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Protective effect of extracts of *Perilla frutescens* treated with sucrose on *tert*-butyl hydroperoxide-induced oxidative hepatotoxicity *in vitro* and *in vivo*

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ABSTRACT

Perilla frutescens leaves are often used in East Asian gourmet food. In this study, we investigated the hepatoprotective effects of *P. frutescens* leaves grown in different concentrations of sucrose (0, 115, 175 and 235 mM sucrose) leading to four samples of perilla leaf extracts (PLEs). Based on caffeic acid level and antioxidant activities, further experiments were conducted using perilla leaf extracts treated with 6% sucrose compared with non-treated perilla leaf extracts as a control. Oral intubation with non-treated perilla leaf extracts or perilla leaf extracts treated with 6% sucrose (1000 mg/kg b.w. rat) for 5 days was conducted before treatment with a single dose of *tert*-butyl hydroperoxide (0.5 mmol/kg b.w., i.p.) led to a significant reduction of hepatic toxicity in the perilla leaf extracts treated with 6% sucrose. We demonstrated that *P. frutescens* with higher contents of caffeic acid was produced, and that sucrose could play a role in the induction of this secondary metabolite. Sucrose-treated perilla leaves, which had better antioxidant activities than untreated leaves, can be used as a potential dietary source.

1. Introduction

Perilla frutescens (L.) Britt. *var. japonica* (Hassk.) Harais is an annual herbaceous plant native to Southeast Asian countries. The leaves of this plant are often used in sushi, garnish, soups, and to wrap and eat cooked food. Our previous *in vivo* study showed that aqueous perilla leaf extract (PLE) had antioxidant activities against *t*-butyl hydroperoxide-induced oxidative hepatotoxicity (Kim et al., 2007). In another study, our group demonstrated that caffeic acid (CA) isolated from aqueous PLE caused a significant increase in intracellular γ-glutamylcysteine synthetase (γ-GCS) activity in hepatocytes, and that this elevation appears to result in *de novo* elevation of the levels of the endogenous antioxidant, glutathione (GSH) content (Park et al., 2010). CA is a dietary non-flavonoid phenolic compound that is naturally occurring in a large number of vegetables and medicinal herbs (Marques & Farah, 2009). This phytochemical also possesses antioxidant, immunomodulatory, carcinogenic, and anti-inflammatory activities (Fesen et al., 1994; Johnson, Marchand, & Pommier, 2004; Nardini, Natella, Gentili, DiFelice, & Scaccini, 1997).

It was recently reported that sucrose treatment increased secondary phenylpropanoid metabolites, such as anthocyanins in gravine cells and Arabidopsis seed cultures (Ferri, Righetti, & Tassoni, 2011; Kwon et al., 2011). It has long been known that, in addition to providing energy and serving as a structural unit, sucrose also generates physiological signals that regulate the expression of genes involved in both primary and secondary metabolism in plants (Rolland, Moore, & Sheen, 2002; Smeekens, 2000). Moreover, the synthesis of rosmarinic acid (RA), an ester of CA found in many medicinal plants including rosmary, mint and perilla, has been shown to be upregulated by elevation of sucrose levels from 1% to 5% (Gertlowski & Petersen, 1993; Petersen, 1991). As CA is a compound produced by the common phenylpropanoid pathway (Croteau, Kutchan, & Lewis, 2000), we proposed that CA biosynthesis in the perilla leaf could be also promoted by increasing the sucrose concentration during cultivation.

In this study, we report for the first time, a method of inducing biosyntheses of CA in *P. frutescens* by treatment with different sucrose levels. These findings indicate that sucrose concentrations can play a role in the induction of CA. Additionally, we investigated the sucrose-treated PLE to determine if it had enhanced effects on liver injury induced by *tert*-butyl hydroperoxide (*t*-BHP) when

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compared untreated PLE. Various biochemical measurements such as antioxidant parameters, including free radical-scavenging activity, lipid peroxidation and GSH status, were also assessed as an index of oxidative stress.

2. Materials and methods

2.1. Materials

Minimum Essential Medium Eagle cell culture media, penicillin streptomycin, trypsin–EDTA and bovine serum albumin were purchased from GIBCO (Grand Island, NY). 3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), trifluoroacetic acid (TFA), *tert*-butyl hydroperoxide, GSH, L-buthioninesulfoximine, 2,2-diphenyl-1-picrylhydrazyl (DPPH·), Folin and Ciocalteu's phenol reagent, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) diammonium salt, potassium persulphate, *N*-ethylmaleimide (NEM), 2,4-dinitrofluorobenzene (FDNB), iodoacetic acid (IAA), trifluoroacetic acid (TFA), caffeic acid and rosmarinic acid were purchased from Sigma Chemical (St. Louis, MO). Kits for the measurement of lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were purchased from Siemens (New York, NY). XTerra RP_{18} HPLC column was purchased from Waters (Milford, MA) and HPLC-grade reagents were purchased from American Burdick & Jackson (Muskegon, MI). Bicinchoninic acid (BCA) protein assay kit was purchased from Thermo Scientific (Rockford, IL).

2.2. Plant material and preparation of extract

P. frutescens was grown in a greenhouse facility with an area of approximately 330 m^2 at the Miryang City Agricultural Technology Center (Miryang, Korea). The perilla plants were cultivated, and treated with different levels of sucrose. Specifically, the plants were divided into the following groups: non-sucrose, 115 mM (4%), 175 mM (6%) and 235 mM (8%) sucrose. To apply the treatments, 800 l of water containing different sucrose concentrations were applied twice a week via irrigation. After 7 days, the fresh leaves of perilla were collected, washed, and immediately dried at −70 °C. The dried *perilla* leaves were then ground, and soaked in distilled water (30 g/l), after which they were subjected to refluxing at 100 °C for 3 h to give the following four aqueous PLEs based on sucrose treatments: PLE-I (no sucrose), PLE-II (115 mM sucrose), PLE-III (175 mM sucrose) and PLE-IV (235 mM sucrose). Undissolved residues were removed by filtration through Whatman 42 filter paper (Clifton, NJ, USA), followed by filtration through a 0.45 μm membrane filter (Millipore, Billerica, MA, USA). The filtrate was then freeze-dried.

2.3. Quantification of caffeic acid and rosmarinic acid

A high performance liquid chromatography system (HPLC) (Varian Prostar 210, Santa Clara, CA, USA) was used for analysis of the caffeic acid and rosmarinic acid. Analyses were conducted using an XTerra RP_{18} column (150 mm × 3.9 mm, 5 μm diameter particle size, Waters, Milford, MA, USA), with a mobile phase composed of 0.05% TFA buffer and methanol. The caffeic acid and rosmarinic acid measurements were analyzed using gradient elution with a gradient profile composed of 0% methanol at the initial stage, followed by a linear increase to 100% methanol over 40 min. A constant flow rate of 1.0 ml/min was applied, and the spectrum was detected at 340 nm using a UV detector.

2.4. Antioxidant activity measurements

Total polyphenol (TP) was measured by the modified Folin–Denis method (Appel, Govenor, D'Ascenzo, Siska, & Schultz, 2001). A reaction mixture consisting of 100 μl of PLE, 0.25 ml of Folin–Ciocalteu reagent, 0.4 ml of D.W. and 1.25 ml of 20% Na_2CO_3 was prepared and shaken vigorously. After incubation for 40 min at 37 °C, the absorbance was measured at 725 nm.

The total flavonoid (TF) content was measured using a previously described method (Maksimovic, Malencic, & Kovacevic, 2005), in which 100 μl of PLE and 100 μl of 2% $AlCl_3{\cdot}6H_2O$ were incubated for 5 min at 37 °C, and the absorbance was measured at 430 nm. The FRAP value was measured using a modified version of the method described by Bezie and Strain (Wang, Zhang, & Yang, 2005). FRAP reagent was produced by mixing 2.5 ml of 10 mM 2,4,6-triptyridyl-s-triazine (TPTZ) solution in 40 mM HCl, 2.5 ml of 120 mM $FeCl_3{\cdot}6H_2O$ and 25 ml of 0.3 M acetate buffer (pH 3.6). Next, 100 μl of extract was added to 3 ml of working FRAP regent, after which the samples were incubated for 5 min, at which time the absorbance was recorded at 593 nm.

$DPPH^{\cdot}$ was dissolved in ethanol at a final concentration of 200 μM, after which 100 μl of PLE was mixed with 100 μl of DPPH regent. After incubation in the dark for 30 min, the absorbance was measured at 515 nm. The ABTS radical scavenging activity was then measured using a modified version of the method described by Re et al. (1999). Briefly, ABTS was dissolved in distilled water to concentration of 7 mM, after which the radical cation, $ABTS^{\cdot+}$, was produced by reacting ABTS solution with 2.45 mM potassium persulphate. The solution was then allowed to stand overnight in the dark at room temperature, after which it was diluted with ethanol to an absorbance of 1.40 ± 0.02 at 750 nm. Finally, 20 μl of PLE were added to 160 μl of $ABTS^{\cdot+}$ solution, after which the mixture was incubated in the dark for 6 min and the absorbance was measured at 750 nm.

2.5. Cell culture

Human hepatoma HepG2 cells were obtained from ATCC (Rockville, MD, USA) and cultured in MEM medium containing 10% (v/v) FBS, 2.2 g/l sodium bicarbonate, 100 U/ml penicillin and 100 U/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. HepG2 were split 1:5 every 5 days, after which they were seeded at a density of 1×10^6 cells/well in 6-well plates to measure the GSH content and TBAR assay. In addition, the cells were seeded at a density of 1.5×10^5 cells/well in a 24-well plate to measure the cytotoxicity. Cells were routinely grown in MEM medium containing 10% FBS, but the medium was changed to serum-free medium 20 h before the assay.

2.6. Cell viability assay

After attachment, the cells were washed twice with PBS and incubated with PLE-I, PLE-III and *t*-BHP for 2 h. The medium was then removed, after which 5 mg/ml of MTT in a MEM medium was added and the samples were incubated for 4 h at 37 °C. The medium was removed by aspiration, after which 200 μl of DMSO was added to each well to dissolve the crystal. Finally, the optical density at a wavelength of 540 nm was measured using a multiplate reader.

2.7. Rats and treatment

Male Sprague–Dawley rats, 7-weeks-old, were purchased from Samtako Bio Korea Co. (Gyeonggi, Korea) and were housed with free access to a standard rodent diet (Samyang Feed Co., Ltd., Incheon, Korea) and tap water. The experimental animals were

treated in compliance with the Guide for the Care and Use of Laboratory Animals (Committee on Care and Use of Laboratory Animals, 1985). This experimental protocol was reviewed and approved by the Korea University Animal Care Committee (No. KUIACUC-20100319-2).

The rats were divided into the following four groups, which each contained six rats. To investigate the hepato-protective activity against *t*-BHP-induced oxidative stress, 1000 mg/kg of PLE-I or PLE-III was administered to the animals daily by gavage, while the other groups received oral dosing by vehicle only. On day 5, 0.5 mmol/kg of *t*-BHP was injected (i.p.) into each animal, and 18 h later the rats were sacrificed. Blood samples were collected from the vena cava for the ALT, AST and LDH assays. After collecting the bloods, the livers were removed, rinsed with PBS, and stored at −80 °C for later biochemical analysis.

2.8. GSH assay and lipid peroxidation assay

The GSH content of the HepG2 and rat liver tissues was determined using a modified version of the method reported by Reed et al. (1980). Intracellular lipid peroxidation was assayed using a modified thiobarbituric acid fluorometric method (Lee, Chiang, Chen, & Hsu, 2000). The protein concentration was measured using a BCA protein assay kit with BSA as the standard.

2.9. Pathological histology

After the rat liver tissues were removed, they were fixed in formaldehyde, processed for histological evaluation according to conventional methods, and stained with hematoxylin & eosin (H&E).

2.10. Statistical analysis

Statistical analyses were conducted using the Statistical package for Social Science (SPSS) version 12.0 (SPSS Inc., IL, USA). Differences among groups were evaluated by one-way analysis of variance (ANOVA) and Duncan's multiple-range tests. Values were expressed as means ± standard deviation (SD).

3. Results

3.1. Antioxidant activities and levels of flavonoid and polyphenolic compounds of PLEs

The antioxidant effects of aqueous extract of *P. frutescens* leaves (PLE) from plants subjected to treatment with varying sucrose levels during cultivation (PLE-I, PLE-II, PLE-III, PLE-IV; 0, 115 mM, 175 mM, and 235 mM sucrose, respectively) were evaluated based on the FRAP value, DPPH-SC_{50} value and ABTS-IC_{50} value, as well as the levels of antioxidants (Table 1). Furthermore, flavonoid and polyphenolic compound contents, which related to protective effect on *t*-BHP-induced oxidative stress, were dependently enhanced with sucrose-concentration during cultivation. PLE-III antioxidant activities (FRAP value, DPPH-SC_{50}, ABTS-IC_{50}) are similar to PLE-IV. However, PLE-III has higher amounts of flavonoid and polyphenol compounds, including caffeic acid and rosmarinic acid, than PLE-IV. To effectively visualize and analyze these results, the measurements obtained from each PLE were displayed together on a radar chart (Fig. 1). In this chart, each value is presented on a 0–100 axis and connected to each other. The blackened area depicts the relative antioxidant activities of the various PLEs, and the order of the integrated area was as follows: PLE-III > PLE-IV > PLE-II > PLE-1. Based on these findings, we next compared the effects of PLE-III to those of PLE-I (non-sucrose- treated control) on *in vitro* and *in vivo* hepato-protective activities.

3.2. Effects of PLE-I and PLE-III on in vitro t-BHP induced cytotoxicity

HepG2 cells were treated with PLE-I and PLE-III for 2 h. In the presence of PLE-I or PLE-III at concentrations ranging from 0 to 1000 μg/ml, the cell viability was greater than 95% (data not shown). The oxidative stress induced by 0.3 mM *t*-BHP reduced the viability to 24% after 2 h of incubation, but treatment of the cells with 1000 μg/ml PLE-I or 1000 μg/ml PLE-III resulted in 43% and 52% cell viability, respectively (Fig. 2). Furthermore, the recovery of cells treated with PLE-III was significantly higher than that of cells treated with PLE-I.

3.3. Effects of PLE-I and PLE-III on lipid peroxidation and GSH level in HepG2

MDA is used as the index of lipid peroxidation, and GSH plays a hepato-protective role against *t*-BHP-induced oxidative stress. As shown in Fig. 3A, MDA formation increased by about 3.8-fold in HepG2 cells treated with 0.3 mM *t*-BHP when compared to the control group. In addition, treatment with PLE-I and PLE-III led to a significant reduction in the amount of MDA by 2.3- and 2.9-fold, respectively, when compared with *t*-BHP alone, indicating that PLE-III was more significantly effective than PLE-I for the prevention of lipid peroxidation.

Treatment of the cells with 0.3 mM of *t*-BHP also reduced the GSH level by 0.3-fold when compared with the control group (Fig. 3B). However, the GSH levels of the PLE-I and PLE-III treatment groups increased by 1.6- and 1.8-fold, respectively, when compared to the group treated with *t*-BHP. Furthermore, the GSH level in the PLE-III treated group was significantly higher than that in the PLE-I group.

3.4. Effects of PLE-I and PLE-III on t-BHP-induced oxidative stress in rat liver

The levels of AST and ALT were used as biomarkers of hepatic damage. Treatment with *t*-BHP is known to cause LDH to leak from

Table 1
Contents of total polyphenol (TP), total flavonoids (TF), caffeic acid and rosmarinic acid, ferric-reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) SC_{50}, and 2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS)-IC_{50} of water extracts from various *Perilla frutescens* leaf aqueous extracts (PLEs).[a]

	Total polyphenol (μg GAE/mg DM)	Total flavonoids (μg quercetin/mg DM)	Caffeic acid (mg/100 g fresh wt.eq.)	Rosmarinic acid (mg/100 g fresh wt.eq.)	FRAP (mM $FeSO_4$/mg DM)	DPPH-SC_{50} (μg DM/ml)[b]	ABTS-IC_{50} (μg DM/ml)[b]
PLE-I[c]	39.6 ± 1.34[a]	13.1 ± 2.04[a]	2.9 ± 0.04[a]	5.9 ± 0.18[a]	0.4 ± 0.01[a]	739.7 ± 62.84[a]	601.1 ± 31.06[a]
PLE-II	46.2 ± 2.78[b]	16.1 ± 4.83[a]	3.5 ± 0.05[b]	15.1 ± 0.03[b]	0.7 ± 0.01[b]	225.9 ± 2.11[b]	415.1 ± 30.29[b]
PLE-III	70.9 ± 1.07[b]	26.5 ± 0.87[b]	4.8 ± 0.05[b]	28.5 ± 0.66[b]	1.1 ± 0.02[b]	306.4 ± 0.28[b]	363.6 ± 19.74[b]
PLE-IV	59.0 ± 1.21[c]	35.1 ± 1.18[b]	3.8 ± 0.02[c]	21.1 ± 0.11[c]	1.1 ± 0.02[b]	207.5 ± 13.00[c]	491.1 ± 23.38[c]

[a] Each value is the SD of three replicate experiments. DM, dry matter; GAE, gallic acid equivalents.
[b] Amounts of sample necessary to decrease the initial DPPH concentration and ABTS concentration by 50%, respectively.
[c] PLE-I, untreated; PLE-II, treated with 115 mM sucrose; PLE-III treated with 175 mM sucrose; PLE-IV treated with 235 mM sucrose. Means with different superscript letters are significantly different at $p < 0.05$ as determined by Duncan's multiple range tests.

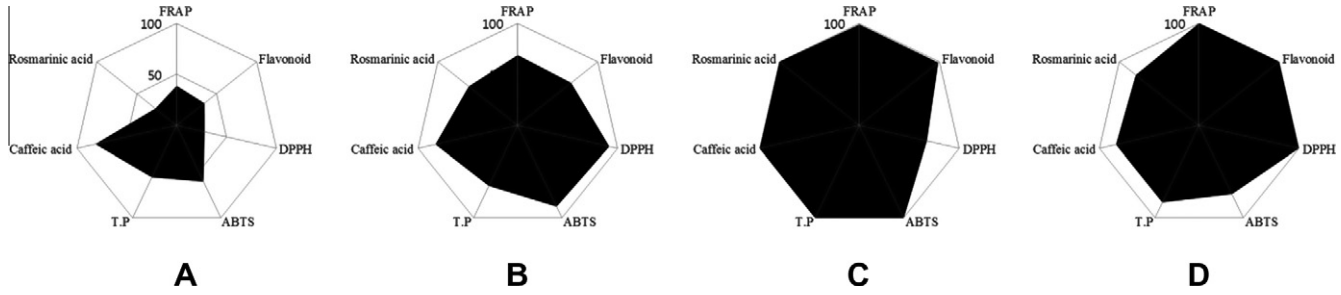

Fig. 1. Radar charts demonstrating characteristic features of various aqueous *Perilla frutescens* leaf extracts (PLEs) based on the polyphenol compounds and antioxidant activities. Contents of total polyphenol (TP), total flavonoid (TF), caffeic acid and rosmarinic acid, ferric-reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS)) assays were conducted as described in Section 2. The percentage scales of the rader charts are as follows: % scale = value of each sample/highest value of each assay × 100. (A) PLE-I, untreated; (B) PLE-II, treated with 115 mM sucrose; (C) PLE-III, treated with 175 mM sucrose; (D) PLE-IV, treated with 235 mM sucrose.

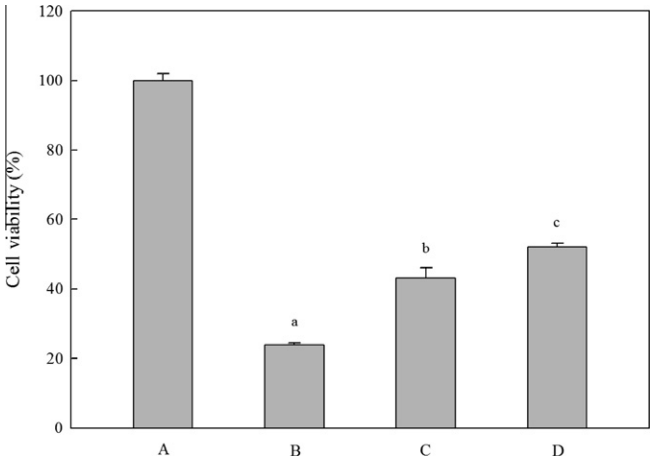

Fig. 2. Effects of PLE-I and PLE-III on *tert*-butyl hydroperoxide (*t*-BHP)-induced oxidative hepatotoxicity in HepG2 cells. A, negative control; B, *t*-BHP (0.3 mM); C, *t*-BHP with PLE-I (no sucrose treatment); D, *t*-BHP with PLE-III (175 mM sucrose treatment). Values are expressed as means ± SD ($n = 3$). Means with different small letters are significantly different at $p < 0.05$ as determined by Duncan's multiple range tests.

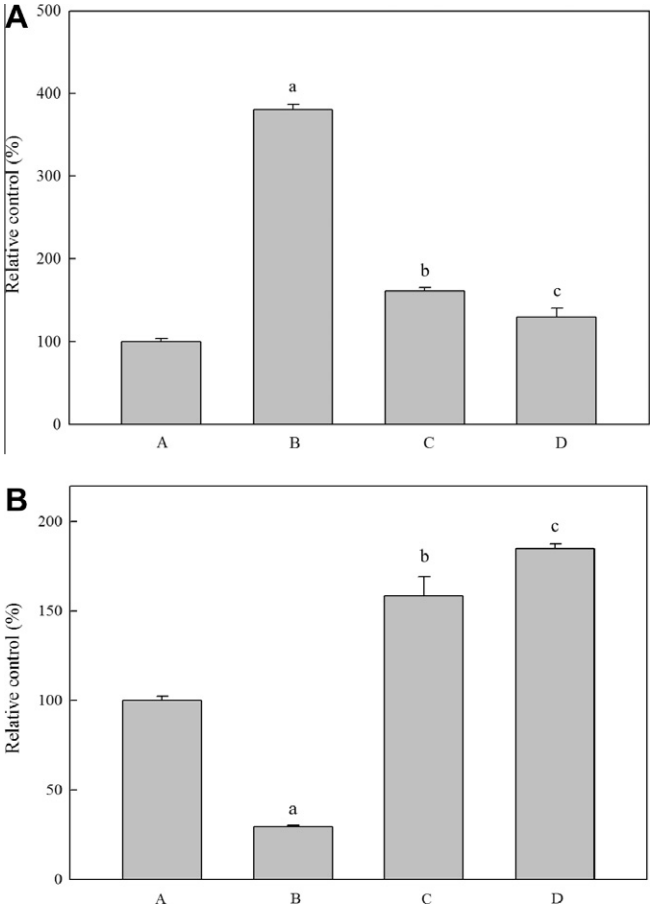

Fig. 3. Effects of PLE-I and PLE-III on the formation of *t*-BHP-induced lipid peroxidation (A) and glutathione level (B) in HepG2 cells. A, negative control; B, *t*-BHP (0.3 mM); C, *t*-BHP with PLE-I (no sucrose treatment); D, *t*-BHP with PLE-III (175 mM sucrose treatment). Values are expressed as means ± SD ($n = 3$). Means with different small letters are significantly different at $p < 0.05$ as determined by Duncan's multiple range tests.

the liver. In the present study, a single dose of *t*-BHP (0.5 mmol/kg b.w., i.p.) led to a remarkable increase in serum AST, ALT and LDH (Table 2). However, pretreatment with PLE-I or PLE-III led to a significant reduction in the serum AST, ALT, and LDH levels, with full recoveries to normal values occurring in response to the PLE-III treatment.

The level of MDA and GSH were similar to those observed in a previously conducted *in vitro* study. Only the group treated with 1000 μg/ml *t*-BHP group showed a significantly increased amount of MDA (2.6-fold) when compared to the control group. However, the groups treated with both 1000 mg/kg of PLE-I or PLE-III and *t*-BHP showed significantly reductions of 1.3- and 1.7-fold in the level of MDA, respectively, when compared with the *t*-BHP group. Interestingly, the reduction in the GSH level in the group pretreated with 1000 mg/kg of PLE-III was even higher than the changes in the GSH levels in the PLE-I treated group and the untreated control ($p < 0.05$). Taken together, these findings confirmed that PLE-III had a hepato-preventative effect against oxidative stress in the liver of rats treated with *t*-BHP (Table 2).

3.5. Histopathology of the liver

Treatment with *t*-BHP causes confluent necrosis associated with neutrophilic infiltration, which is observed in the case of swelling of the liver cells. Histopathological studies showed that *t*-BHP induced hepatocyte degeneration (asterisk) and neutrophilic infiltration (arrow) (Fig. 4B). However, microscopic examinations showed that the severe lesions caused by *t*-BHP were considerably reduced by the administration of PLE-I or PLE-III.

4. Discussion

It has been established that functional foods containing physiologically active compounds from plants may improve health and

Table 2
Effects of PLEs on serum enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), hepatic malondialdehyde (MDA) and glutathione (GSH) levels in rats treated with *t*-BHP.

Treatment	AST (IU/L)	ALT (IU/L)	LDH (U/L)	MDA (nmol/mg protein)	GSH (nmol/mg protein)
Control	135.4 ± 8.11[a]	31.3 ± 4.68[a]	2527.6 ± 378.56[a]	256.9 ± 10.99[a]	155.6 ± 8.95[a]
t-BHP (0.5 mmol/kg)	304.0 ± 52.89[b]	60.7 ± 6.11[b]	3945.0 ± 538.82[b]	671.4 ± 31.63[b]	100.7 ± 3.98[b]
PLE-I[a]	184.7 ± 19.86[c]	36.0 ± 4.00[a]	3540.3 ± 891.72[ab]	491.3 ± 6.46[c]	266.3 ± 22.89[c]
PLE-III	140.0 ± 24.00[a]	37.7 ± 7.57[a]	1933.7 ± 655.38[a]	382.5 ± 12.69[d]	286.1 ± 30.55[c]

[a] Animals were pretreated with PLE-I or PLE-III (1000 mg/kg) by gastric tube for 5 consecutive days before administration of *t*-BHP. The rats were sacrificed 18 h later, after which the serum AST, ALT, LDH, and hepatic MDA and GSH levels were determined. Means with different superscript letters are significantly different at $p < 0.05$ as determined by Duncan's multiple range tests.

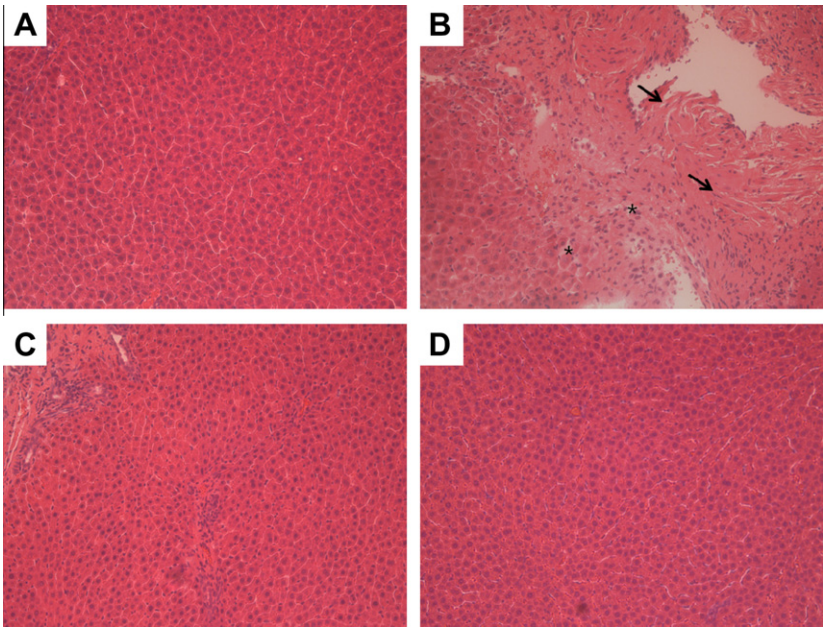

Fig. 4. Effects of PLE-I and PLE-III pretreatment on *t*-BHP induced liver damage. Rats were pretreated with PLE-I or PLE-III (1000 mg/kg) once a day by gavage for 5 consecutive days. The control rats were given saline. Three hours after the final treatment, the rats were treated with *t*-BHP (0.5 mmol/kg, i.p.). A, control ($n = 6$); B, rats ($n = 6$) treated with *t*-BHP (0.5 mmol/kg) showed liver cell death (arrow) and several areas of ballooning degeneration of hepatocytes (asterisk); C, rats ($n = 6$) pretreated with 1000 mg/kg of PLE-I (no sucrose treatment), and then *t*-BHP; D, rats pretreated with 1000 mg/kg of PLE-III (no sucrose treatment), and then *t*-BHP. Hematoxylia/eosin staining; magnification 200×.

longevity. The food industry appears to expect that consumers will welcome foods that emphasize the positive benefits of newly added components. Thus, new food products are being developed with beneficial active components. With the continuously growing acceptance of functional foods, scientists have searched for specific substances or combinations of substances that have been shown to reduce the risk of disease.

In this regard, our group has investigated the antioxidant and hepato-protective activities of *P. frutescens* (Kim et al., 2007; Park et al., 2010), and shown that a natural herbal phytochemical, CA, present in *perilla* leaves, is responsible for increasing hepatic GSH content by enhancing its *de novo* synthesis (Park et al., 2010). Therefore, developing methods of increasing the CA levels in this plant might provide a candidate material for development of a functional food, making supplementation with CA more available for general public consumption. CA is derived from phenylpropanoid metabolism providing plant phenolic compounds (Croteau et al., 2000). It has been suggested that secondary metabolites can be strongly influenced by the sugar content of the medium (Zenk, Elshagi, & Ulbrich, 1977). Moreover, a recent report demonstrated that sugar dose-dependently increased the levels of flavonoids in *Melissa officinalis* (Hossain, Kim, Kim, Lee, & Lee, 2009). Accumulation of sugar-induced anthocyanin has also been reported in various plants. Furthermore, sugars act as signaling molecules, activating the PAP1 gene by means of a sucrose-signaling pathway. Sucrose increases mRNA level of PAP1 and other anthocyanin biosynthetic genes and triggers increased anthocyanin synthesis (Nagira, Ikegami, Koshiba, & Ozeki, 2006; Nagira & Ozeki, 2004; Solfanelli, Poggi, Loreti, Alpi, & Perata, 2006). In the present study, *perilla* treated with sucrose showed increased levels of CA and RA (Table 1 and Fig. 1). These enhanced levels of CA and RA production could have been due to changes in the surrounding environment such as osmolarity and disturbed cellular metabolism (Ferri et al., 2011).

t-BHP is a well-known oxidant that is often used as a model to investigate the mechanism of cell damage initiated by acute oxidative stress (Rush et al., 1985). Two kinds of pathways are involved in the metabolism of *t*-BHP in hepatotoxicity. The first mechanism is microsomal cytochrome P-450 system leading to the production of reactive oxygen species (ROS) that initiate lipid peroxidation (Hogberg, Orrenius, & Obrien, 1975). The second concerns a reaction involving GSH peroxidase and GSH, which converts glutathione disulfide (GSSG). Due to the NADPH oxidation mechanism, the glutathione disulphide (GSSG) which oxidized form of GSH is reduced to GSH by GSH reductase. Decreased GSH and oxidized NADPH contribute to altered Ca^{2+} homeostasis, which is considered to be a major event in *t*-BHP-induced hepatotoxicity. Moreover, the model of *t*-BHP induced acute oxidative stress in

animals has been used in several studies (Hwang, Choi, & Jeong, 2009; Liu, Wang, Chu, Cheng, & Tseng, 2002). In the present study, *t*-BHP led to a dose-dependent decrease in the viability of HepG2 (data not shown). Treatment with non-cytotoxic concentrations (1000 μg/ml) of PLEs increased the viability of HepG2 exposed to *t*-BHP to induce oxidative damage, suggesting that they exerted a hepatic protective effect (Fig. 2). Based on the results of an *in vivo* study, although there were no significant differences between organ weights of samples treated with *t*-BHP in the presence of PLEs (data not shown), there were significant differences in the relative ALT and AST levels in these groups (Table 2). We further studied the hepatic protective effect using several other biomarkers of oxidative stress such as lipid peroxidation and GSH levels *in vitro* and *in vivo*. GSH is known to play a protective role that occurs via *t*-BHP-induced oxidative stress (Joyeux, Rolland, Fleurentin, Mortier, & Dorfman, 1990). *t*-BHP can react with GSH to increase ROS formation, which leads to lipid peroxidation. A previous study showed that *t*-BHP caused an increase in lipid peroxidation and decrease in GSH level in rats (Chou, Ko, & Yang, 2000; Duh, Wang, Liou, & Lin, 2010; Hwang et al., 2008; Kim et al., 2007; Vidyashankar, Mitra, & Nandakumar, 2010). Pretreatment of PLEs led to a significant increase in the intracellular GSH level in HepG2. Moreover, the results of the *in vivo* study showed that *t*-BHP reduced the GSH level, but that PLEs recovered the decrease. Furthermore, the GSH levels of the cells and tissues pretreated with PLEs were even higher than the non-treated group. Interestingly, our subsequent preliminary experiment showed that the sucrose-treated PLE-III also had a similar protective effect against *t*-BHP-induced oxidative hepatotoxicity in a different human hepatic stellate cell line, LX-2 (data not shown). Previously, GSH was found to be related to the induction of γ-GCS enzyme activity, and the results of our study showed that γ-GCS activity was increased by PLE treatment (Huber et al., 2002; Huber et al., 2003). Our group also isolated caffeic acid from PLE and found that it played roles in the enhancement of γ-GCS synthetase activity and GSH level (Park et al., 2010). Therefore, the increased concentration of CA in *perilla* in response to the addition of sucrose might play a role in the increased GSH level. Previous studies have shown that polyphenol compounds from plants increased the γ-GCS activity and GSH contents (Chen, Yang, Jiao, & Zhao, 2004; Jeon et al., 2003; Ramirez-Mares & de Mejia, 2003). These subunits regulated the GSH synthetase expression (Huang, Yang, Chen, Zeng, & Lu, 2000), and the promoter region of the heavy subunit of γ-GCS (γ-GCS-HS) has been shown to be controlled by the activator protein-1 (AP-1) transcription factor binding site (Rahman & MacNee, 2000; Urata et al., 1999). However, no studies have yet been conducted to determine if CA can increase γ-GCS mRNA expression and activate the AP-1 DNA binding site.

In our *in vitro* study, no cytotoxicity of PLEs against HepG2 cells was observed at doses up to 3000 μg/ml. Similarly, administration (rat: 1000 mg/kg b.w.) of PLEs did not show any sign of toxicity, and showed a hepatoprotective effect in our *in vivo* study. According to Reagan-Shaw, Nihal, and Ahmad (2008), the Food and Drug Administration has suggested that the extrapolation of animal dose to human dose is properly done only through normalization to body surface area, which corresponds to in mg/m^2. According to its recommended calculation, we converted the dose in a rat to a dose based on surface area for humans, multiply 1000 mg/kg (PLEs dose) by K_m factor (b.w. (kg)) divided by body surface area (m^2)) (6) for a rat, and then divided by the K_m factor (37) for a human. This conversion leads to human equivalent dose for PLEs of 162 mg/kg b.w., which equates to 9.7 g of PLEs for a 60 kg person (or about 205 g of perilla fresh leaves in 60 kg b.w. adult). While this may not be practically achievable through consumption of perilla fresh leaves, this level can be provided through a daily oral supplement. On the other hand, regarding the toxicity of CA, this compound is still listed under an older hazard data sheet as a potential carcinogen 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 in rats (U.S. National Library of Medicine). However, considering that a rat consumes a daily food intake of 30 g with 1% caffeic acid (300 mg of CA per day) in the diet it would correspond to a daily oral ingestion of 4.8 kg of perilla fresh leaves (or approximately 2100 fresh leaf sheets of perilla per day) on a wet basis, suggesting that the daily intake of perilla leaves would become unphysiologically and excessively high to cause carcinogenic effects in some animal studies. 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-oxde (Tanaka et al., 1993).

In summary, we demonstrated for the first time that proper treatment of *P. frutescens* with sucrose led to increased levels of CA, conferring better protection from hepatotoxicity *in vitro* and *in vivo* when compared with untreated control perilla. These findings indicate that these leaves can be used as a candidate health dietary source.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2012.01.037.

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