlipid model systems.

Morel et al. (*30*) reported that polyphenolic compounds such as flavonoids form complexes with metal ions, whereas they have been considered as a partial mechanism in the antioxidant action (*30, 31*). Polyphenolic compounds are the most abundant group of compounds in the tea extract (*32*). The polyphenolic compounds in tea extract can form a complex with iron ions to give a blue or purple color (*33*). **Figure 6** shows the chelating effect of WEPT and other tea extracts on ferrous ions. As expected, the chelating effect increased with increasing the concentraction of extract. Of the four samples at 500 μg/mL,

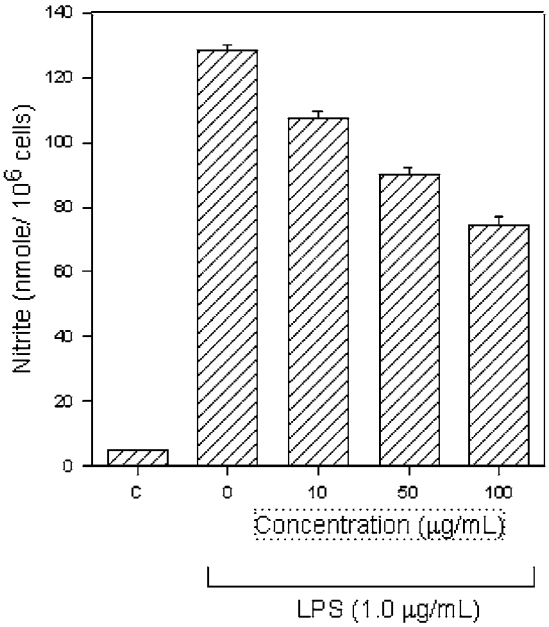

Figure 7. Effect of water extracts of pu-erh tea (WEPT) on nitrite accumulation in cell supernatants of LPS-activated RAW 264.7 macrophages. LPS, lipopolysaccharide; C, control.

WEPT showed the highest chelating effect on ferrous ions. Pannala et al. (*34*) noted that the chelating effect of tea extract on transition metal ions can retard iron-mediated free radical production Obviously, WEPT and other tea extracts showed remarkable chelating ability on ferrous ions, which caused the lipid peroxidation via Fenton reaction.

Nitric oxide (NO) reacts with superoxide ($O_2^{\bullet -}$) to form the peroxynitrite anion, which is a potential strong oxidant as the decomposition of this molecule produces hydroxyl radical and nitrogen dioxide (*35*). As a consequence, during recent years, it has become increasingly apparent that NO contributes significantly to oxidative cell damage (*36*). Therefore, it might be beneficial to human health if consumed foods could scavenge NO.

In scavenging nitric oxide production in LPS-activated RAW 264.7 cells performed in this study, the cell viability generally exceeded 95% at a WEPT concentration of 10−100 μg/mL. Thus, WEPT showed no cytotoxicity to RAW 264.7 cells under the tested concentration.

The inhibitory activity of WEPT toward LPS-induced nitric oxide (NO) production in RAW 264.7 is shown in **Figure 7**. LPS-treated cells produced a high level of nitrite (128 nmol/10^6 cell) that was 25.6-fold higher than that produced by the unstimulated cells (5.0 nmol/10^6 cell). Moreover, this LPS-activated nitrite production could be significantly ($P < 0.05$) reduced by incubation with WEPT in a dose-dependent manner. WEPT at 10, 50, and 100 μg/mL showed 16.4, 29.7, and 42.2% inhibition of nitrite production, respectively. Obviously, LPS-activated nitrite production was significantly reduced by incubation with WEPT. Many reports (*5, 37*) have demonstrated that flavonoids, flavones, flavonols, and catechins inhibited the NO production of inducible nitrite oxide synthase (iNOS). Kim et al. (*6*) noted that some edible Japanese plants at 200 μg/mL had 90% inhibitory effect on NO production in LPS-activated RAW 264.7 cells. Chan et al. (*5*) noted that EGCG could inhibit iNOS activity and its mRNA expression in LPS-activated macrophage. In the present study, WEPT exhibited an inhibitory effect on NO production in LPS-activated RAW 264.7 cells that may be attributed to some active compounds in WEPT.

Table 2. Analysis of Total Phenolics, Flavonoids, and Ascorbic Acid Contents of Water Extracts of Pu-erh Tea (WEPT)

sample	total phenolics (mg/mL)	flavonoid (mg/mL)	ascorbic acid (mg/mL)
WEPT	229.5	10.2	4.40
green tea	415.3	390.4	21.3
oolong tea	211.8	39.0	17.2
black tea	164.5	118.6	6.60

Table 3. Catechins Content of Water Extracts of Pu-erh Tea (WEPT)[a]

sample	EC (mg/mL)	ECG (mg/mL)	EGC (mg/mL)	EGCG (mg/mL)	total catechins (mg/mL)
WEPT	8.01	0	0	0	8.01
green tea	1.85	1.47	27.0	48.8	79.1
oolong tea	4.35	1.48	30.5	31.0	67.3
black tea	0.54	1.26	6.81	11.2	19.8

[a] Tea (50 g) was extracted with boiling water (500 mL) for 5 min, and the filtrate was freeze-dried.

Several components that are responsible for the antioxidant activity have been isolated from tea. Catechins are a main constituent of tea having such antioxidant activity (*38*). To determine the main antioxidant compounds in WEPT and other tea extracts, their contents of total phenolics, flavonoid, ascorbic acid, and catechins were assayed. From the results shown in **Table 2**, the content of total phenolics of green tea extract is the highest among all of the samples followed by WEPT, oolong tea extract, and black tea extract. It is well-known that plant polyphenolic compounds act as both free radical scavengers and as antioxidants (*39*). The high potential of phenolic compounds to scavenge free radicals may be explained by their ability to donate a hydrogen atom from their phenolic hydroxyl groups. In the present study, WEPT is found to contain a large number of potential polyphenolic compounds, which may contribute to their antioxidant activity. The flavonoid contents of the four tea extracts decreased in the order green tea extract > black tea extract > oolong tea extract > WEPT. The content of ascorbic acid in the four tea extract is in the order green tea extract > oolong tea extract > black tea extract > WEPT. Flavonoids are strong antioxidants that occur naturally in food and can inhibit carcinogenesis in rodents, and their presence in regularly consumed foods might reduce the risk of death from coronary heart disease in elderly people (*40*). As for the ascorbic acid, it is a water-soluble vitamin that is a potent antioxidant in some in vitro model and inhibits cytotoxicity or apoptotic cell death induced in vitro by various oxidants (*41*). Although the contents of flavonoid and ascorbic acid in WEPT are the least among the four tea extracts, WEPT still has 10.2 mg/mL flavonoids and 4.40 mg/mL ascorbic acid. The significantly lower level of flavonoid and ascorbic acid in WEPT may be as a result of the degree of oxidation of fermentation during the preparation of pu-erh tea.

Tea catechins consist primarily of four components: (−)-epicatechin (EC) (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigallocatechin gallate (EGCG). These amount to 10−16% of the mass on a dry basis (*38*, *42*). According to the data in **Table 3**, EGCG and EGC are the main constituents in the green tea extract, oolong tea extract, and black tea extract, which is in agreement with the report of Unno et al. (*38*). Moreover, green tea extract has the highest amount of EGCG and EGC among the four extracts. However, EGCG and EGC were not detected in WEPT. As shown in **Table 3**, the content of total catechins in various tea extracts is in the order green tea extract (79.1 mg/mL) > oolong tea extract (67.3 mg/mL) > black tea extract (19.8 mg/mL) > WEPT(8.01 mg/mL). These findings are in agreement with the observations of Yen and Chen (*25*). They reported that the content of tatal catechinsin various tea is in the order green tea > paucha tea > oolong tea > black tea. In addition, the content of total catechins in the tea is related to the degree of fermentation of tea during preparation. Oolong tea, black tea, and pu-erh tea are fermented during preparation, but the latter is usually preserved for a long period, which may explain the content of total catechins of oolong tea extract, black tea extract, and WEPT being less than that of green tea extract. This observation implies that the higher the degree of the fermentation, the greater is the decrease in total catechins. Sato et al. (*39*) noted that the features of fermented tea (oolong tea, black tea, and pu-erh tea) differ greatly from those of green tea, which is nonfermented and is preferably taken as fresh as possible.

Due to the fermentation and preservation for a long period, the content of flavonoid, ascorbic acid, and total catechins in WEPT is the least among all of the tea extracts (**Tables 2** and **3**). According to the data in **Table 1** and **Figures 1**−**3**, WEPT showed marked antioxidant activity on lipid and nonlipid oxidation damage; thus, the antioxidant activity of WEPT may not be mainly attributed to the content of catechins, ascorbic acid, and flavonoid. Unno et al. (*38*) suggested that the components of pu-erh tea leaves are oxidized by the enzymes during fermentation. As a consequence, some active substances may be formed in pu-erh tea during fermentation. We suggest that some active substances formed during fermentation could complement the biological effects of flavonoid, ascorbic acid, and EC in WEPT. These observations seem to be responsible for the antioxidant activity of WEPT.

Nitric oxide has been found to be beneficial in maintaining proper physiological homeostasis; NO may contribute to many diseases (*43*). Therefore, determination of cellular NO release is important because cytokines or endotoxin was found to induce overproduction of NO and its metabolites, in particular the deleterious molecule peroxynitrite; both seem to lead to numerous pathological conditions associated with inflammatory disorders (*44*, *45*). Nakagawa and Yokozawa (*7*) noted that green tea directly scavenges NO. *Ginkgo biloba* extract and EGCG were shown not only to directly act as NO scavengers but also to inhibit NO production in LPS/IFN-r-activated macrophages by the concomitant inhibition of the induction of iNOS mRNA and the enzyme activity of iNOS (*5*, *37*). Our results (**Figure 7**) showed that LPS-activated nitrite production in RAW 264.7 cells reduced by WEPT may be attributed directly to the NO scavenging ability.

In conclusion, WEPT exerted significant inhibitions on lipid and nonlipid oxidation similar to that of green tea extract, oolong tea extract, and black tea extract. Although ascorbic acid, EC, flavonoids and polyphenolic compounds, which are well-known as antioxidants, were found in WEPT, some unknown active compounds with water-soluble characteristics might exist in WEPT. Most likely they exert their action as a result of various mechanisms; among these are prevention of chain initiation, terminating radical chain reactions, binding of transition metal ions, and radical scavenging. In addition, directly scavenging NO radicals by WEPT contributed to suppress NO production in RAW 264.7 macrophages. These results may indicate possible protective effects against oxidative damage and chronic inflammatory diseases through the intake of pu-erh tea.

NOTE ADDED AFTER ASAP PUBLICATION

The caption of Figure 4 has been modifed from the original ASAP publication of December 7, 2004; the corrected version was published ASAP December 8, 2004.

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