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# Polyphenol contents of Pu-Erh teas and their abilities to inhibit cholesterol biosynthesis in Hep G2 cell line

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ABSTRACT

Thirty samples of Pu-Erh tea (a microbial fermented Chinese tea) were collected and assayed for cholesterol synthesis inhibitory activity and polyphenol composition. All samples were able to inhibit the cholesterol biosynthesis in Hep G2 cell model and the inhibition ratios ranged from 7% to 35%. The inhibition abilities of tea polyphenol standards were in the order of gallocatechin gallate (GCG) > epigallocatechin gallate (EGCG) > epicatechin gallate (ECG) > gallic acid > epigallocatechin (EGC) > myricetin > quercetin > catechin (C) > epicatechin (EC). It appears that catechins with a galloyl structure on the B ring or a gallic acid moiety in the structure would have better inhibitory activity. In summary, tea polyphenol may play a role on the cholesterol biosynthesis inhibitory ability of Pu-Erh tea.



## 1. Introduction

Atherosclerosis is the major cause of cardiovascular disease, and a high level of low density lipoprotein (LDL) is the risk factor in promoting atherosclerosis. Cholesterol balancing in hepatocytes is controlled by LDL uptake from serum and biosynthesis within the cell. If cholesterol biosynthesis is inhibited, the LDL uptake by liver will be increased, and the level of LDL in serum, and thus the risk of atherosclerosis, will be lowered (Brown & Goldstein, 1986). A hepatoma cell line, Hep G2, has been applied widely to evaluate this inhibiting effect due to its similar cholesterol biosynthesis pathway to that in normal hepatocytes, many chemical compounds were therefore screened for the ability to inhibit cholesterol biosynthesis (Javitt, 1990).

Pu-Erh tea, a microbially fermented Chinese tea made from the leaves of large-leaf species tea plant, can be classified into green-tea-like (GTL), fermented (F), and post-processed (PP) Pu-Erh tea according to their processing procedures. The first type, GTL, is processed without microbial fermentation or high humidity storage. It is the large-leaf green tea stored at room temperature. The second type, F, is produced by microbial fermentation of piled large-leaf green tea. The third type, PP, is also called wet-stored tea because the aging process is accelerated by high humidity storage of the large-leaf green tea in order to simulate the naturally aged Pu-Erh tea so that it can be sold for a higher price (He, 2002).

Polyphenols, especially phenolic acids, flavanols, and flavonols, have been considered as active components in the prevention of cardiovascular diseases, cancers, neurodegenerative diseases, or diabetes (Higdon & Frei, 2003; Wu, Juan, Ho, Hsu, & Hwang, 2004). Tea contains many naturally occurring polyphenols including catechin (C), epicatechin (EC), epigallocatechin (EGC), gallocatechin gallate (GCG), epicatechin gallate (ECG), epigallocatechin gallate (EGCG), quercetin, myricetin, kaempferol, and gallic acid. Although there are some reports on the contents of flavanol compounds in Pu-Erh teas (Duh, Yen, Yen, Wang, & Chang, 2004; Lin, Lin, Liang, Lin-Shiau, & Juan, 1998; Lin, Tsai, Tsay, & Lin, 2003; Shao, Powell, & Clifford, 1995; Zuo, Chen, & Deng, 2002), these studies did not mention the type of Pu-Erh tea used.

Previous studies indicated that the Pu-Erh tea exhibited lipid-lowering abilities both *in vitro* and *in vivo* (Hwang, Lin, Chen, Liuchang, & Shiao, 2003; Kuo et al., 2005; Sano et al., 1986). Our laboratory found that the serum cholesterol and triacylglycerol contents were significantly reduced in male hamsters fed with Pu-Erh tea water extract (Hwang et al., 2003). However, the cholesterol biosynthesis inhibiting abilities of different Pu-Erh teas were not reported. Also, contents of the possible bioactive components (tea polyphenols) in Pu-Erh teas were not investigated thoroughly. Therefore, we attempted to investigate the cholesterol synthesis inhibitory effects of tea polyphenol standard compounds in Hep G2 cell model. We also conducted survey on the polyphenol contents and the cholesterol biosynthesis inhibiting abilities of different Pu-Erh teas in order to elucidate their relationships.

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## 2. Materials and methods

### 2.1. Materials

Pu-Erh tea samples were obtained from Da-You Tea Co. (Yingge, Taipei, Taiwan). EC, ECG, EGC, EGCG, GCG, C, gallic acid, kaempferol, myricetin, quercetin and MTT were purchased from Sigma Chemical Co. (St. Louis, MO). Lovastatin was generously provided by Dr. Ming-Shi Shiao (Department of Life Science, Chang Gung University, Taoyuan, Taiwan). 2-$^{14}$C acetate was purchased from Amersham Biosciences Co. (Buckinghamshire, UK). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Co. (Grand Island, NY, USA). All other chemicals and reagents were of analytical grade.

### 2.2. Preparation of tea extracts

Pu-Erh tea samples were ground into powder (30-mesh) and extracted with boiling water (1:50; w/v) for 5 min, and the aqueous extracts were filtered through Whatman No. 2 filter papers. For the analysis of flavanol and flavonol compounds, 1 ml of each aqueous extract was filtered through 0.45 μm filter prior to the HPLC analysis. For the assay of the inhibition of the cholesterol biosynthesis, 50 ml of each aqueous extract were lyophilized to powder and reconstituted into culture medium.

### 2.3. HPLC analysis of gallic acid, and flavanol compounds

Gallic acid, EGC, C, EC, EGCG, GCG, and ECG in tea extracts were analyzed by HPLC (Merken & Beecher, 2000), performed with a Hitachi HPLC system (Hitachi, Tokyo, Japan) consisting of a model L7610 degasser, a model L-7100 pump, a model L-7200 autosampler, and a model L-7420 UV–Vis detector. A Luna C18 column (5 μm, 250 mm × 4.6 mm, Phenomenex, Torrance, CA) connected to a C18 Securityguard cartridge (4 mm × 3 mm, Phenomenex, Torrance, CA) was used for analysis. The volume injected was 20 μl. The mobile phases, acetonitrile (A) and 1.1% acetic acid aqueous solution (B), were degassed by sonication and run by gradient elution at a flow rate of 1.0 ml/min. The wavelength of UV detection was set at 280 nm. The gradient elution program was as follows: 0–5 min, 5% A; 5–6 min, 5% A to 13% A; 6–26 min, 13% A; 26–27 min, 13% A to 30% A; 27–40 min, 30% A. The contents of gallic acid and flavanol compounds were quantified from the respective standard curve.

### 2.4. HPLC analysis of flavonol compounds

Myricetin, quercetin, and kaempferol in tea extracts were analyzed by HPLC (Merken & Beecher, 2000), performed with the Hitachi HPLC system. The column, column temperature, and injection volume were the same as above. The mobile phase, 1.1% acetic acid aqueous solution/acetonitrile (75/25, v/v), was degassed by sonication and run by an isocratic elution at a flow rate of 1.0 ml/min. The wavelength of UV detection was set at 370 nm. The contents of flavonol compounds were quantified from the respective standard curve.

### 2.5. Cell culture

Hep G2 cell line (BCRC 60025) was purchased from the Food Industry Research and Development Institute in Taiwan. Hep G2 cells were cultured in complete Dulbecco's Modified Eagle Medium (c-DMEM) and were maintained in humidified 5% $CO_2$ at 37 °C. Each liter of c-DMEM contained 10% fetal bovine serum (FBS), 100 μM non-essential amino acid, $1 \times 10^5$ IU penicillin, $1 \times 10^5$ μg streptomycin, 2 mM L-glutamine, and 3.7 g sodium bicarbonate (Hwang et al., 2003).

### 2.6. MTT assay

Hep G2 cells were cultured in 96 well plates ($5 \times 10^3$ cells/100 μl c-DMEM/well) in 5% $CO_2$ at 37 °C for 24 h, and then the c-DMEM was replaced with serum-free DMEM (SFM) that contained sample. The Hep G2 cells were treated with the samples for 2 h. The sample-containing SFM was discarded, and then MTT stock solution (5 mg/ml in PBS) was diluted by SFM (1:4, v/v) and added into each well (100 μl/well). After the 4 h incubation of MTT, MTT lysis buffer (20% sodium dodecyl sulphate in 50% dimethylformamide aqueous solution, 100 μl/well) was added to each well and incubated for 16 h. Results were read on an Anthos 2001 microplate reader (Cambridge, UK) at 570 nm. Cell viability was calculated by the following equation: (absorbance of sample treated well/absorbance of SFM treated well) × 100% (Hwang et al., 2003).

### 2.7. Inhibition of cholesterol synthesis in Hep G2 Cells

The potentials of Pu-Erh teas, flavanol compounds, and flavonol compounds to inhibit cholesterol biosynthesis were determined by the inhibition of the synthesis of cholesterol from acetate in Hep G2 cell using 2-$^{14}$C acetate (Cheng, Yang, & Shiao, 1993). Hep G2 cells were cultured in 24-well culture dishes ($1 \times 10^5$ cells/1 ml c-DMEM/well) at 37 °C in a 5% $CO_2$ atmosphere for 24 h, and then the c-DMEM was replaced with SFM and cultured for another 24 h. After being transferred to another fresh SFM that contained samples and labeled precursors (2-$^{14}$C acetate 1 μCi/well), the cells were treated with various kinds and doses of samples or SFM (control). Lovastatin, in hydroxy acid form, was used as a positive control. The inhibition experiment was carried out at 37 °C in a 5% $CO_2$ atmosphere for 2 h. After removal of the medium followed by extensive washing, the cells were harvested by 0.25% trypsin-EDTA treatment and transferred into test tubes. Saponification of lipids in the cells was performed by adding 2 ml saponification solution (saturated KOH aqueous solution: absolute ethanol = 1:3, v/v) and incubating the test tubes in a 56 °C water bath for 2 h. Unlabeled cholesterol (0.1 mg/tube) was used as a carrier in saponification. Cholesterol in the non-saponifiable fraction of the cell lipid was recovered by extracting the mixture with 5 ml *n*-hexane, and the cholesterol-containing *n*-hexane was transferred into scintillation vials. After evaporation of *n*-hexane, 10 ml Aquasol-2 (Perkin Elmer Co., Wellesley, MA) was added into each vial and vortexed. Then the radioactivity was measured by using a liquid scintillation counter (LS 6500, Beckman Coulter Co., Fullerton, CA). The inhibition ratio (%) was calculated by the following equation: 100%-(dpm of $^{14}$C-cholesterol in sample treated group/dpm of $^{14}$C-cholesterol in control group) × 100%.

### 2.8. Statistical analysis

All experiments were performed in duplicate and results were expressed as mean ± standard error. All statistical analyses were performed on SAS software. A *p*-value less than 0.05 was considered as statistically significant.

## 3. Results and discussion

### 3.1. Polyphenol contents in Pu-Erh tea samples

The polyphenolic compounds known to be present in tea samples, such as gallic acid, C, GCG, EC, ECG, EGC, EGCG, myricetin, quercetin, and kaempferol, were quantified in GTL ($n = 13$), F ($n = 10$), and PP ($n = 7$) samples. Results are shown in Table 1.

**Table 1**
Polyphenol contents of Pu-Erh tea samples[A,B]

| | Gallic acid (mg/g) | C (mg/g) | GCG (mg/g) | EC (mg/g) | ECG (mg/g) | EGC (mg/g) | EGCG (mg/g) | Myricetin (ng/g) | Quercetin (ng/g) |
|---|---|---|---|---|---|---|---|---|---|
| *Range* | | | | | | | | | |
| GTL | 1.2–12.6 | 2.3–8.8 | 0.3–4.3 | 1.8–16.2 | 10.7–32.9 | 8.5–33.1 | 6.7–44.7 | ND-42.4 | ND-47.1 |
| F | 1.0–16.7 | 0.5–3.0 | ND-2.5 | 0.4–3.8 | 3.6–22.3 | ND-12.5 | ND-6.5 | ND | ND-53.6 |
| PP | 0.2–6.6 | 0.3–2.5 | ND-1.1 | ND-4.6 | 2.2–13.9 | ND-10.7 | ND-3.4 | ND | ND |
| *Median* | | | | | | | | | |
| GTL | 3.0 | 5.0 | 1.6 | 8.2 | 27.6 | 16.2 | 19.0 | 30.9 | 29.6 |
| F | 5.9 | 1.6 | 0.4 | 2.7 | 8.1 | 7.1 | 1.0 | ND | 30.7 |
| PP | 0.4 | 0.7 | ND | ND | 5.6 | 1.0 | ND | ND | ND |
| *Mean ± SE*[C] | | | | | | | | | |
| GTL | $4.1 \pm 0.9^{a}$ | $5.0 \pm 0.5^{a}$ | $2.0 \pm 0.4^{a}$ | $8.3 \pm 1.1^{a}$ | $25.5 \pm 1.9^{a}$ | $18.0 \pm 1.8^{a}$ | $19.8 \pm 2.9^{a}$ | $25.3 \pm 4.1^{a}$ | $24.4 \pm 4.1^{a}$ |
| F | $6.8 \pm 1.8^{ab}$ | $1.7 \pm 0.2^{b}$ | $0.7 \pm 0.3^{b}$ | $2.7 \pm 0.4^{b}$ | $9.9 \pm 1.8^{b}$ | $6.9 \pm 1.1^{b}$ | $1.7 \pm 0.6^{b}$ | $ND^{b}$ | $22.9 \pm 6.7^{a}$ |
| PP | $1.5 \pm 0.9^{b}$ | $1.0 \pm 0.3^{b}$ | $0.2 \pm 0.2^{b}$ | $1.1 \pm 0.7^{b}$ | $6.0 \pm 1.4^{b}$ | $3.6 \pm 1.6^{b}$ | $0.6 \pm 0.5^{b}$ | $ND^{b}$ | $ND^{b}$ |

[A] Abbreviations: C: catechin; GCG: gallocatechin gallate; EC: epicatechin; ECG: epicatechin gallate; EGC: epigallocatechin; EGCG: epigallocatechin gallate; GTL: green-tea-like Pu-Erh tea ($n = 13$); F: fermented Pu-Erh tea ($n = 10$); PP: post-processed Pu-Erh tea ($n = 7$); ND: not detected.
[B] Data are expressed on per gram dried tea leaves basis. All data are the results of duplicate analyses.
[C] Different superscripts indicate significant difference ($p \leqq 0.05$) within the same column.

Kaempferol was not detected in any of these tea samples, so it was not shown in the table.

The range in the content of a specific compound was quite large across the GTL, or F, or PP samples; for example, the content of gallic acid in GTL, F, and PP samples ranged from 1.2 to 12.6 mg/g, 1.0–16.7 mg/g, and 0.2–6.6 mg/g, respectively. The composition of tea may vary with species, season, age of the leaf (Lin et al., 1998), processing procedure, storage condition, and storage duration. These factors may be the causes of the large range of tea polyphenol contents.

Medians of GTL and F were close to means, but PP did not show similar trend. The skewed distribution might due to the smaller sample size ($n = 7$) and the harsh processing method of PP tea. In general, GTL had higher quantities of C, GCG, EC, ECG, EGC, EGCG, and myricetin than F and PP while gallic acid and quercetin were high in F. It is postulated that the wet storage of Pu-Erh teas caused the loss of polyphenols in PP. The slightly higher amount of gallic acid in F might be arisen from the enzymatic cleavage of gallic acid from GCG, ECG, and EGCG during microbial fermentation (Bhat, Singh, & Sharma, 1998). GTL or F samples might be collected in previous studies (Duh et al., 2004; Lin et al., 1998, 2003; Zuo et al., 2002) since similar or higher gallic acid quantities were reported in Pu-Erh tea compared to green tea, oolong tea, and black tea.

### 3.2. Cholesterol biosynthesis inhibitory activities of different Pu-Erh teas

Incorporation of the water extract of Pu-Erh tea in the high cholesterol diet of hamsters could significantly reduce the levels of plasma cholesterol and triacylglycerol (Hwang et al., 2003), but the cholesterol biosynthesis inhibitory abilities among various kinds of Pu-Erh teas were not investigated. Therefore, the cholesterol biosynthesis inhibitory abilities of GTL, F, and PP samples were examined in Hep G2 cells. The inhibition ratios of the 30 tea samples, ranging from 7% to 35%, indicated that all of the collected Pu-Erh tea samples could inhibit cholesterol biosynthesis. Although the inhibition ability of PP samples was significantly lower than that of GTL and F samples (Fig. 1), there was no statistically significant difference between GTL and F samples.

Correlation coefficients between contents of tea polyphenols and inhibition ratios of all tea samples were calculated, and the results are shown in Table 2. All of the correlation coefficients were statistically significant and in the order: EC > gallic acid > EGC > ECG > C > EGCG > Myricetin > Quercetin > GCG. It shows that the polyphenol contents in Pu-Erh teas were positively correlated with the cholesterol inhibitory abilities, but the cholesterol biosynthesis inhibitory abilities of some of these compounds have not been reported. Therefore, we tested the cholesterol biosynthesis inhibitory activities of tea polyphenols.

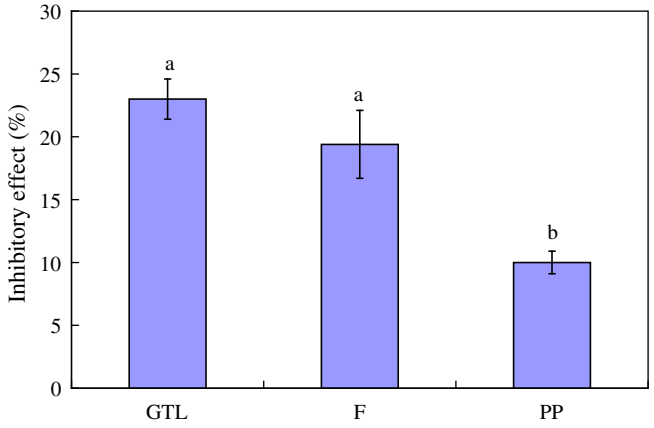


**Fig. 1.** Cholesterol biosynthesis inhibitory effects of green-tea-like (GTL), fermented (F), and post-processed (PP) Pu-Erh teas. GTL, $n = 13$; F, $n = 10$; PP, $n = 7$. Different alphabet indicates significant difference ($p < 0.05$).

**Table 2**
Pearson correlation coefficients between inhibition effects and polyphenol contents ($n = 30$)

| Compound[a] | Correlation coefficients | *p*-Value |
|---|---|---|
| Gallic acid | 0.65637 | <0.0001 |
| C | 0.53161 | 0.0025 |
| GCG | 0.36445 | 0.0477 |
| EC | 0.65906 | <0.0001 |
| ECG | 0.55492 | 0.0015 |
| EGC | 0.60661 | 0.0004 |
| EGCG | 0.51854 | 0.0033 |
| Myricetin | 0.45509 | 0.0115 |
| Quercetin | 0.39264 | 0.0319 |

[a] Abbreviations: same as in Table 1.

### 3.3. Cholesterol biosynthesis inhibitory activities of tea polyphenols

Gallic acid, EGC, C, EC, EGCG, GCG, ECG, myricetin, quercetin, and kaempferol standards were tested in Hep G2 cell system. MTT assays were performed prior to the cholesterol inhibition

**Table 3**
Cholesterol biosynthesis inhibitory effects of lovastatin and tea polyphenols

| Compound | Regression equation[a] | *R*-square | $IC_{50}$ (μM)[b] | Relative potency (%)[c] |
|---|---|---|---|---|
| Lovastatin | $y = 0.1134\text{Ln}(x) - 0.0144$ | 0.9788 | 0.47 | 100 |
| Gallic acid | $y = 0.0255x + 0.1053$ | 0.8954 | 90.99 | 0.52 |
| C | $y = 0.0069x + 0.0322$ | 0.9151 | 233.58 | 0.20 |
| GCG | $y = 0.0126x + 0.1082$ | 0.9704 | 67.85 | 0.69 |
| EC | $y = 0.0042x + 0.0946$ | 0.915 | 332.52 | 0.14 |
| ECG | $y = 0.0121x + 0.0285$ | 0.9625 | 88.09 | 0.53 |
| EGC | $y = 0.0115x + 0.108$ | 0.9814 | 111.31 | 0.42 |
| EGCG | $y = 0.0135x + 0.0498$ | 0.971 | 72.76 | 0.65 |
| Myricetin | $y = 0.0108x + 0.0786$ | 0.974 | 122.61 | 0.38 |
| Quercetin | $y = 0.0107x + 0.0279$ | 0.9534 | 130.43 | 0.36 |

[a] *x*: dosage (μg/ml) of the standard compound; *y*: inhibition ratio (%).
[b] The $IC_{50}$ values were calculated from the regression equation when the inhibition ratio (y) was equal to 50%.
[c] The relative potency values were calculated from $IC_{50}$ values.

assays, and the results showed that tea polyphenols did not cause cell death within the test range. The cholesterol biosynthesis inhibitory results are shown in Table 3. Lovastatin was used as a positive control, and the relative potency of each compound was calculated on the basis of $IC_{50}$ values. According to the relative potencies, the cholesterol biosynthesis inhibitory activities of tea polyphenols were low (0.14–0.69% of lovastatin) and in the order of GCG > EGCG > ECG > gallic acid > EGC > myricetin > quercetin > C > EC. Kaempferol showed no dose-dependent activity in inhibiting the biosynthesis of cholesterol in Hep G2 cell model.

Catechins with a galloyl structure on the B ring or a gallic acid moiety in the structure seemed to have better inhibitory activities. Comparisons of the inhibitory activities of EGCG versus ECG and EGC versus EC indicated that the presence of the galloyl structure on the B ring showed higher inhibitory effect on cholesterol synthesis. The existence of the gallic acid moiety in the structure was also found to be important for the inhibitory activity, as observed in the comparisons of EGCG versus EGC and ECG versus EC and for gallic acid. EC and C, lacking both the gallic acid moiety and galloyl structure, were poor inhibitors. GCG and EGCG, possessing both the gallic acid moiety and the galloyl structure, were the strongest inhibitors of cholesterol synthesis among the tea polyphenols tested. A similar trend was also observed in biological activity studies of tea catechins concerning scavenging effects of DPPH radicals (Nanjo et al., 1996), inhibition of activator protein 1 activity (Chung, Huang, Meng, Dong, & Yang, 1999), inhibition of fatty acid synthase (Wang, Song, Guo, & Tian, 2003), and inhibition of cysteine proteinases (Okamoto et al., 2004).

The mevalonate pathway, the major cholesterol biosynthesis pathway in cells, starts from acetyl-CoA, and then HMG-CoA, mevalonate, squalene, and cholesterol are sequentially synthesized. Many drugs for lowering cholesterol have been developed to inhibit this pathway. For example, statin compounds are inhibitors of HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis (Goldstein & Brown, 1990). In this study, $^{14}$C-acetate, the precursor of acetyl-CoA, was used to produce $^{14}$C-containing cholesterol, and any step in that pathway might have been inhibited. Therefore, the exact step that was inhibited could not be determined. Gallic acid, EGCG, GCG, and ECG, however, were found to be potent in direct inhibiting the rat squalene epoxidase (Abe, Seki, & Noguchi, 2000; Abe et al., 2000), and the studies also showed that catechins without gallic acid moiety were less potent than that with gallic acid moiety. Accordingly, squalene epoxidase might be one of steps in the mevalonate pathway inhibited by tea catechins.

## 4. Conclusions

Although different Pu-Erh tea samples have very different polyphenol contents, all of these teas showed cholesterol biosynthesis inhibitory activities, which may be related to the polyphenolic compounds present. Tea catechins with a galloyl structure on B ring or a gallic acid moiety in the structure seemed to exhibit better inhibitory activity. It is postulated that tea polyphenols are the important component of Pu-Erh tea to inhibit cholesterol biosynthesis.

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