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Isolation of caffeic acid from *Perilla frutescens* and its role in enhancing γ-glutamylcysteine synthetase activity and glutathione level

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ABSTRACT

Perilla frutescens is an annual herbaceous plant native to Asia, where its leaves are used in Asian gourmet food. Our previous study showed that the inhibition of γ-glutamylcysteine synthetase (γ-GCS) activity was remarkably recovered by pretreatment with perilla leaf extract (PLE). The objective was to fractionise PLE, and to identify the active component that is responsible for the enhancement of γ-GCS activity and glutathione (GSH) concentration. Among the five fractions from PLE, PLE-III of the ethyl acetate fraction showed the highest γ-GCS activity in a HepG2 cell experiment, and was further chromatographed. The purified compound, which enhanced γ-GCS activity, was finally identified as caffeic acid. We first report the enhancement of γ-GCS activity and GSH level in HepG2 cells by caffeic acid obtained from PLE. Our results suggest that caffeic acid may be a key factor in the chemopreventive potential of perilla leaf components by increasing *de novo* synthesis of GSH.

1. Introduction

It is well recognised that oxidative and free-radical-mediated reactions contribute to aging and related diseases, such as cancer, coronary disease, and neurogenerative disorders (Smith et al., 1996; Weinbrenner et al., 2003). Endogenous or dietary factors play an important role in the antioxidative defenses of organisms against the reactive oxygen species (ROS) generated during normal cellular aerobic respiration (Kohen & Nyska, 2002). Current epidemiological data support that increased intakes of dietary antioxidants may help the tipping of the balance towards a proper antioxidant status (Halliwell, Murcia, Chirico, & Aruoma, 1995).

As a candidate dietary source, our research group has focused on *Perilla* [*Perilla frutescens* (L.) Britt. var. *japonica* (Hassk.) Hara]. This plant is an annual herbaceous plant native to Southeast Asian countries. Its leaves are often used in sushi, garnishes, and soups, and to wrap and eat cooked foods. The antioxidative, anti-allergic, anti-inflammatory, and anti-tumor promoting substances contained in perilla plants have earned considerable attention (Banno et al., 2004; Kim et al., 2007; Makino et al., 2003; Ueda, Yamazaki, & Yamazaki, 2002, 2003). Our previous *in vivo* study showed a protective effect of aqueous perilla leaf extract (PLE) on *tert*-butyl hydroperoxide (*t*-BHP)-induced oxidative hepatotoxicity (Kim et al., 2007). While the administration of *t*-BHP significantly decreased glutathione (GSH) level, the pretreatment of PLE remarkably increased GSH to an even higher level compared to that of the untreated control. Also, we observed a significant increase in hepatic γ-glutamylcysteine synthetase (γ-GCS) activity by the PLE treatment.

GSH is a ubiquitous molecule that plays an important role in intracellular free radical metabolism as well as xenobiotic detoxification (Biaglow et al., 1989). The enzyme catalysing the first and rate-limiting step in *de novo* GSH synthesis is γ-GCS (Dringen, 2000). To the best of our knowledge, the active component of PLE that is responsible for enhancing hepatic γ-GCS activity and GSH concentration has not been identified. Therefore, this research aimed to identify the functional compound of aqueous PLE possessing the aforementioned activities using the human hepatocellular cell line HepG2.

Abbreviations: γ-GCS, γ-glutamylcysteine synthetase; GSH, glutathione; PLE, perilla leaf extract; *t*-BHP, *tert*-butyl hydroperoxide; MEM, minimum essential medium; FBS, fetal bovine serum; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium; ATP, adenosine 5′-triphosphate; PEP, phospho(enol)pyruvate; NADH, β-nicotinamide adenine dinucleotide reduced dipotassium salt; BSA, bovine serum albumin; EtOAc, ethyl acetate; BuOH, *n*-butanol; MeOH, methanol; BSO, buthionine sulfoxide.

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2. Materials and methods

2.1. Chemicals

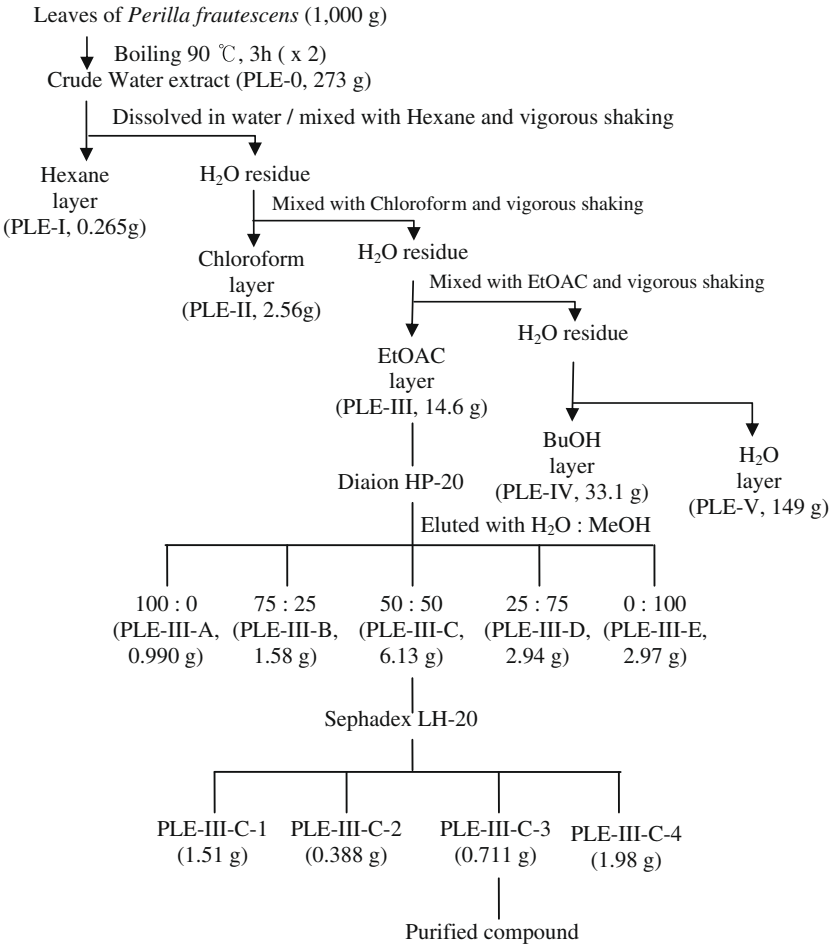
Minimum essential medium (MEM) and fetal bovine serum (FBS) were purchased from GIBCO® Life Technologies (Carlsbad, CA, USA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium (MTT), streptomycin, penicillin, adenosine 5′-triphosphate (ATP), phospho(enol)pyruvate (PEP), $MgCl_2$, L-glutamate, L-α-aminobutylate, β-nicotinamide adenine dinucleotide reduced dipotassium salt (NADH), pyruvate kinase, lactate dehydrogenase, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Diaion HP-20 and Sephadex LH-20 were purchased from Mitsubishi Chemical Co. (Tokyo, Japan) and Amersham Biosciences (Uppsala, Sweden), respectively. The other chemicals were of the highest grade from commercial sources.

2.2. Plant materials

The edible leaves of a green type of perilla were purchased locally (Kyungdong Herb-Market, Seoul, Korea) and identified by Dr. B.W. Kang (College of Life Sciences and Biotechnology, Korea University). The fresh leaves were washed, and then blanched at 100 °C for 5 min, and immediately dried in a freeze dryer.

2.3. Extraction and isolation preparations

The dried perilla leaves (1.0 kg) were ground in a mortar and then soaked in distilled water (1 g/10 ml), followed by refluxing twice at 100 °C for 3 h, and then cooled. The undissolved remains were removed by filtration through a Whatman 41 filter paper (Clifton, NJ, USA) followed by a membrane filter with a 0.45 μm pore size (Millipore, Billerica, MA, USA). The filtrate was concentrated with a rotary vacuum evaporator (N-1000S, EYELA, Tokyo, Japan), and then lyophilised yielding a dried residue. For further fractionation of the dried PLE (273 g), the extract was resuspended in H_2O, and then extracted successively with *n*-hexane, chloroform, ethyl acetate (EtOAc), and *n*-butanol (BuOH) (Scheme 1). The yields of the *n*-hexane (PLE-I), chloroform (PLE-II), EtOAc (PLE-III), *n*-BuOH (PLE-IV), and water residue (PLE-V) extracts were 0.0971%, 0.938%, 5.35%, 12.1%, and 54.6%, respectively. The EtOAc-soluble portion (14.6 g) was applied to a Diaion™ HP-20 (250 × 45 mm; Mitsubishi Chemical Co., Tokyo, Japan) column, and then eluted by a gradient with increasing methanol (MeOH) in H_2O (100/0, 75/25, 50/50, 25/75, and 0/100, v/v; 237 ml of each eluent). The active fraction (PLE-III-C) eluted by the H_2O and MeOH (50/50, v/v) was further purified on a Sephadex™ LH-20 (Amersham Biosciences, Uppsala, Sweden) column (400 × 30 mm) in which the column was eluted with MeOH–H_2O (7:3) at 0.3 ml/min, and 3 ml/tube was collected. Finally, the active compound from fraction (PLE-III-C-3) was obtained by SymmetryPrep C18 (300 × 7.8 mm, 7 μm; Waters, Milford, MA, USA) column chromatography, in which elution was affected using a linear gradient of solvent mixtures of H_2O (solvent A) and MeOH (solvent B). The composition of B was held to 50% for 10 min, increased to 55% over 40 min and held for 10 min, and then returned to the initial concentration over 5 min at 1 ml/min. The eluted fraction was monitored at 320 nm.



Scheme 1. Isolation and purification procedure for *P. frutescens*.

2.4. Cell culture

The HepG2 cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). The cells were grown at 37 °C in humidified air with 5% CO_2 in MEM with 10% FBS, 2.2 g/l sodium bicarbonate, 100 μg/ml of streptomycin and 100 U/ml of penicillin. The cells were split 1:4 every 7 days. The cells were seeded into 6-well plates (Falcon, Franklin Lakes, NJ, USA) at 1×10^6 cells/well, and incubated for 30 h prior to treatment. After treatment they were washed twice with 0.1 M PBS, scraped with lysis buffer (50 mM Tris buffer and 0.5% triton X-100), sonicated, and centrifuged at 12,000*g* for 15 min to collect the cytosolic fraction.

2.5. GSH assay

The intracellular reduced GSH of the HepG2 cells was determined by HPLC as described previously (Reed et al., 1980). The protein concentrations were determined according to the method of Bradford (Bradford, 1976). The amount of protein in each sample was calculated from a standard curve constructed with bovine serum albumin.

2.6. Enzymatic assay

The activity of γ-GCS was determined by the rate of formation of ADP by the lactate dehydrogenase–pyruvate kinase system according to a coupled enzyme assay as described by Ray, Misso, Lenzo, Robinson, and Thompson (1999) with a slight modification. Briefly, 990 μl of pre-warmed assay medium containing Tris–HCl buffer (0.1 M, pH 8.0), 10 mM L-glutamate, 10 mM L-α-aminobutylate, 5 mM ATP, 20 mM $MgCl_2$, 150 mM KCl, 2 mM EDTA, 2 mM phosphoenolpyruvate and 0.2 mM NADH, 17 μg of pyruvate kinase and 17 μg of lactate dehydrogenase were pre-incubated for 5 min at 37 °C. Immediately after the pre-incubation, the γ-GCS reaction was initiated at 37 °C by addition of 10 μl of cytosol making the final volume of the assay media to 1.0 ml. An equal volume of Tris–HCl buffer instead of cytosol was added to the reference cuvette as a blank control. The change in absorbance at 340 nm was monitored for 3 min and the rate of NADH oxidation determined. The activity of the enzyme was determined by the time-dependent change in [NADH] ($\varepsilon = 6270\ M^{-1}\ cm^{-1}$).

2.7. Statistical analysis

The experiments for all of the *in vitro* assessments were performed in triplicate. The obtained results were expressed as the mean ± standard deviation (SD). The statistical significance of the differences was determined using the unpaired Student's *t*-test and one-way ANOVA to evaluate the significant differences at *p*-values < 0.05. The statistical calculations were conducted using SigmaStat (Systat Software Inc.; Point Richmond, CA, USA).

3. Results

3.1. γ-GCS activity of PLE in HepG2 cells

Because of an observed significant increase in hepatic γ-GCS activity by PLE treatment in a previous study (Kim et al., 2007), this study was undertaken to examine γ-GCS activity in HePG2 cells treated with PLE. The cells treated with PLE-0 (50, 100, 500, and 1000 μg/ml) for 24 h had increased γ-GCS activity compared to the untreated cells (Fig. 1A). The cells treated with PLE at the dose of 500 μg/ml showed the highest level of γ-GCS activity at 124%. Treatment with 2.5 mM buthionine sulfoxide (BSO), a selective

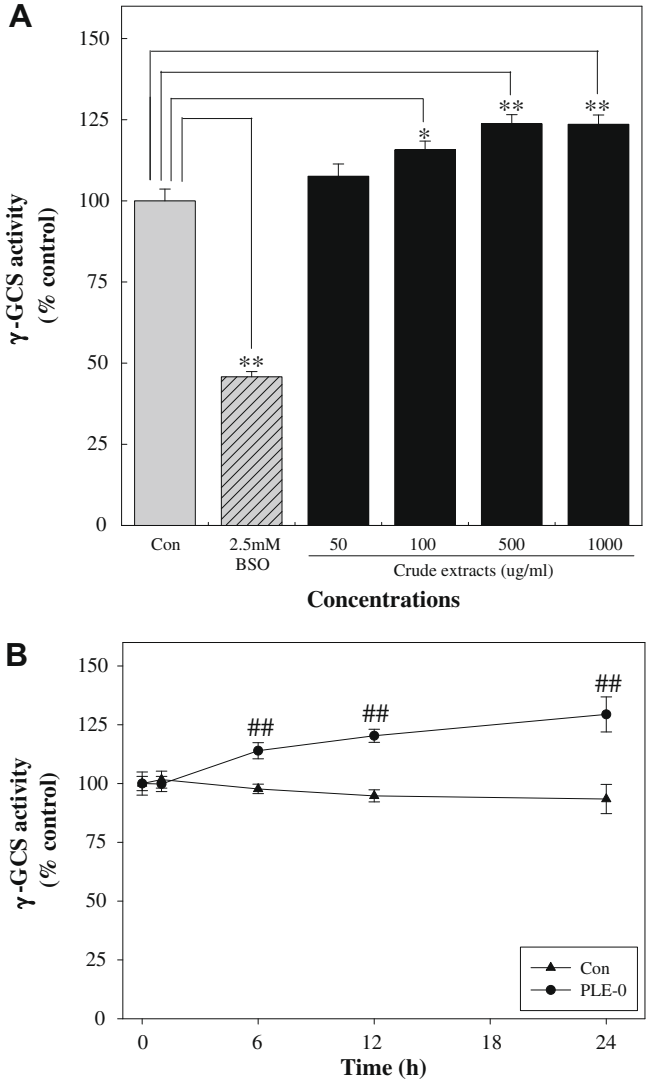


Fig. 1. Effects of perilla leaf extract (PLE) on γ-glutamylcysteine synthetase (γ-GCS) activity in HepG2 cells. (A) HepG2 cells were treated with a 2.5 mM buthionine sulfoxide (BSO) or 50, 100, 500, and 1000 μg/ml of PLE. Results are represented as means ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the untreated control group. (B) HepG2 cells were treated with 500 μg/ml of PLE for 0, 1, 6, 12, and 24 h. The γ-GCS activity of the lysates was measured immediately after each period of incubation. The results represent means ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\#\#}p < 0.01$, compared with the lysate of 0 h incubated cells ($n = 3$).

inhibitor of GSH synthesis, decreased the activity of γ-GCS up to 45% compared to the control group. On the other hand, the difference in the enzyme activity percentage between the treatments and control increased with time in the HepG2 cells (Fig. 1B), by 23%, 25%, and 28% when the cells were treated with 500 μg/ml for 6, 12, and 24 h, respectively.

3.2. Identification of the active constituent of PLE that enhances γ-GCS activity

We next attempted to isolate the active component from PLE with strong γ-GCS activity. After the PLE (273 g) was sequentially partitioned into hexane (PLE-I), chloroform (PLE-II), EtOAc (PLE-III), BuOH (PLE-IV), and D.W. (PLE-V), the five portions were assayed for γ-GCS activity. The active EtOAc-soluble fraction (PLE-III), which showed the highest enzyme activity of 133% (Table 1), was further chromatographed on a Diaion HP-20 column

eluted with H_2O/MeOH (50/50, v/v) to produce the active fraction (PLE-III-C), which had a γ-GCS activity of 136% (Table 1). PLE-III-C was further purified on a Sepadex LH-20 column and eluted with H_2O/MeOH (30/70, v/v), which yielded four fractions, namely PLE-III-C-1, 2, 3, and 4 (Fig. 2A). Among them, PLE-III-C-3 gave the highest γ-GCS activity of 171% (Table 1). A pure active compound of PLE-III-C-3 was confirmed by RP-HPLC (Fig. 2B).

Table 1
Increases in γ-GCS activity in HepG2 cells as obtained from each fraction.

Purification step		γ-GCS activity (% control)[a]	Yields (%, w/w)[b]	Purity (%)[c]
Aqueous extracts	(PLE-0)	122 ± 7*	27.3	0.260
Solvent extraction	(PLE-I)	99.8 ± 3.5	0.0265	
	(PLE-II)	77.4 ± 9.4*	0.256	
	(PLE-III)	133 ± 5**	1.46	4.79
	(PLE-IV)	120 ± 4*	3.31	
	(PLE-V)	114 ± 10	14.9	
Diaion HP-20 chromatogram	(PLE-III-A)	102 ± 9	0.0990	
	(PLE-III-B)	129 ± 9**	0.158	
	(PLE-III-C)	136 ± 7**	0.613	11.5
	(PLE-III-D)	119 ± 7*	0.294	
	(PLE-III-E)	102 ± 9	0.297	
Sephadex LH-20 chromatogram	(PLE-III-C-1)	101 ± 5	0.151	
	(PLE-III-C-2)	99.9 ± 5.5	0.0388	
	(PLE-III-C-3)	171 ± 13**	0.0711	99.9
	(PLE-III-C-4)	125 ± 8*	0.198	

The cells were treated with 500 μg/ml of each extract for 24 h. Results are expressed as the mean ± SD ($n = 3$).
[a] Compared with the untreated control group (100% γ-GCS activity).
[b] Yields of total soluble extract from dried *P. frutescens* by each step of separation and purification.
[c] Percentage of caffeic acid content.
* $p < 0.05$.
** $p < 0.01$.

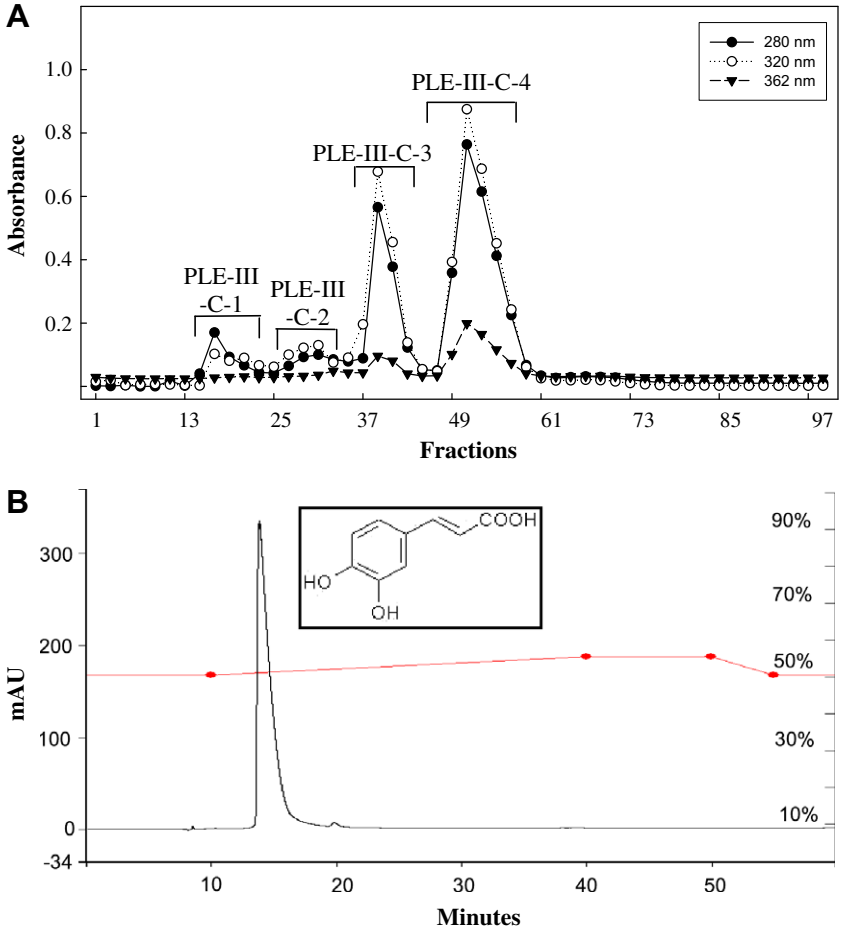


Fig. 2. Sephadex LH-20 chromatogram (A) of the subfraction from Diaion chromatography, and SymmetryPrep C18 HPLC chromatogram (B) of PLE-III-C-3 and its chemical structure (caffeic acid) (Materials and methods for chromatography elution details).

3.3. Structural determination of the active compound

This compound isolated from *P. frutescens* had a yield of 0.0711%, and was obtained as an amorphous powder. The EI-MS spectra of PLE-III-C-3 were recorded on a JMS-SX102A (JEOL, Tokyo, Japan) spectrometer, and exhibited $[M-OH]^+$ and $[M-COOH]^+$ ions at m/z 163 and 135, respectively, and an $[M]^+$ ion peak at *m/z* 180 as a base peak with a molecular mass of 180.16 g/mol. The NMR spectra were measured at 500 MHz for ^1H NMR, 13C NMR and HMBC in MeOH-*d*4 (10:90, v:v) at 25 °C using a Bruker DRX 500 NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). In the ^1H NMR spectrum of the component there were aromatic methine protons at 7.03, 6.93, and 6.77 (chemical shifts in ppm from tetramethylsilane as the internal standard); asymmetrical methylene protons at 7.54 (1H, *d*, *J* = 16.0 Hz) and 6.22 (1H, *d*, *J* = 16.0 Hz); and five broad exchangeable protons quenched by the addition of D_2O were observed. The ^{13}C NMR spectrum revealed nine carbons, which were comprised of a carbonyl carbon, two sp^2 carbons and three methines, two methylenes and a methane. In the HMBC NMR spectrum, a powerful cross peak was shown between the H5 doublet peak and C7, and between H5 of the aromatic and the sp^2 C7 methine there were three bonds. Also, between the C7 olefin and C9 carboxylate there were three bonds owing to the powerful cross peak between the H7 doublet peak and C9 carboxylate (Table 2). Based on the above spectroscopic data and an extensive literature search, the active compound was identified as caffeic acid (insert in Fig. 2B).

Table 2
^1H NMR and ^{13}C NMR data for PLE-III-C-3.

	δ_C	δ_H	HMBC
1	127.95		
2	115.23	7.03 (1H, *d*, 1.5)	C4, C6, C7
3	147.16		
4	149.59		
5	116.63	6.93 (1H, *d*, 8.0)	C1, C3
6	122.98	6.77 (1H, *dd*, 8.0, 1.5)	C2, C4, C7
7	146.78	7.54 (H, *d*, 16)	C1, C2, C6, C9
8	115.69	6.22 (H, *d*, 16)	C1, C9
9	171.17		

Chemical shifts in ppm from tetramethylsilane as internal standard.
^1H and ^{13}H NMR spectra were measured at 500 MHZ.

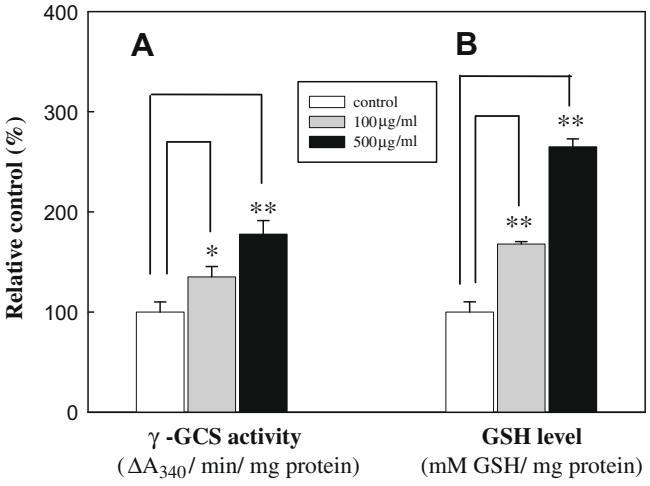


Fig. 3. Effects of caffeic acid on γ-GCS activity and glutathione (GSH) level in HepG2 cells. (A) γ-GCS activity. (B) GSH level. The HepG2 cells were treated with 100 μg/ml and 500 μg/ml of caffeic acid. The results represent means ± SD ($n = 3$). $^*p < 0.05$, $^{**}p < 0.01$, compared with the untreated control group.

3.4. Enhancements of γ-GCS activity and GSH level by caffeic acid in HepG2 cells

To confirm the potential of caffeic acid, originating from PLE-III-C-3, for increasing γ-GCS activity and GSH level, the corresponding measurements were assayed in HepG2 cells. As shown in Fig. 3, treatment of the cells with 100 and 500 μg/ml of caffeic acid increased γ-GCS activities by 1.4- and 1.8-fold compared to the control group, respectively (Fig. 3A). Also, at the same doses of caffeic acid, the treated cells showed increased levels of GSH by 1.7- and 2.7-fold compared to the control, respectively (Fig. 3B).

4. Discussion

In our previous study (Kim et al., 2007), we indicated that a single dose of *t*-BHP (0.2 mmol/kg), a short-chain analog of lipid peroxide to induce acute oxidative damage, administered to rats by i.p. injection caused a significant reduction in the level of GSH (57.0 ± 1.6 nmol/mg protein). However, oral pretreatment with an extract of *P. Frutescens* leaves (1000 mg/kg) protected against this reduction in GSH level caused by *t*-BHP, and maintained the GSH concentration (75.1 ± 11 nmol/mg protein) at a level that was even higher than that in the untreated control (67.7 ± 4.7 nmol/mg). Considering the step of combining cysteine with glutamate generating γ-glutamyl cysteine, which is catalysed by γ-GCS, and is the rate-limiting step for *de novo* GSH synthesis (Wild & Mulcahy, 2000), the activity of γ-GCS was measured from the rate of formation of ADP (assumed to be equal to the rate of oxidation of NADH by linking the γ-GCS reaction to the lactate dehydrogenase-pyruvate kinase system according to a coupled enzyme assay procedure (Seelig & Meister, 1984)) as calculated from the change in absorbance at 340 nm in the reaction mixture. *t*-BHP induced the inhibition of γ-GCS activity (by 40%) when compared to the control group, however, this inhibition was prevented by the pretreatment of 1000 mg/kg of PLE in which γ-GCS activity was recovered (by 93%) compared to the untreated control. As a next stage, in the present study we aimed to investigate which compound from PLE was responsible for enhancing γ-GCS activity and the GSH level.

We utilised HepG2 cells, a widely employed hepatoblastoma line of human origin, to investigate the effects of sub-fractions of PLE on γ-GCS activity and on the resulting GSH levels. HepG2 cells are considered a model to study *in vitro* xenobiotic metabolism, including GSH synthesis and toxicity to the liver, since they retain many of the specialised functions that characterise normal human hepatocytes (Knasmüller et al., 1998; Scharf, Prustomersky, Knasmüller, Schulte-Hermann, & Huber, 2003). The HepG2 cell line has been proposed to be a useful model as an alternative approach to human studies to investigate γ-GCS induction and the resulting enhancement of GSH levels by potential chemoprotectants (Scharf et al., 2003). Our present results suggest that the active EtOAc-soluble fraction (PLE-III) from PLE had the highest γ-GCS activity among the five different solvent-soluble portions, and further separations using Diaion HP-20 and Sephadex LH-20 column chromatography yielded the active compound of PLE-III-C-3, which was identified as caffeic acid (Table 1 and Fig. 2). Caffeic acid is found in plant-based foods such as grapes, olives, spinach, sweet potatoes, coffee, etc. (Larson, 1988; Rice-Evans, Miller, & Paganga, 1996), and has been reported to have a wide range of biological effects, e.g. antioxidant activity, anti-inflammatory and anti-carcinogenic properties (Fernandez, Saenz, & Garcia, 1998; Olthof, Hollman, & Katan, 2001; Tanaka et al., 1993; Vieira, Laranjinha, Madeira, & Almeida, 1998).

To our knowledge, we are the first to report on the enhancement of γ-GCS activity and GSH level by caffeic acid derived from

PLE. It was previously reported that plant-derived components such as kahweol/cafestol, α-angelicalactone, benzyl isothiocyanate, diallyl sulfide, and quercetin showed increases in both γ-GCS activity and GSH concentration in *vivo and in vitro* (Huber et al., 2002; Scharf et al., 2003). γ-GCS consists of a catalytic heavy subunit (γ-GCS-HS) and a regulatory light subunit (γ-GCS-LS) (Huang, Chang, Anderson, & Meister, 1993). It has been reported that phenolic antioxidants such as β-naphthoflavone can increase GSH levels by inducing γ-GCS-HS, and the promoter region of the γ-GCS-HS gene is regulated by the active protein-1 (AP-1) binding site (Rahman & MacNee, 2000). The AP-1 transactivation in response to the phenolic compound, apocyanin, lead to the up-regulation of γ-GCS-HS resulting increased GSH synthesis (Lapperre et al., 1999). However, it remains to be investigated whether caffeic acid can increase the expression of γ-GCS-HS mRNA via AP-1 DNA binding in our study. Depending on the compounds tested, and with doses in the single-digit to three-digit micromolar range, accentuated increases in both γ-GCS activity and GSH level were observed in HepG2 cells with a time optimum between 12 and 24 h of incubation (Scharf et al., 2003). In our results, the 100 and 500 μg/ml of caffeic acid-treated cells had an 1.4- and 1.8-fold increase in GCS activities, respectively, and 1.7- and 2.7-fold increased in GSH levels, respectively, after 24 h of incubation. However, further investigations on the time course and dose dependence of the effects of caffeic acid on γ-GCS and GSH in HepG2 cells is required.

Despite there being no known ill-effects of caffeic acid in humans, caffeic acid is still listed under older hazard data sheets as a potential carcinogen (Group 2B) due to earlier experiments on animals showing that oral administration of only high doses of caffeic acid (1–2% caffeic acid in the diet) caused forestomach papillomas ("Hazardous Substances Data Bank: caffeic acid," 2009). However, animals that were fed a diet containing a low dose of caffeic acid (0.5%) did not produce tumors, and 0.04% caffeic acid inhibited rat tongue carcinogenesis induced by 4-nitroquinoline-1-oxide (Tanaka et al., 1993). Based on our data, the perilla leaves contained approximately 9 mg of caffeic acid/kg of the plant leaves on a wet basis (data not shown). Assuming that a rat consumes a daily food intake of 30 g with 1% caffeic acid in the diet it would correspond to a daily oral ingestion of ~33,000 g of perilla leaves on a wet basis, suggesting that the daily intake of perilla leaves would become excessively high. In cell culture experiments, there have been reports that caffeic acid possessed anti-carcinogenic properties such as suppressing tumor angiogenesis and protecting against the deleterious effects of UVB radiation (Jung et al., 2007; Staniforth, Chiu, & Yang, 2006).

In conclusion, the present study demonstrated that caffeic acid derived from aqueous PLE caused a sustained elevation of intracellular γ-GCS activity in HepG2 cells. This elevation appeared to result in a *de novo* increase of GSH content. While the benefits of applying caffeic acid would depend on a cautious administration strategy, caffeic acid from *P. frutescens,* as a natural herbal complement, could be a beneficial factor in the chemopreventive potential of perilla leaf components by increasing *de novo* synthesis of GSH.

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References

Banno, N., Akihisa, T., Tokuda, H., Yasukawa, K., Higashihara, H., Ukiya, M., et al. (2004). Triterpene acids from the leaves of *Perilla frutescens* and their anti-inflammatory and antitumor-promoting effects. *Biosci. Biotechnol. Biochem., 68*(1), 85–90.

Biaglow, J. E., Varnes, M. E., Epp, E. R., Clark, E. P., Tuttle, S. W., & Held, K. D. (1989). Role of glutathione and other thiols in cellular response to radiation and drugs. *Drug Metab. Rev., 20*(1), 1–12.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem., 72*, 248–254.

Dringen, R. (2000). Glutathione metabolism and oxidative stress in neurodegeneration. *Eur. J. Biochem., 267*(16), 4903.

Fernandez, M. A., Saenz, M. T., & Garcia, M. D. (1998). Anti-inflammatory activity in rats and mice of phenolic acids isolated from *Scrophularia frutescens*. *J. Pharm. Pharmacol., 50*(10), 1183–1186.

Halliwell, B., Murcia, M. A., Chirico, S., & Aruoma, O. I. (1995). Free radicals and antioxidants in food and in vivo: What they do and how they work. *Crit. Rev. Food Sci., 35*(1–2), 7–20.

Huang, C. S., Chang, L. S., Anderson, M. E., & Meister, A. (1993). Catalytic and regulatory properties of the heavy subunit of rat kidney gamma-glutamylcysteine synthetase. *J. Biol. Chem., 268*(26), 19675–19680.

Huber, W. W., Scharf, G., Rossmanith, W., Prustomersky, S., Grasl-Kraupp, B., Peter, B., et al. (2002). The coffee components kahweol and cafestol induce γ-glutamylcysteine synthetase, the rate limiting enzyme of chemoprotective glutathione synthesis, in several organs of the rat. *Arch. Toxicol., 75*(11), 685–694.

Jung, J. E., Kim, H. S., Lee, C. S., Park, D.-H., Kim, Y.-N., Lee, M.-J., et al. (2007). Caffeic acid and its synthetic derivative CADPE suppress tumor angiogenesis by blocking STAT3-mediated VEGF expression in human renal carcinoma cells. *Carcinogenesis, 28*(8), 1780–1787.

Kim, M.-K., Lee, H.-S., Kim, E.-J., Won, N.-H., Chi, Y.-M., Kim, B.-C., et al. (2007). Protective effect of aqueous extract of *Perilla frutescens* on tert-butyl hydroperoxide-induced oxidative hepatotoxicity in rats. *Food Chem. Toxicol., 45*(9), 1738–1744.

Knasmüller, S., Parzefall, W., Sanyal, R., Ecker, S., Schwab, C., Uhl, M., et al. (1998). Use of metabolically competent human hepatoma cells for the detection of mutagens and antimutagens. *Mutat. Res., 402*(1–2), 185–202.

Kohen, R., & Nyska, A. (2002). Oxidation of biological systems: Oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol. Pathol., 30*(6), 620–650.

Lapperre, T. S., Jimenez, L. A., Antonicelli, F., Drost, E. M., Hiemstra, P. S., Stolk, J., et al. (1999). Apocynin increases glutathione synthesis and activates AP-1 in alveolar epithelial cells. *FEBS Lett., 443*, 235–239.

Larson, R. A. (1988). The antioxidants of higher plants. *Phytochemistry, 27*(4), 969–978.

Makino, T., Furuta, Y., Wakushima, H., Fujii, H., Saito, K., & Kano, Y. (2003). Anti-allergic effect of *Perilla frutescens* and its active constituents. *Phytother. Res., 17*(3), 240–243.

Medicine USNLo (2009). Hazardous substances data bank: Caffeic acid. Vol. 2009. US National Library of Medicine.

Olthof, M. R., Hollman, P. C., & Katan, M. B. (2001). Chlorogenic acid and caffeic acid are absorbed in humans. *J. Nutr., 131*(1), 66–71.

Rahman, I., & MacNee, W. (2000). Oxidative stress and regulation of glutathione in lung inflammation. *Eur. Respir. J.*(3), 534–554.

Ray, S., Misso, N. L., Lenzo, J. C., Robinson, C., & Thompson, P. J. (1999). Gamma-glutamylcysteine synthetase activity in human lung epithelial (A549) cells: Factors influencing its measurement. *Free Radic. Biol. Med., 27*(11–12), 1346–1356.

Reed, D. J., Babson, J. R., Beatty, P. W., Brodie, A. E., Ellis, W. W., & Potter, D. W. (1980). High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. *Anal. Biochem., 106*(1), 55–62.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med., 20*(7), 933–956.

Scharf, G., Prustomersky, S., Knasmüller, S., Schulte-Hermann, R., & Huber, W. W. (2003). Enhancement of glutathione and γ-glutamylcysteine synthetase, the rate limiting enzyme of glutathione synthesis, by chemoprotective plant-derived food and beverage components in the human hepatoma cell line HepG2. *Nutr. Cancer, 45*(1), 74–83.

Seelig, G., & Meister, A. (1984). Gamma-glutamylcysteine synthetase. Interactions of an essential sulfhydryl group. *J. Biol. Chem., 259*(6), 3534–3538.

Smith, M. A., Perry, G., Richey, P. L., Sayrec, L. M., Anderson, V. E., Beal, M. F., et al. (1996). Oxidative damage in Alzheimer's. *Nature, 382*(6587), 120–121.

Staniforth, V., Chiu, L.-T., & Yang, N.-S. (2006). Caffeic acid suppresses UVB radiation-induced expression of interleukin-10 and activation of mitogen-activated protein kinases in mouse. *Carcinogenesis, 27*(9), 1803–1811.

Tanaka, T., Kojima, T., Kawamori, T., Wang, A., Suzui, M., Okamoto, K., et al. (1993). Inhibition of 4-nitroquinoline-1-oxide-induced rat tongue carcinogenesis by the naturally occurring plant phenolics caffeic, ellagic, chlorogenic and ferulic acids. *Carcinogenesis, 14*(7), 1321–1325.

Ueda, H., Yamazaki, C., & Yamazaki, M. (2002). Luteolin as an anti-inflammatory and anti-allergic constituent of *Perilla frutescens*. *Biol. Pharm. Bull., 25*(9), 1197–1202.

Ueda, H., Yamazaki, C., & Yamazaki, M. (2003). Inhibitory effect of *Perilla* leaf extract and luteolin on mouse skin tumor promotion. *Biol. Pharm. Bull., 26*(4), 560–563.

Vieira, O. I., Laranjinha, J., Madeira, V. O., & Almeida, L. (1998). Cholesteryl ester hydroperoxide formation in myoglobin-catalyzed low density lipoprotein

oxidation: Concerted antioxidant activity of caffeic and *p*-coumaric acids with ascorbate. *Biochem. Pharmacol., 55*(3), 333–340.

Weinbrenner, T., Cladellas, M., Isabel Covas, M., Fitó, M., Tomás, M., Sentí, M., et al. (2003). High oxidative stress in patients with stable coronary heart disease. *Atherosclerosis, 168*(1), 99–106.

Wild, A. C., & Mulcahy, R. T. (2000). Regulation of gamma-glutamylcysteine synthetase subunit gene expression: Insights into transcriptional control of antioxidant defenses. *Free Radic. Res., 32*(4), 281–301.