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# Free Radical Scavenging Effect of Pu-erh Tea Extracts and Their Protective Effect on Oxidative Damage in Human Fibroblast Cells

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In the present study, we successively extracted the Pu-erh tea with acetone, water, chloroform, ethyl acetate, and *n*-butanol, and the extracts were then isolated by column chromatography. Our study demonstrates that the Pu-erh tea ethyl acetate extract, *n*-butanol extract, and their fractions had superoxide anion and hydroxyl radical scavenging activity: fractions 2 and 8 from the ethyl acetate extract and fractions 2, 4, and 5 from the *n*-butanol extract showed protective effects against hydrogen peroxide-induced damage in human fibroblast HPF-1 cells and increased the cells' viability under normal cell culture conditions. In addition, it is found that these fractions, except fraction 5 from the *n*-butanol extract, decreased the accumulation of intracellular reactive oxygen species in hydrogen peroxide-induced HPF-1 cells. Interestingly, the antioxidant effect of fraction 8 from the ethyl acetate extract on the above four systems was much stronger than that of the typical green tea catechin (−)-epigallocatechin-3-gallate, but there were almost no monomeric polyphenols, theaflavins, and gallic acid in fraction 8.

**KEYWORDS: Pu-erh tea; tea extraction; free radicals; oxidative damage; natural antioxidants; human fibroblast; HPF-1**

## INTRODUCTION

Tea is one of the most popular beverages in the world. According to the degree of fermentation, generally, tea is classified into three major categories: nonfermented green tea, partially fermented oolong or paochong tea, and fully fermented black tea and Pu-erh tea (*1*).

Interest in the health properties of tea and related scientific investigation is increasing (*2*). The potential for the consumption of tea or tea polyphenols to prevent or ameliorate chronic disease is currently the subject of considerable scientific investigations (*3*).

Pu-erh tea, a kind of postfermented tea, produced mainly in the Yunnan province of China, is consumed widely in southeast Asia. A number of studies have shown that pu-erh tea has a wide range of biological effects, such as antioxidative (*4*, *5*) and hypocholesterolemic effects in rats (*6*), microbicidal activity against *Mycoplasma pneumoniae* and *Mycoplasma orale* (*7*), bactericidal activity against *Bordetella pertussis* (*8*), and anti-obese activity, reducing plasma triglyceride, cholesterol, and LDL-cholesterol in rat (*9*).

Reactive oxygen species (ROS) are forms of activated oxygen that include free radicals such as superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical ($HO^{\bullet}$), as well as non-free radical species such as hydrogen peroxide ($H_2O_2$) (*10*). Because of their high reactivity, free radicals can damage diverse cellular macromolecules, including proteins, carbohydrates, lipids, and nucleic acids. Free radical-caused damage to these molecules has been implicated in the causation of some degenerative diseases. For example, destructive effects on proteins may play a role in cataract formation, oxidative damage to DNA may be involved in the development of certain cancers, and lipid oxidative damage can contribute to the occurrence and progression of vascular disease (*11*).

Although a number of mechanisms have been proposed for the beneficial effects of tea in different models of chronic diseases, the radical scavenging and antioxidant properties of tea polyphenols are frequently cited as important contributors (*12*).

In this study, we successively extracted the Pu-erh tea with acetone, chloroform, ethyl acetate, and *n*-butanol, and the extracts were then isolated by column chromatography. The

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chemical compounds of Pu-erh tea extract's fractions were identified by high-performance liquid chromatography (HPLC) and spectrum technology. We tested the antioxidative activities of Pu-erh tea extract's fractions for their inhibitory effect on Fenton reaction system and superoxide anion-generating system. In addition, we assessed the protective effect and ROS-inhibiting effect of Pu-erh tea extract's fractions on $H_2O_2$-induced oxidative damage in the HPF-1 cells (*13*).

## MATERIALS AND METHODS

**Materials.** Dulbecco's modified Eagle medium (DMEM), newborn calf serum, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) were purchased from Gibco BRL (Grand Island, NY). Embryonic human lung fibroblasts (HPF-1) were purchased from Peking Union Medical College. Trypsin, penicillin, streptomycin, 2′,7′-dichlorofluorescein diacetate (DCF-DA) and 3-aminophthalhydrazide (Luminol) were purchased from Sigma Chemical Co. (St. Louis, MO). Pu-erh tea was purchased from Shuangjiang, Yunnan, China. EGCG was a generous gift from the Tea Department of Zhejiang University; the purity was above 98% (analyzed by HPLC). All other chemicals made in China were of analytical grade.

**Preparation of Pu-erh Tea Extracts and Pu-erh Tea Fractions.** The Pu-erh tea (10 kg) was minced for adequatey extraction and then extracted with 60% acetone (acetone/water, 60:40 v/v) four times (each time, Pu-erh tea/60% acetone, 1:5 w/v, at room temperature for 7 days). The four times extracted solutions were combined and concentrated under low pressure, till the acetone was eliminated. After centrifugation to separate the insoluble material, the solution was then successively extracted with chloroform, ethyl acetate, and *n*-butyl alcohol. After concentration under low pressure to get out the organic solvent, we got the Pu-erh tea chloroform extract, Pu-erh tea ethyl acetate extract (PEF), Pu-erh tea butyl alcohol extract (PBF), and the residual water extract. Then, a portion of the PEF was dissolved in methanol and the solution was eluted by Sephadex LH-20 column chromatography, A gradient elution was performed by varying the proportion of methanol and water (started with water, followed by an increase of 10% acetone per degree, 500 mL per degree, at a flow rate of 9.5 mL/min) and finally eluted with 1000 mL of 50% acetone. A portion of the PBF was dissolved in methanol, and the solution was eluted by varying the proportion of methanol and water (started with 10% methanol, followed by an increase of 10% per degree, 300 mL per degree, at a flow rate of 9.5 mL/min)and finally eluted with 1000 mL of 50% acetone. The eluted solution was successively collected in many flasks. According to the thin-layer chromatography (TLC) results, flasks with the same atlas were combined into one fraction. The Pu-erh tea ethyl acetate extract was fractionated into eight fractions, and the Pu-erh tea butyl alcohol extract was fractionated into five fractions. In this study, we assigned these fractions as PEF1−8 and PBF1−5.

**Assay for the Scavenging Effect on Hydroxyl Radical Generated from a Fenton Reaction System.** The HO• scavenging activity was assessed according to the method of Cheng et al. (*14*) with a slight modification. HO• was generated by a Fenton-type reaction at room temperature. The reaction mixture (1.0 mL) contained 600 $\mu$L of luminol [0.1 mM, diluted in the carbonic acid-uffered saline solution (CBSS), pH 10.2], 100 $\mu$L of sample solution (with different concentrations, replaced with CBSS in the control), 200 $\mu$L of $Fe^{2+}$-EDTA (3 mM), and 100 $\mu$L of $H_2O_2$ (1.2 mM). Initiation of the reaction was achieved by adding $Fe^{2+}$-EDTA and then $H_2O_2$ into the mixture.

The HO• scavenging abilities of Pu-erh tea extractions were assessed on an ultraweak luminescence analyzer (BPLC). The detection parameters were as follows: measure time, 180 s; interval, 1 s; noise, 10; high voltage, 1000; light source, 200; number, 500; The chemiluminescence intensity (CL) integral was recorded and the inhibitory rate ($I_R$) was obtained according to the formula inhibitory rate (%)= [[CL-(control) − CL(sample)] × 100]/CL(control).

**Assay for the Scavenging Effect on the Superoxide Radical Generated from Pyrogallol Autoxidation.** The $O_2^{\bullet -}$ scavenging activity was assessed according to the slightly modified method of Yu et al. (*15*). Briefly, $O_2^{\bullet -}$ was generated by pyrogallol autoxidation. The reaction mixture (1.0 mL) contained 810 $\mu$L of luminol (0.1 mM, diluted in CBSS, pH 10.2), 100 $\mu$L of sample solution (with different concentration, replaced with CBSS in the control), 80 $\mu$L of NaOH (4 M), and 10 $\mu$L of pyrogallol (3 mM).

The testing procedure and scavenging rate formula were similar to the HO• assay noted above.

**High-Performance Liquid Chromatographic Analysis.** Gallic acid, monomeric polyphenols from the catechins, and theaflavins, maybe contained in the Pu-erh tea fractions, were analyzed by HPLC. HPLC analysis was conducted on a LC-2010 liquid chromatograph. A C18 reversed-phase column was used for separation. Gradient elution was performed by varying the proportion of solvent A (acetic acid/acetonitrile/water, 0.5:3:96.5 v/v/v) to solvent B (acetic acid/acetonitrile/water, 0.5:30:69.5 v/v/v), with a flow rate of 1 mL/min. The mobile phase composition started at 100% solvent A, followed by a linear increase of solvent B to 100% in 45 min, and then solvent B for 15 min. All the prepared solutions were filtered through 0.45 $\mu$m membranes, and the mobile phase was degassed before being injected onto HPLC.

**Cell Culture and Treatment.** Embryonic human lung fibroblasts (HPF-1), purchased from Peking Union Medical College, were normal diploid fibroblasts. The HPF-1 cells were maintained in DMEM supplemented with 10% (v/v) heat-inactivated newborn calf serum, 100 IU/mL penicillin, and 100 $\mu$g/mL streptomycin. Cultures were incubated at 37 °C in humidified 5% $CO_2$/95% air atmosphere. Culture medium was refreshed every 3 days. Cultures were maintained for 7 days prior to experimentation.

Cells were cultured at a density of $2 \times 10^5$ cells/mL on 96-well plates and cultured 24 h before treatment. Initially, the culture medium was replaced with fresh medium containing various concentrations of $H_2O_2$, to determine the optimal oxide damage concentration of $H_2O_2$ for the following experiments (*16*). Pu-erh tea fractions were added 1 h before treatment with $H_2O_2$ for a subsequent 24 h.

**Assessment of Cell Viability.** Cell viability was measured by quantitative colorimetric assay with MTT, showing the mitochondrial activity of living cells as described in the literature (*17*, *18*). After treatment with $H_2O_2$ for 24 h, the medium was removed and fresh medium containing 0.5 mg/mL MTT was added to each well, followed by incubation for 3 h at 37 °C. Finally the medium containing MTT was removed, and cells were lysed with dimethyl sulfoxide (DMSO). The absorbance at 595 nm was measured on a Bio-Rad 3350 microplate reader. Control cells were treated in the same way without $H_2O_2$, and the value of different absorbance was expressed as a percentage of control.

**Measurement of Intracellular Reactive Oxygen Species.** The level of intracellular ROS was quantified by fluorescence with 2′,7′-dichlorofluorescein diacetate (DCF-DA) as described by Yamamoto et al. (*19*). DCF-DA is cell-permeable and nonfluorescent. Viable cells can deacetylate DCF-DA to 2′,7′-dichlorofluorescein (DCFH) without fluorescence. This compound reacts quantitatively with oxygen species within the cell to produce a fluorescent dye, 2′,7′-dichlorofluorescein (DCF), which remains trapped within the cell and can be measured to provide an index of ROS level.

In the present study, cells ($1.5 \times 10^4$ cells/well) were incubated with 5 $\mu$M DCF-DA [disolved in Hallam's physiological saline (HPS)] in a 96-well microplate for 30 min at 37 °C. After the incubation, the excess probes were washed out with HPS three times, then incubated with different concentrations of Pu-erh tea fractions (dissolved in HPS) for 30 min, and incubated with $H_2O_2$ (final concentration 600 $\mu$M) for the indicated time periods. The intracellular ROS levels were measured by using a fluorescence plate reader (Fluoroskan Ascent 2.4), at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. The measured fluorescence values were expressed as a percentage of the fluorescence in control cells.

**Statistical Analysis.** All experiments were performed in triplicate. One-way ANOVA was used to estimate overall significance followed by posthoc Turkey's tests corrected for multiple comparisons (*20*). The statistical analysis software we used was the Origin program (Origin 7.5, OriginLab Corp.). Data were presented as mean ± SEM. A probability level of 5% ($p < 0.05$) was considered significant.

**Table 1.** Quenching Ability of Pu-erh Tea Fractions on the Chemiluminescence Caused by $HO^{\bullet}$

| sample[a] | regression equation[b] | R | linear range ($\mu$g/ mL) | $IC_{50}$ ($\mu$g/mL) |
|---|---|---|---|---|
| ascorbic acid | $y = 23.03x - 18.49$ | 0.9492 | 5.0–50.0 | 19.7 |
| EGCG | $y = 51.05x + 16.57$ | 0.9878 | 1.0–4.0 | 1.9 |
| PEF2 | $y = 40.95x + 39.4$ | 0.9645 | 0.8–4.0 | 1.3 |
| PEF5 | $y = 35.65x + 52.19$ | 0.9629 | 0.5–4.0 | 0.9 |
| PEF6 | $y = 48.25x + 39.06$ | 0.9853 | 0.8–3.0 | 1.3 |
| PEF7 | $y = 43.51x + 42.00$ | 0.9842 | 0.8–3.0 | 1.2 |
| PEF8 | $y = 30.87x + 66.39$ | 0.9735 | 0.3–2.0 | 0.6 |
| PBF2 | $y = 40.17x + 33.40$ | 0.9854 | 1.0–4.0 | 1.5 |
| PBF3 | $y = 35.14x + 45.75$ | 0.9756 | 1.0–4.0 | 1.1 |
| PBF4 | $y = 33.79x + 41.32$ | 0.9898 | 1.0–4.0 | 1.3 |
| PBF5 | $y = 33.86x + 41.93$ | 0.9910 | 1.0–4.0 | 1.3 |

[a] Ascorbic acid and EGCG were used as controls. [b] $y$, inhibitory rate (percent); $x$, logarithm values of corresponding concentrations of samples.

**Table 2.** Quenching Ability of Pu-erh Tea Fractions[a] on the Chemiluminescence Caused by $O_2^{\bullet -}$

| sample[a] | regression equation[b] | R | linear range ($\mu$g/mL) | $IC_{50}$ ($\mu$g/mL) |
|---|---|---|---|---|
| ascorbic acid | $y = 23.49x - 7.13$ | 0.9928 | 5.0–100.0 | 11.4 |
| EGCG | $y = 16.89x + 34.33$ | 0.9060 | 1.0–50.0 | 2.5 |
| PEF2 | $y = 7.76x + 44.09$ | 0.9003 | 1.0–200.0 | 2.1 |
| PEF5 | $y = 7.61x + 55.16$ | 0.9304 | 0.01–50.0 | 0.5 |
| PEF6 | $y = 6.02x + 58.19$ | 0.9496 | 0.1–50.0 | 0.3 |
| PEF7 | | | | |
| PEF8 | $y = 13.37x + 63.62$ | 0.9872 | 0.2–5.0 | 0.4 |
| PBF2 | | | | |
| PBF3 | $y = 32.67x - 63.72$ | 0.9041 | 20.0–200.0 | 32.1 |
| PBF4 | | | | |
| PBF5 | $y = 10.56x + 41.83$ | 0.9036 | 2.0–160.0 | 2.2 |

[a] Ascorbic acid and EGCG were used as controls. [b] $y$, inhibitory rate(percent); $x$, logarithm values of corresponding concentrations of samples.

## RESULTS

**Evaluation of the Scavenging Effects on Free Radicals.** Scavenging abilities of Pu-erh tea fractions on the CL signal, which indicate their potentials to scavenge free radicals, were ranked by their 50% inhibition concentration ($IC_{50}$). The lower the $IC_{50}$ value, the higher activity for scavenging effect the Pu-erh tea fractions possessed on the free radicals. Upon linear regression analysis of $I_R$ (percent) and the sample concentration, some fractions of PEF and PBF showed a good linear relationship between these two parameters, and the regression equations and correlation coefficients are listed in **Tables 1** and **2**.

**Scavenging Effect on Hydroxyl Radicals.** The scavenging effects of Pu-erh tea fractions on the $HO^{\bullet}$ were evaluated by means of Fenton-type reaction. With regression equations derived, $IC_{50}$ values of Pu-erh tea fractions were calculated. As shown in **Table 1**, fractions PEF2, PEF5, PEF6, PEF7, PEF8, PBF2, PBF3, PBF4, and PBF5 showed a good linear relationship between their $I_R$ (percent) and the logarithm of their concentration. On the basis of the comparison among the $IC_{50}$ values of each fraction, the five fractions of Pu-erh tea ethyl acetate extract and the four fractions of butyl alcohol extract exhibit higher antioxidant potency than that of EGCG and even higher than that of ascorbic acid. PEF8 shows the strongest scavenging effect on the hydrogen radicals.

**Scavenging Effect on Superoxide Anions.** The scavenging effects of Pu-erh tea fractions on $O_2^{\bullet -}$ were evaluated by means of pyrogallol autoxidation, and the results are shown in **Table 2**. Upon linear regression analysis of $I_R$ (percent) and sample concentration, fractions PEF2, PEF5, PEF6, PEF8, PBF3 and PBF5, showed a good linear relationship between their two parameters. Like the scavenging effect on $HO^{\bullet}$, the Pu-erh tea ethyl acetate extract's fractions also exhibit stronger $O_2^{\bullet -}$ scavenging effects than that of EGCG and even stronger than that of ascorbic acid. A little difference was shown in the fractions PEF7, PBF2 and PBF4; they showed scavenging effect on $O_2^{\bullet -}$, but no good relationship between their $I_R$ (percent) and concentration. PBF3, unlike its scavenging effect on $HO^{\bullet}$, showed a lower $O_2^{\bullet -}$ scavenging effect even than that of ascorbic acid. Consistent with its scavenging effect on $HO^{\bullet}$, PEF8 also showed the strongest scavenging effect on the $O_2^{\bullet -}$.

**Ameliorated $H_2O_2$-Induced Loss of HPF-1 Cell Viability.** The cell viability was expressed as MTT conversion rate. The effect of Pu-erh tea fractions on $H_2O_2$-induced loss of HPF-1 cell viability is depicted in **Figure 1**. Treatment with 600 $\mu$M $H_2O_2$ for 24 h decreased the viability of HPF-1 cells about 30−35% relative to the negative control. After pretreatment with PEF2, PEF8, PBF2, PBF4, and PBF5 at different concentrations (0.6, 1.2, 2.4, 4.8, or 9.6 $\mu$g/mL), the cell viability was almost dose-dependently ameliorated. PEF2, at the concentration of 4.8 $\mu$g/mL, showed the best protective effect on the damaged HPF-1 cell. The cell viability after pretreatment with PEF8 gradually increased in accordance with the concentration: at 9.6 $\mu$g/mL, the viability was about 18.9% higher than that of the negative control group. The cell viability after pretreatment with PBF2 and PBF5 also significantly increased at different concentrations. PBF4 showed the strongest protective effect at the concentration of 2.4 $\mu$g/mL; then the increased viability decreased in accordance with the concentration.

In addition, we also checked the cell viability when the cells were treated with Pu-erh fractions alone. As shown in **Figure 2**, the cells that were treated with PEF2 and PBF4 at concentrations of 0.6−9.6 $\mu$g/mL had no improvements in viability. But after treatment with 4.8 $\mu$g/mL PEF8, the increased viability was about 18.9% higher that of the negative control. PBF2 and PBF5 can distinctly increase the cell viability at all five concentrations of the experiment.

**Decreased $H_2O_2$-Induced Accumulation of ROS in HPF-1 Cells.** ROS are the main factor that causes oxidative stress, which results in decreasing cell viability. The level of DCF fluorescence is an indicator of ROS production. After treatment with different doses of $H_2O_2$ in the HPF-1 cells, the level of ROS in the cells increased dose-dependently. After treatment with 600 $\mu$M $H_2O_2$ for 1 h, the DCF fluorescence intensity increased about 70−80% in comparison with that of the negative control. As shown in **Figure 3**, the increase in the DCF fluorescence intensity was eliminated partly when the cells were cotreated with different concentrations of EGCG or PEF8.The fluorescence intensity of cells treated with EGCG and PEF8 at a concentration of 1.6 $\mu$g/mL was decreased by about 31% and 33%, respectively. When the cells were cotreated with PEF8, the decrease in fluorescence intensity was dose-dependent. In addition, even with no exposure to $H_2O_2$, treatment with EGCG or PEF8 at concentrations of 0.2−1.6 $\mu$g/mL for 1 h resulted in decreased DCF fluorescence intensity in the HPF-1cells (**Figure 3B**).

As shown in **Figure 3A**, under the condition of exposure to $H_2O_2$ for 1 h, the increase in DCF fluorescence intensity was also decreased when the cells were cotreated with different concentrations of PEF2, PBF2, or PBF4, but no significant difference to the positive control was observed. Under the condition of no exposure to $H_2O_2$ but treatment with different concentrations of PEF2, PBF2, or PBF4 for 1 h, the DCF

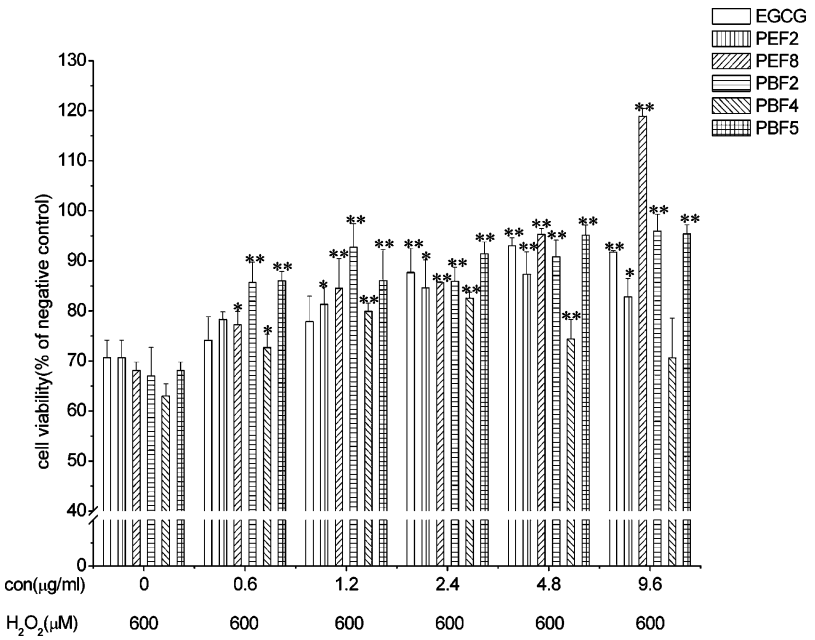


**Figure 1.** Effects of Pu-erh tea fractions on $H_2O_2$-induced decrease of HPF-1 cell viability. Cell viability was estimated by MTT assay after treatment with $H_2O_2$ (600 $\mu$M) and/or Pu-erh tea fractions for 24 h (at indicated concentrations). EGCG was used as a control. Data were expressed as means (OD value) ± SEM ($n = 8$), showed by percentage of the negative control (untreated cells); *$p < 0.05$, **$p < 0.01$ when compared with that of the positive control group (treated only with 600 $\mu$M $H_2O_2$).

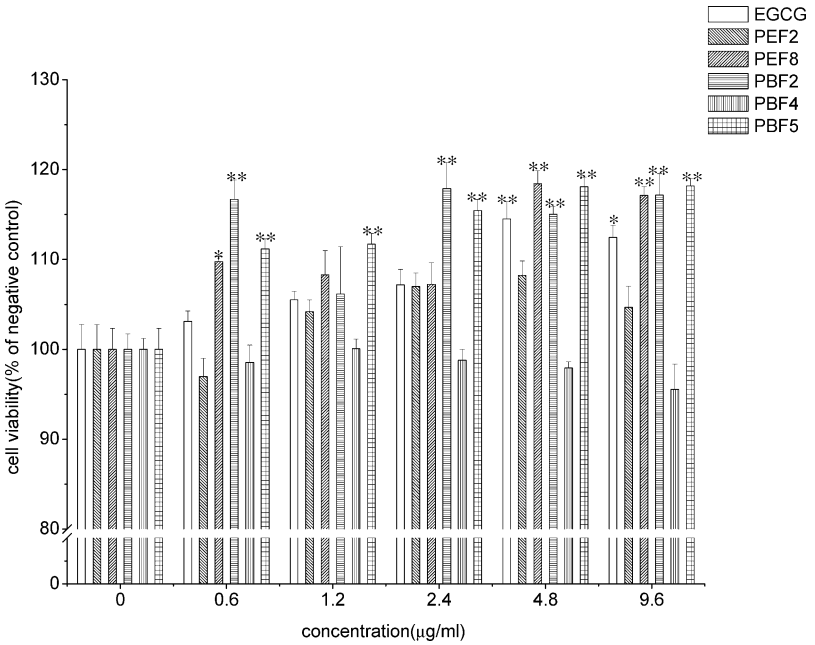


**Figure 2.** Effects of Pu-erh tea fractions on HPF-1 cell viability. Cell viability was estimated by MTT assay after treatment with Pu-erh tea fractions for 24 h (at indicated concentrations). EGCG was used as a control. Data were expressed as mean (OD value) ± SEM ($n = 8$), showed by percentage of the negative control (untreated cells); *$p < 0.05$, **$p < 0.01$ when compared with that of the negative control.

fluorescence intensity in the HPF-1 cells was also decreased (**Figure 3B**).

**Identification of Pu-erh Tea Fractions.** The total content of tea polyphenols was 63% in ethyl acetate extract and 44.5% in *n*-butanol extract. The total content of monomeric polyphenols from the catechins and the content of gallic acid were 20.1% and 1.1% in ethyl acetate extract and 6.0% and 0.4% in the *n*-butanol extract, respectively. No theaflavins were found in the ethyl acetate or *n*-butanol extract. The HPLC chromatogram (detected at 280 nm) for PEF8 is shown in **Figure 4**, in which it can be seen that the total content of monomeric polyphenols from the catechins was less than 1%, and no theaflavins or gallic acid were found in PEF8.

## DISCUSSION

In a previous study of the inhibitory effect of Pu-erh tea on free radicals, we used the Fenton reaction system and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging method in vitro and observed that the Pu-erh tea ethyl acetate extract and *n*-butanol extract had strong scavenging effects on $HO^{\bullet}$ and DPPH radicals (*21*).

In the present study, we have demonstrated that some fractions in the Pu-erh tea ethyl acetate extract and in the

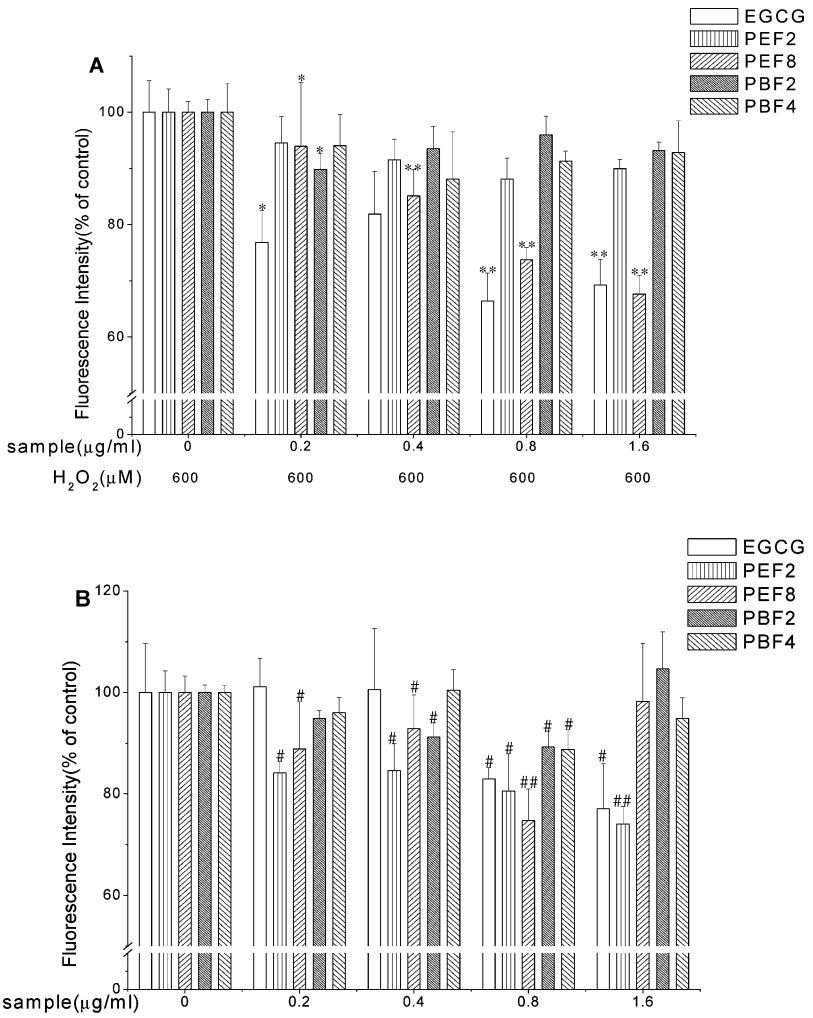


**Figure 3.** Pu-erh tea fractions reduced $H_2O_2$-induced accumulation of ROS in HPF-1 cells. The fluorescence intensity of DCF was measured after HPF-1 cells were exposed to Pu-erh tea fractions (indicated concentration) and/or $H_2O_2$ (600 $\mu$M) for 1 h. Data were expressed as mean (fluorescence intensity value) ± SEM ($n = 8$), shown as (**A**) percentage of negative control (untreated cells) or (**B**) percentage of positive control (treated only with 600 $\mu$M $H_2O_2$); $^{*}p < 0.05$, $^{**}p < 0.01$ compared with that of the positive control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with that of the negative control.



*n*-butanol extract had strong inhibitory effects on the Fenton reaction system and pyrogallol autoxidation.

Many studies have shown that green and black teas have powerful scavenging effects on the ROS (*22*). Green tea is heated and dried to avoid enzymatic oxidation, and therefore it contains high concentrations of monomeric polyphenols from the catechins, which are mainly responsible for green tea antioxidant actions. Black tea is thoroughly oxidized enzymatically; it is generally believed that polyphenols such as theaflavins, thearubigins, and catechins are major constituents of black tea that are mainly responsible for antioxidant actions (*23*).

Pu-erh tea is processed by a special postfermentation, different from the fermentation of black tea, which is referred to as natural brewing reactions induced by oxidative enzymes within the plant cell. The fresh leaves of Pu-erh tea are heated to avoid enzymatic oxidation and then oxidized mainly by hygrothermal condition in the process of piling and long time preservation. According to research by Shao et al. (*24*), there is little monomeric polyphenol content from the catechins in Pu-erh tea, which is different from green tea, and little thearubigin and no theaflavin content, which is different from black tea. In the present study, the content of monomeric polyphenols from the catechins and gallic acid are 20.1% and 1.1% in ethyl acetate extract and 6.0% and 0.4% in the *n*-butanol extract, respectively, and no theaflavins were detected by HPLC; the results were consistent with the report of Shao (*24*).

HPF-1, human normal diploid fibroblasts, exhibit finite proliferative potential in vitro, the so-called Hayflick limit (*25*). They undergo a limited number of population doublings before entering a state of permanent growth arrest, referred to as "replicative senescence," "cellular senescence", or "cellular aging" (*26*), in which they remain alive and metabolically active but are completely refractory to mitogenic stimuli. HPF-1 offers the typical model for studying the process of aging in vitro. Various oxidative stresses have been used to study the onset of cellular senescence. The early onset of cellular senescence induced by oxidative stresses is termed stress-induced premature senescence (SIPS) (*27*), and $H_2O_2$ is the most commonly used inducer of SIPS (*28*, *29*), which shares features of replicative senescence: similar morphology, senescence-associated $\beta$-galactosidase activity, cell cycle regulation, etc. (*30*, *31*). In the present study, oxidative stresses in the HPF-1 cells were induced by treatment with $H_2O_2$, and the level of ROS in the cells were increased dose-dependently. After pretreatment with PEF2, PEF8, PBF2, PBF4, and PBF5, the $H_2O_2$-induced loss of HPF-1 cell viability were ameliorated and the increased ROS were partly eliminated. These results showed that PEF2, PEF8, PBF2, and PBF4 may ameliorate cell viability by decreasing the

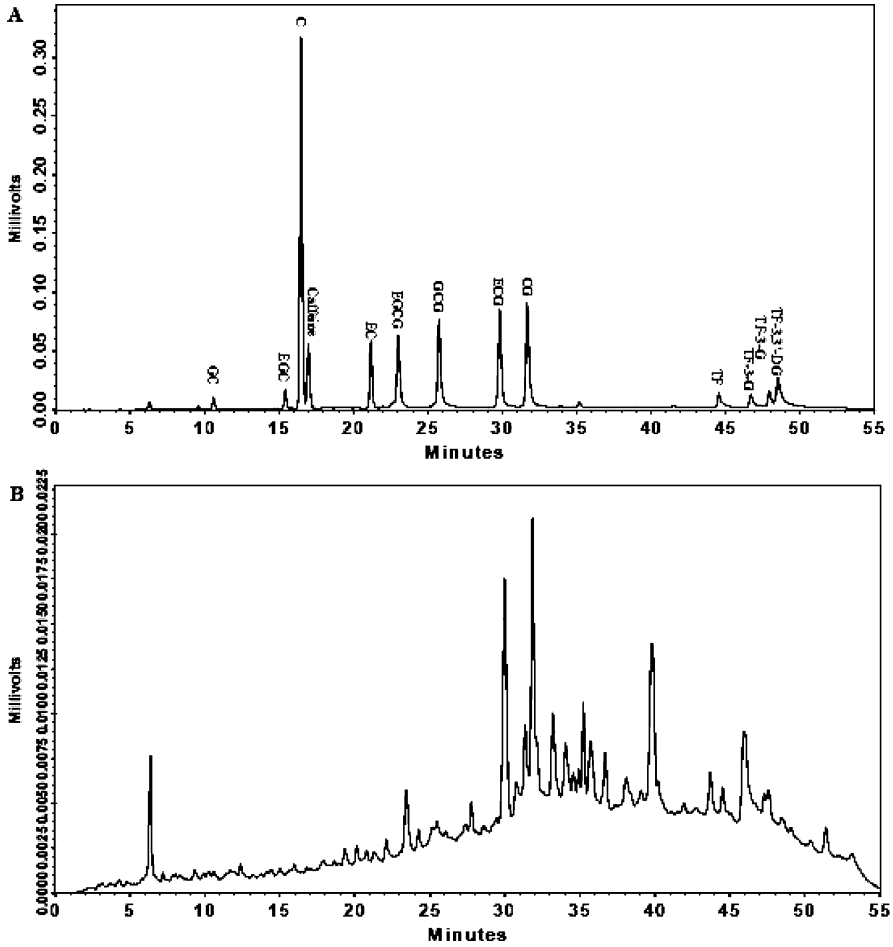


**Figure 4.** HPLC chromatograms (detected at 280 nm) of criterion (**A**) and PEF8 (**B**).

accumulation of intracellular ROS in $H_2O_2$-induced HPF-1 cells. In addition, after treatment with PEF2, PEF8, PBF2, PBF4, or PBF5 but not $H_2O_2$, cell viability increased under normal culture conditions. The cell culture environment in the present study was a balanced system. PEF2, PEF8, PBF2, PBF4 and PBF5 may have activity in promoting cell division. But the detailed mechanism of promoting cell growth requires further study.

The antioxidant characteristics of the Pu-erh tea fractions were determined by multiple factors including their hydrophobicity/hydrophilicity, the experimental systems we used, and the total polyphenolic components present in the individual fractions. Among the four systems we used, only PEF2 and PEF8 showed positive antioxidant effects on all of them. PEF8 showed the strongest antioxidant effects. Interestingly, the antioxidant effect of this fraction on the four systems was much stronger than that of typical green tea catechin EGCG, but the HPLC analysis (**Figure 4**) showed that the content of monomeric polyphenols from the catechins was less than 1%, and no theaflavins or gallic acid were contained in PEF8. Further experiments are now in progress to separate and identify the specific components that are responsible for the relatively high antioxidative activities of PEF8.

The antioxidant effects of Pu-erh tea require further examination. Especially, animal experiments involving treatment with the separated Pu-erh tea fractions are important for the evaluation of Pu-erh tea antioxidant effect. Moreover, the isolation, identification, and quantification of certain unknown constituents should be emphasized.



## ABBREVIATIONS USED

PEF2, PEF5, PEF6, PEF7, and PEF8, Pu-erh tea ethyl acetate extract's fractions 2, 5, 6, 7, and 8; PBF2, PBF3, PBF4, and PBF5, Pu-erh tea butyl alcohol extract's fractions 2, 3, 4, and 5; ROS, reactive oxide species; MTT, 3-(4,5-dimethylthiazol-2-yl)-(2,5-diphenyl)tetrazoliumbromide; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; EGCG, (−)-epigallocatechin-3-gallate; CBSS, carbonic acid-buffered saline solution; CL, chemiluminescence intensity; $I_R$, inhibitory rate.

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**Supporting Information Available:** Processing and fractionation of Pu-erh tea. This material is available free of charge via the Internet at http://pubs.acs.org.

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