BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 24(3), 338-345 (2016)

The Neuro-Protective Effect of the Methanolic Extract of *Perilla frutescens* var. *japonica* and Rosmarinic Acid against H_2O_2-Induced Oxidative Stress in C6 Glial Cells

Ah Young Lee[1,†], Ting Ting Wu[1,†], Bo Ra Hwang[1], Jaemin Lee[2], Myoung-Hee Lee[3], Sanghyun Lee[2,*] and Eun Ju Cho[1,*]

[1]Department of Food Science and Nutrition& Kimchi Research Institute, Pusan National University, Busan 46241, [2]Department of Integrative Plant Science, Chung-Ang University, Anseong 17546, [3]Department of Southern Area Crop Science, National Institute of Crop Science, Rural Development Administration, Miryang 50424, Republic of Korea

Abstract

Neurodegenerative diseases are often associated with oxidative damage in neuronal cells. This study was conducted to investigate the neuro-protective effect of methanolic (MeOH) extract of *Perilla frutescens* var. *japonica* and its one of the major compounds, rosmarinic acid, under oxidative stress induced by hydrogen peroxide (H_2O_2) in C6 glial cells. Exposure of C6 glial cells to H_2O_2 enhanced oxidative damage as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and thiobarbituric acid-reactive substance assays. The MeOH extract and rosmarinic acid prevented oxidative stress by increasing cell viability and inhibiting cellular lipid peroxidation. In addition, the MeOH extract and rosmarinic acid reduced H_2O_2-induced expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) at the transcriptional level. Moreover, iNOS and COX-2 protein expression was down-regulated in H_2O_2-indcued C6 glial cells treated with the MeOH extract and rosmarinic acid. These findings suggest that *P. frutescens* var. *japonica* and rosmarinic acid could prevent the progression of neurodegenerative diseases through attenuation of neuronal oxidative stress.

Key Words: *Perilla frutescens* var. *japonica*, Rosmarinic acid, Oxidative stress, Hydrogen peroxide, C6 glial cell

INTRODUCTION

Overproduction of reactive oxygen species (ROS) damages tissue and leads to oxidative stress through lipid peroxidation, protein cross-linking, and DNA cleavage, thereby disrupting cellular function (Gorman *et al*., 1996). ROS are constantly produced and play a key role in the pathogenesis of a wide variety of acute and chronic neurodegenerative diseases. Hydrogen peroxide (H_2O_2) is one of the major ROS and excessive production is associated with pathological process of acute and chronic neuronal toxicity. Previous reports indicated that H_2O_2 is a weak oxidant, but it can be converted to a highly reactive toxic hydroxyl radical. In addition, overgeneration of nitric oxide (NO) acts as neurotoxic effector in the central nervous system, resulting in neurodegeneration (Guix *et al*., 2005). Activation of glial cells by H_2O_2 leads to the up-regulation of inflammatory mediators, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression, and cytokine release, which cause neuronal apoptosis (Minagar *et al*., 2002).

COX is responsible for the conversion of arachidonic acid to prostaglandins. There are two COX isoforms, which are known to COX-1 and COX-2. COX-1 is constitutively expressed in most tissue types and is generally regarded as a "housekeeping enzyme". In contrast, COX-2 expression is induced by cytokines, growth factors, and hormones (Funk *et al*., 1991; Masferrer *et al*., 1994; Smith *et al*., 1996). NOS is expressed in a variety of cells under both normal and pathological conditions and produces high levels of NO (Brown, 2007). Thus, inhibiting the activity or expression of COX-2 and/or iNOS might be a promising method for prevention of degenerative diseases and inflammation. Moreover, Zhang *et*

Open Access **http://dx.doi.org/10.4062/biomolther.2015.135**

Received Aug 25, 2015 **Revised** Jan 23, 2016 **Accepted** Feb 19, 2016
Published Online May 1, 2016

***Corresponding Authors**
E-mail: slee@cau.ac.kr (Lee S), ejcho@pusan.ac.kr (Cho EJ)
Tel: +82-31-670-4688 (Lee S), +82-51-510-2837 (Cho EJ)
Fax: +82-31-676-4686 (Lee S), +82-51-510-3648 (Cho EJ)
[†]The first two authors contributed equally to this work.

Table 1. Primers and conditions used in real-time quantitative PCR

Gene	Primer sequence	PCR conditions
iNOS	F: CCT-CCT-CCA-CCC-TAC-CAA-GT	53°C
	R: CAC-CCA-AAG-TGC-CTC-AGT-CA	Cycles: 35
COX-2	F: AAG-ACT-TGC-CAG-GCT-GAA-CT	53°C
	R: CTT-CTG-CAG-TCC-AGG-TTC-AA	Cycles: 35
GAPDH	F: TCA-TGA-AGT-GTG-ACG-TTG-ACA-TCC-GT	60°C
	R: CCT-AGA-AGC-ATT-TGC-GGT-GCA-CGA-TG	Cycles: 35

al. (2006) reported that anti-oxidants can block or delay neuronal apoptosis, and thus, such substances may prevent neurodegenerative disorders.

Glial cells are believed to play a significant role in host defense and tissue repair in the central nervous system. In addition, glial cells support brain function by carrying nutrients to neurons and removing waste (Ullian *et al*., 2001). C6 glial cells are widely used in studies of protective mechanisms against neuronal oxidative stress, because they protect neurons from extracellular oxidants such as H_2O_2 (Dringen *et al*., 2000). Under pathological conditions, glial cells are activated and produce a large number of neuro-active substances, including cytokines and radicals such as NO (Minghetti and Levi, 1998; Hanisch, 2002). However, it has also been reported that pro-inflammatory mediators released by microglia inhibit the progression of neurodegenerative disorders (Eikelenboom and van Gool, 2004). Therefore, production of toxic inflammatory mediators and cytokines by activated glial cells must be regulated.

Diverse natural products, including foods, have been shown to possess biological activities that might protect neurons from oxidative injury, including anti-oxidative and anti-inflammatory effects (Aruoma *et al*., 2003). *Perilla frutescens* var. *japonica*, an annual herb with a distinctive aroma and taste, has been cultivated for centuries in Asia, especially in Korea and Japan. *P. frutescens* var. *japonica* has been widely used as a folk medicine and food, and it possesses several biological activities (Yang *et al*., 2013). In a previous study, methanolic (MeOH) extract of *Perilla* leaves was shown to produce antimutagenic and anti-oxidative effects (Lee *et al*., 1993). Kim *et al*. (2007) suggested that the leaves of *Perilla* have protective effects against oxidative hepatotoxicity induced by *tert*-butyl hydroperoxide. In addition, oral administration of a *Perilla* decoction and its constituents produced anti-allergic activity in mice (Makino *et al*., 2001). Rosmarinic acid (RA) is a polyphenolic phytochemical found in *Perilla* and other medicinal plants, including rosmary and mint (Makino *et al*., 1998; Al-Sereiti *et al*., 1999; Areias *et al*., 2000). Osakabe *et al*. (2002) reported that *Perilla* and RA reduced liver injury induced by lipopolysaccharides and D-galactosamine due to scavenging of superoxides or peroxynitrite.

The present study was conducted to evaluate the neuroprotective effect of *P. frutescens* var. *japonica* and RA against oxidative stress, as well as to explore their molecular mechanisms by investigating mRNA and protein expression related to oxidative stress.

MATERIALS AND METHODS

Plant materials and chemicals

P. frutescens var. *japonica* was obtained from the Department of Functional Crop, National Institute of Crop Science, Rural Development Administration, Miryang, Korea. RA and malondialdehyde (MDA) were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). All other chemicals used were of analytical grade and obtained from Merck (Darmstadt, Germany) or Sigma-Aldrich Co.

Preparation of sample

Freeze-dried *P. frutescens* var. *japonica* was extracted 3 times with 20 volumes of 100% MeOH at room temperature for 24 h. The extract was obtained by a rotary evaporator and the yield was 23.43%. The MeOH extract of *P. frutescens* var. *japonica* (MP) and RA were dissolved in phosphate buffered saline (PBS) and dimethyl sulfoxide (DMSO), respectively.

Cell culture

C6 glial cells were obtained from the KCLB (Korean Cell Line Bank, Seoul, Korea) and maintained at 37°C in a 5% CO_2 incubator. Cells were cultured with DMEM containing 1% penicillin/streptomycin and 10% fetal bovine serum, and subcultured weekly with 0.05% trypsin-EDTA in PBS.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

After confluence had been reached, the cells were plated in 96-well plates at a density of 2×10^3 cells/well, incubated for 2 h, and treated with H_2O_2 (100 μM). After treatment of H_2O_2 for 24 h, the cells were added with MP (5, 25, 50, and 100 μg/mL) and RA (0.5, 2.5, 5, and 10 μg/mL) for 24 h. Cell viability was determined using the MTT assay. MTT solution was added to each well of the 96-well plate, and the plate was incubated for 4 h at 37°C, after which the medium containing the MTT was removed. The incorporated formazan crystals in the viable cells were solubilized with DMSO, and the absorbance of each well was read at 540 nm (Mosmann, 1983).

Thiobarbituric acid-reactive substance (TBARS) levels

Thiobarbituric acid (TBA)-reactive substance levels were determined using the Fraga assay (Fraga *et al*., 1988). After treatment of the cells with MP and RA, 1% TBA (1 mL) and 25% trichloroacetic acid (1 mL) were added, and the mixture was boiled at 95°C for 20 min. The mixture was cooled with ice and extracted with *n*-butanol (*n*-BuOH). After centrifugation at 4,000 rpm for 30 min, the florescence of the *n*-BuOH layer was measured at 532 nm using a fluorescence spectrophotometer (BMG LABTECH, Ortenberg, Germany).

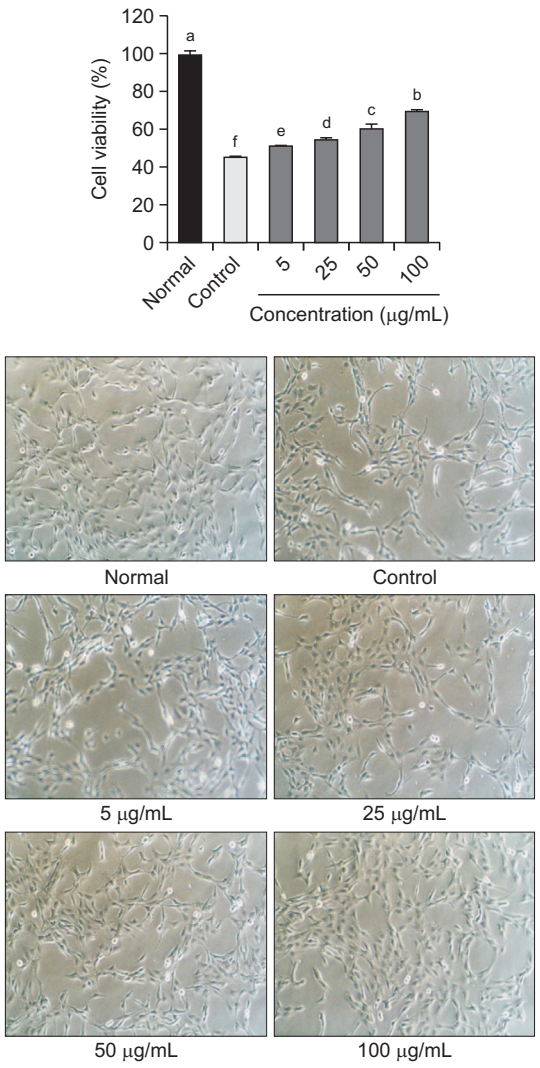

Fig. 1. Effect of MP on C6 glial cell viability after treatment with H_2O_2. Cells were pre-incubated for 24 h in the presence of 100 μM H_2O_2, followed by the addition of MP (5, 25, 50, and 100 μg/mL) for 24 h. Representative morphology of cells exposed to MP under oxidative damage. Values are mean ± SD. [a-f]Means with different letters are significantly different (p<0.05) as determined by Duncan's multiple range test.

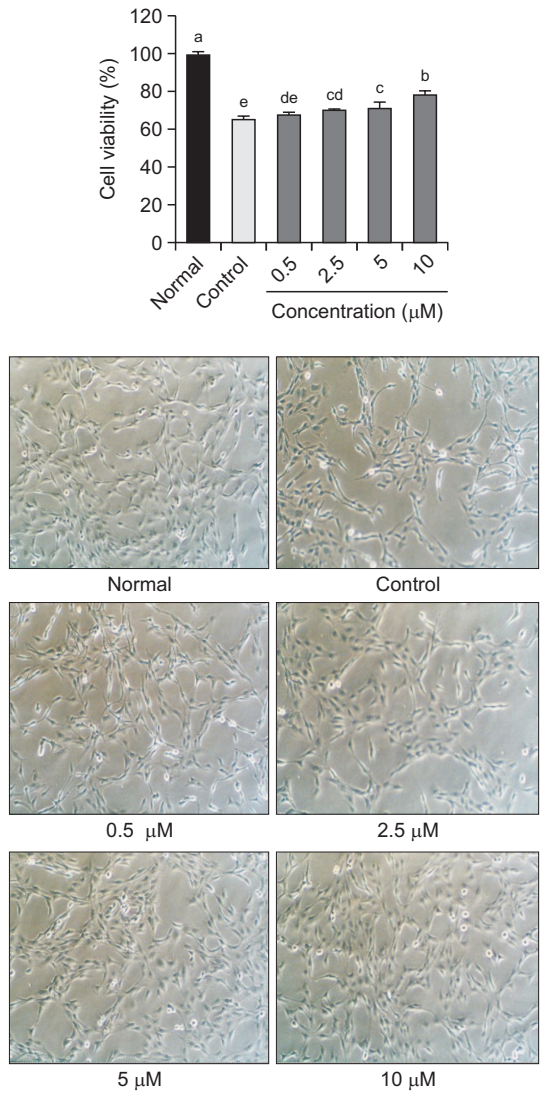

Fig. 2. Effect of RA on C6 glial cells viability after treatment with H_2O_2. Cells were pre-incubated for 24 h in the presence of 100 μM H_2O_2, followed by the addition of RA (0.5, 2.5, 5, and 10 μM) for 24 h. Values are mean ± SD. [a-e]Means with different letters are significantly different (p<0.05) as determined by Duncan's multiple range test.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using a Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Cells were lysed using Trizol reagent and transferred to microfuge tubes. RNA was reverse-transcribed into cDNA and used as a template for RT-PCR amplification. The primers and amplification conditions are listed in Table 1. PCR products were analyzed on 1% agarose gels, and bands were visualized with an LED slider imager (Maestrogen, Las Vegas, NV, USA).

Western blotting

C6 glial cell extracts were prepared according to the manufacturer's instructions using RIPA buffer (Cell Signaling, Danvers, MA, USA) supplemented with 1×protease inhibitor cocktail and 1 mM phenylmethylsulfonyl fluoride. Proteins were separated by electrophoresis in a precast 4-15% Mini-PROTEAN TGX gel (Bio-Rad, Hercules, CA, USA), blocked with 10% skim milk solution for 1 h at 4°C and then blotted onto nitrtocellulose membranes and analyzed with epitope-specific primary and secondary antibodies. Bound antibodies were visualized using enhanced chemiluminesence and an LAS 4000 imaging system (Fuji Film, Tokyo, Japan).

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Significance level was verified by performing Duncan's multiple range test and p-values lower than 0.05 was considered statistically significant using SAS software (version 6.0, SAS

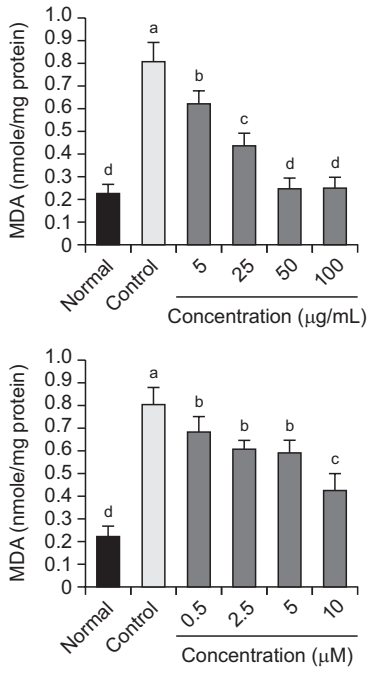

Fig. 3. Effect of MP (A) and RA (B) on TBARS generation in C6 glial cells treated with H_2O_2. Cells were pre-incubated for 24 h in the presence of 100 μM H_2O_2, followed by the addition of MP (5, 25, 50, and 100 μg/mL) and RA (0.5, 2.5, 5, and 10 μM) for 24 h. Values are mean ± SD. [a-d]Means with different letters are significantly different (p<0.05) as determined by Duncan's multiple range test.

Institute, Cary, NC, USA).

RESULTS

Cell viability in H_2O_2-stimulated C6 glial cells

We investigated the effects of MP and RA on cell viability after exposure to H_2O_2. As determined by the MTT assay, the viability of C6 glial cells exposed to H_2O_2 for 24 h was decreased by 45.8% (Fig. 1). However, MP significantly inhibited cell death in a dose-dependent manner, especially it was increased by 70% after treatment 100 μg/mL for 24 h. Treatment with RA also increased cell viability in a concentration-dependent manner (Fig. 2). In particular, cells treated with RA at a concentration of 10 μM showed markedly increased cell viability (78.7%) in comparison with control cells (65.56%).

Lipid peroxidation in H_2O_2-stimulated C6 glial cells

Fig. 3 shows the effect of MP and RA on lipid peroxidation in C6 glial cells stimulated with H_2O_2. MDA values in the control group were 0.81 nmol/mg protein, which was 4 times the level of the untreated group. However, MP dose-dependently suppressed changes in MDA levels induced by H_2O_2. In particular, MDA levels were significantly decreased to 0.25 nmol/mg protein by 50 μg/mL MP. In addition, treatment with RA inhibited lipid peroxidation. Treatment with 10 μM RA inhibited MDA formation by 0.43 nmol/mg protein from 0.81 nmol/mg. This result suggests that MP and RA exhibited inhibitory effects against H_2O_2-induced lipid peroxidation and cell damage.

mRNA expression of iNOS and COX-2 caused by H_2O_2

As illustrated in Fig. 4, C6 glial cells treated with H_2O_2 for 24 h showed significantly increased mRNA expression of iNOS and COX-2. However, treatment with MP or RA suppressed expression of iNOS and COX-2 in comparison with the control group.

Protein expression of iNOS and COX-2 induced by H_2O_2

To determine whether MP and RA inhibit H_2O_2-induced over-expression of iNOS and COX-2 protein, C6 glial cells were treated with MP (25, 50, and 100 μg/mL) or RA (2.5, 5, and 10 μM) for 24 h. Protein expression levels of iNOS and COX-2 are shown in Fig. 5. Treatment with H_2O_2 increased protein expression of iNOS and COX-2 in C6 glial cells, and this effect was suppressed by treatment with MP or RA. In particular, iNOS and COX-2 protein levels were significantly decreased by treatment with 100 μg/mL MP or 10 μM RA.

DISCUSSION

Excessive H_2O_2 can lead to neuronal cell damage by modifying cellular lipids and proteins and inducing DNA oxidation (Whittemore *et al*., 1994). Oxidative damage is mediated by ROS and linked to a variety of degenerative diseases such as coronary artery disease, aging, and cancer (Ames, 1998). Increased ROS formation is considered to be a crucial mediator of cell injury, and neuronal death induced by ROS is observed in patients with neurodegenerative disorders. Natural antioxidants from plant sources can inhibit excessive accumulation of free radicals and attenuate oxidative stress (Hocman, 1989). Therefore, the search for natural antioxidants in foods to replace synthetic antioxidants has attracted considerable attention. *P. frutescens* var. *japonica* is a traditional medicine that has been widely used in East Asian countries for centuries, and several studies have reported that extracts of *P. frutescens* var. *japonica* produce anti-oxidant effects (Chou *et al*., 2009; Meng *et al*., 2009). Previous study demonstrated that RA is the main phenolic compound, and other flavonoids and phenolic acids such as catechin, apigenin, luteolin, caffeic acid, ferulic acid are found in *P. frutescens* (Ishikura, 1981; Masahiro *et al*., 1996). Ueda *et al*. (2002) also reported active constituents from *Perilla* leaf extract, RA, luteolin and caffeic acid. In addition, Gu *et al*. (2009) isolated and identified four antioxidant compounds (RA, luteolin, apigenin, and chrysoeriol) from *P. frutescens*. Among them, RA and luteolin showed significant free radical scavenging activities. RA has four hydroxyl groups that were considered to contribute to scavenging free radicals by functioning as a proton donor (Brand-Williams *et al*., 1995). According to Nakamura *et al*. (1998), RA exhibited antioxidative activity by attenuating both intracellular superoxide and peroxide formation. In addition, RA inhibited ROS formation and lipid peroxidation against amyloid beta peptide, suggesting RA could effectively protect against oxidative stress in neuronal cell (Iuvone *et al*., 2006). However, the neuro-protective effects of MP and RA against oxidative stress have not been reported.

Elevated oxidative stress as a result of ROS generation and MDA formation in glial cells is a primary mediator of neuroinflammation and an important cause of neuronal cell death in neurodegenerative diseases (Mosley *et al*., 2006). In this study, we found that C6 cells treated with MP and RA showed

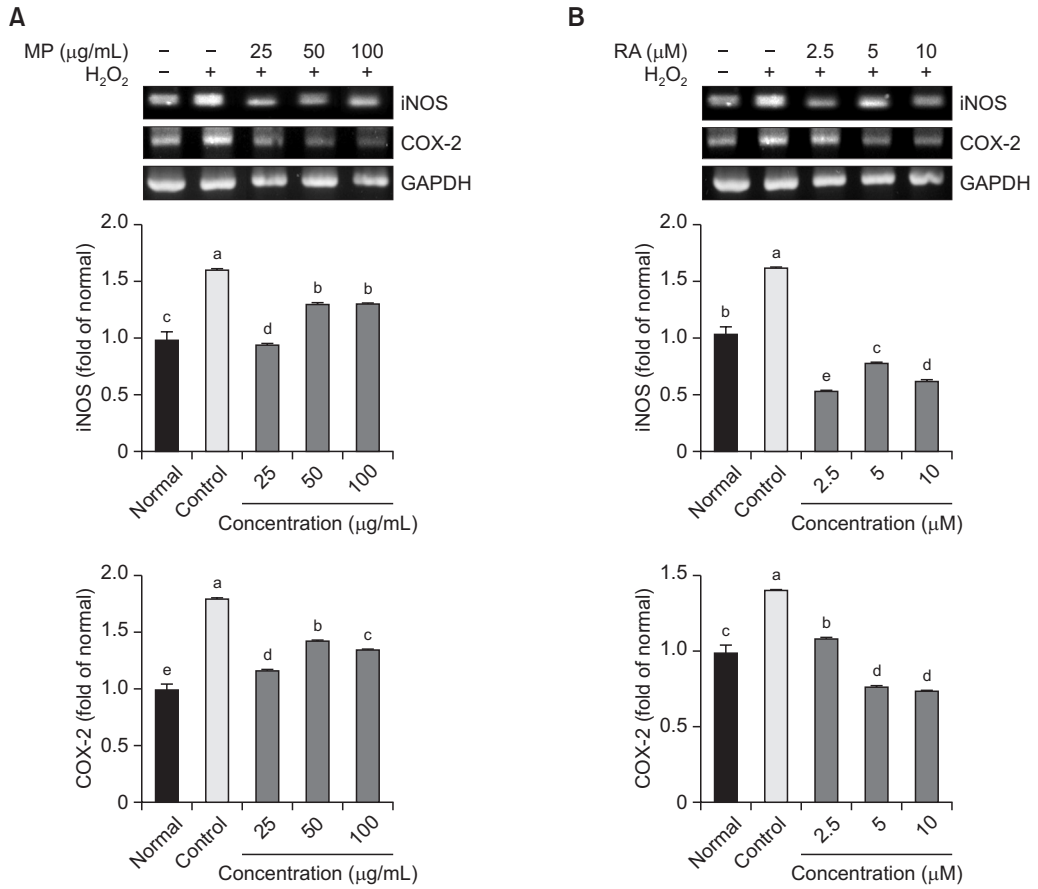

Fig. 4. Effect of MP (A) and RA (B) on mRNA expression of iNOS and COX-2 in C6 glial cells under H_2O_2-induced oxidative stress. Cells were pre-incubated for 24 h in the presence of 100 μM H_2O_2, followed by the addition of MP (25, 50, and 100 μg/mL) and RA (2.5, 5, and 10 μM) for 24 h. Total RNA was isolated, after which RT-PCR was performed using the indicated primers. The amplified PCR products were run in a 1% agarose gel and visualized by staining with ethidium bromide. GAPDH was used as a control gene for normalization of relative gene expression levels. Values are mean ± SD. $^{a-e}$Means with different letters are significantly different (p<0.05) as determined by Duncan's multiple range test.

significantly increased cell viability after exposure to H_2O_2. This result suggests that MP and RA protect C6 glial cells from H_2O_2-induced cytotoxicity.

Determination of MDA content by measuring TBARS is an assay commonly used to assess lipid peroxidation. MDA formation is a key event in oxidative stress and an important cause of cell membrane damage (Gutteridge, 1995). H_2O_2 significantly increased MDA formation in C6 glial cells in comparison with non-stimulated cells. However, MP and RA markedly reduced MDA formation, indicating reduced oxidative stress, and thus, anti-oxidative and neuro-protective effects. Kim *et al*. (2008) also demonstrated that *Perilla* leaves protect DNA against damage and possess anti-oxidative activity. In addition, RA isolated from *Perilla* leaves produced anti-oxidative effects in biological systems by scavenging superoxide radicals, one of the major constituents of ROS (Nakamura *et al*., 1998). These results show that MP and RA possess significant protective capability against H_2O_2-induced cell damage.

Pro-inflammatory cytokines and mediators released by activated glial cells are important pathologic factors in neurodegenerative disorders including Alzheimer's disease and central nervous system inflammation (Hull *et al*., 2000). Yu *et al*. (2012) showed that treatment with H_2O_2 induced over-expressions of iNOS and COX-2, which led to neuro-inflammation. The mechanism through which MP and RA suppress the effects of H_2O_2 in C6 glial cells was investigated by measuring iNOS and COX-2 mRNA and protein expression. Astroglial and microglial cells in a healthy brain do not express iNOS, while neurotoxic or inflammatory damage induces iNOS expression in mice, rats, and humans (Galea *et al*., 1992). Furthermore, there is evidence that increased COX-2 expression contributes to neurotoxicity and brain damage. In the rat brain, COX-2 is expressed in cerebral cortex and the hippocampal formation (Tocco *et al*., 1997). Neurotoxicity causes up-regulation of COX-2 expression, and COX-2 is known to be involved in neuronal death and survival (Ho *et al*., 1999). COX-2 mediates neuronal damage by producing excessive amounts of harmful prostanoids and free radicals (Nogawa *et al*., 1997). Our results showed that exposure of glial cells to H_2O_2 for 24 h resulted in significant induction of iNOS and COX-2 mRNA expression, whereas MP and RA treatment for 24 h after exposure of glial cells to H_2O_2 clearly reduced iNOS and COX-2 expression. These results demonstrated that MP and RA inhibit inflammatory processes by suppressing iNOS

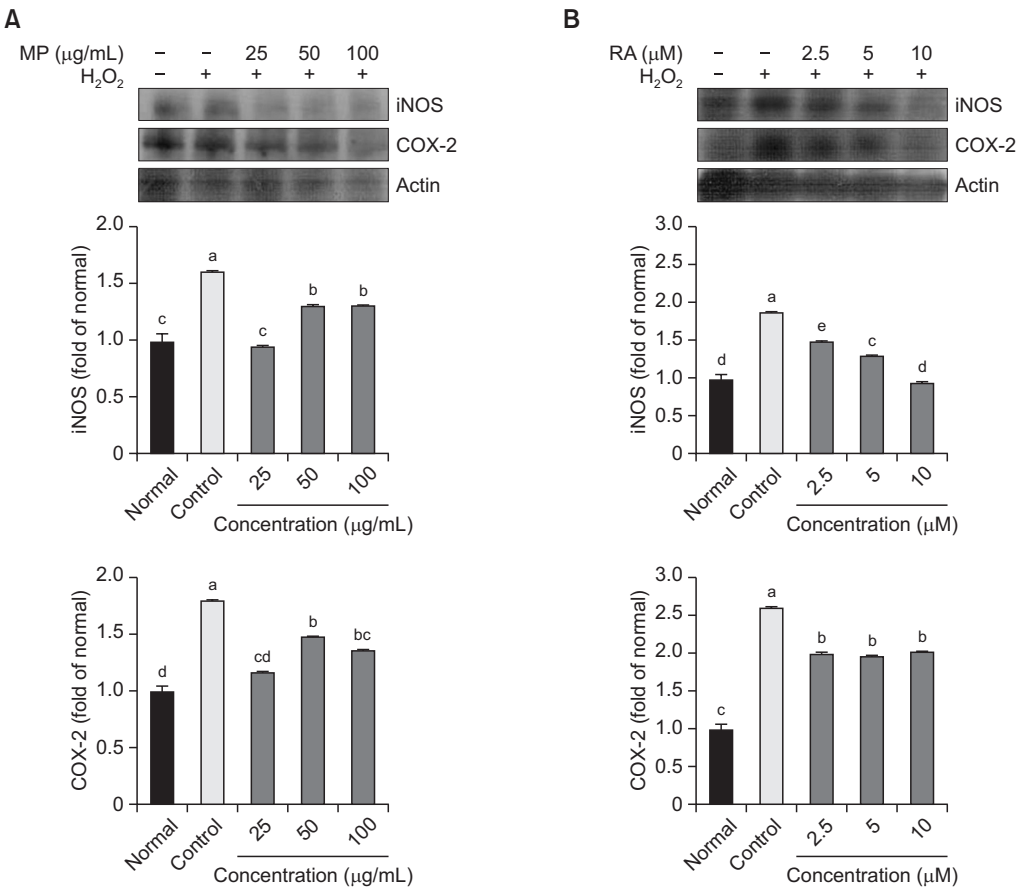

Fig. 5. Effect of MP (A) and RA (B) on protein expression of iNOS and COX-2 in C6 glial cells under H_2O_2-induced oxidative stress. Cells were pre-incubated for 24 h in the presence of 100 μM H_2O_2, followed by the addition of MP (25, 50, and 100 μg/mL) and RA (2.5, 5, and 10 μM) for 24 h. Cellular protein was separated by SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was probed with anti-iNOS and anti-COX-2 antibodies. Protein was visualized using an ECL detection system. Actin was used as an internal control. Values are mean ± SD. [a-e]Means with different letters are significantly different (p<0.05) as determined by Duncan's multiple range test.

and COX-2 expression in H_2O_2-treated C6 glial cells.

Several natural antioxidants directly inhibit expression of iNOS and COX-2, and thus, reduce inflammation (Surh *et al*., 2001). To investigate the protective mechanisms of MP and RA against oxidative stress, iNOS and COX-2 expression was measured. Exposure of C6 glial cells to H_2O_2 increased protein expression of iNOS and COX-2. However, treatment of MP or RA down-regulated iNOS and COX-2 expression. These results indicate that decreased iNOS and COX-2 protein expression by MP and RA may contribute to their attenuation of oxidative stress.

Ghaffari *et al*. (2014) demonstrated that RA prevents H_2O_2-induced oxidative stress in N2A neuroblastoma cells through mitochondrial membrane potential change and regulation of tyrosine hydroxylase and brain derived neurotropic factor gene expression. In addition, Huang *et al*. (2009) studied anti-inflammatory activity of RA from *Prunella vulgaris* using RAW 264.7 macrophage cells stimulated by lipopolysaccharide. However, the neuro-protective effect of RA on NO production against H_2O_2-induced stress in C6 glial cells has not been studied yet. Glial cells, the abundant cell type in mammalian brain, are support neuron by releasing antioxidant protection (Sagara *et al*., 1993). Glial cell lines have been widely used as a model for studies to estimate neurodegenerative condition, because these cells possess closely resembles the actual mechanisms of inflammatory and apoptosis reaction (Grobben *et al*., 2002; Furman *et al*., 2012). In addition, glial cells quickly respond to H_2O_2 exposure, which can increases oxidative-nitrosative stress, iNOS expression and dysfunction of antioxidant defenses (Quincozes-Santos *et al*., 2010; Quincozes-Santos *et al*., 2013). Excessive NO release from glial cells mimics pathological situation which is associated with neurodegenerative disease including AD (Rampe *et al*., 2004). Some studies investigated that whether dietary components have possible value for inhibition of excessive production of NO by down-regulation of iNOS and COX-2 expression in C6 glial cells (Soliman and Mazzio, 1998; Kim *et al*., 2001; Niranjan *et al*., 2010). In present study, we focused on the effects of MP and RA on iNOS and COX-2 expression as the underlying mechanisms of NO production using C6 glial cells, which were activated with H_2O_2. The present results showed MP and RA treatment down-regulated both iNOS and COX-2 expression under NO-induced oxidative stress in C6 glial cells, suggesting neuroprotective effect of MP and RA.

This study showed that H_2O_2-induced cell damage occurred as a result of ROS over-production and lipid peroxidation. However, MP and RA reduced cell damage and lipid peroxidation in C6 glial cells through the regulation of mRNA and protein expression of iNOS and COX-2. Regulation of oxidative stress-related gene expression might be involved in the neuro-protective effects of MP and RA against oxidative stress. Although further study is necessary to determine whether *P. frutescens* var. *japonica* has protective effects against neurodegeneration *in vivo*, these findings show that *P. frutescens* var. *japonica* may exert protection against inflammatory brain damage induced by oxidative stress.

ACKNOWLEDGMENTS

This work was carried out with the support of Cooperative Research Program for Agriculture Science & Technology Development (PJ01015603), Rural Development Administration, Republic of Korea.

REFERENCES

Al-Sereiti, M. R., Abu-Amer, K. M. and Sen, P. (1999) Pharmacology of rosemary (*Rosmarinus officinalis* Linn.) and its therapeutic potentials. *Indian J. Exp. Biol.* **37**, 124-130.

Ames, B. N. (1998) Micronutrients prevent cancer and delay aging. *Toxicol. Lett.* **102-103**, 5-18.

Areias, F., Valentao, P., Andrade, P. B., Ferreres, F. and Seabra, R. M. (2000) Flavonoids and phenolic acids of sage: influence of some agricultural factors. *J. Agric. Food Chem*. **48**, 6081-6084.

Aruoma, O. I., Bahorun, T. and Jen, L. S. (2003) Neuroprotection by bioactive components in medicinal and food plant extracts. *Mutat. Res.* **544**, 203-215.

Brand-Williams, W., Cuvelier, M. E. and Berset, C. (1995) Use of a free radical method to evaluate antioxidant activity. *LWT-Food Sci. Technol.* **28**, 25-30.

Brown, G. C. (2007) Mechanisms of inflammatory neurodegeneration: iNOS and NADPH oxidase. *Biochem. Soc. Trans.* **35**, 1119-1121.

Chou, H. J., Kuo, J. T. and Lin, E. S. (2009) Comparative antioxidant properties of water extracts from different parts of Beefsteak plant (*Perilla frutescens*). *J. Food Drug Anal.* **17**, 489-496.

Dringen, R., Gutterer, J. M. and Hirrlinger, J. (2000) Metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species. *Eur. J. Biochem.* **267,** 4912-4916..

Eikelenboom, P. and van Gool, W. A. (2004) Neuroinflammatory perspectives on the two faces of Alzheimer's disease. *J. Neural. Transm. (Vienna)* **111**, 281-294.

Fraga, C. G., Leibovitz, B. E. and Tapped, A. L. (1988) Lipid peroxidation measured as thiobarbituric-reactive substances in tissue slices: characterization and comparison with homogenates and microsomes. *Free Radic. Biol. Med.* **4**, 155-161.

Funk, C. D., Funk, L. B., Kennedy, M. E., Pong, A. S. and Fitzgerald G. A. (1991) Human platelet/erythroleukemia cell prostaglandin G/H synthase: cDNA cloning, expression and gene chromosomal assignment. *FASEB J.* **5**, 2304-2312.

Furman, J. L., Sama, D. M., Gant, J. C., Beckett, T. L., Murphy, M. P., Bachstetter, A. D., Van Eldik, L. J. and Norris, C. M. (2012) Targeting astrocytes ameliorates neurologic changes in a mouse model of Alzheimer's disease. *J. Neurosci.* **32**, 16129-16140.

Galea, E., Feinstein, D. L. and Reis, D. J. (1992) Induction of calcium-independent nitric oxide synthase activity in primary rat glial cultures. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10945-10949.

Ghaffari, H., Venkataramana, M., Ghassam, B. J., Nayaka, S. C., Nataraju, A., Geetha, N. P. and Prakash, H. S. (2014) Rosmarinic acid mediated neuroprotective effects against H_2O_2-induced neuronal cell damage in N2A cells. *Life Sci.* **113**, 7-13.

Gorman, A. M., McGowan, A., O'Neill, C. and Cotter, T. (1996) Oxidative stress and apoptosis in neurodegeneration. *J. Neurol. Sci.* **139**, 45-52.

Grobben, B., Deyn, P. D. and Sleger, H. (2002) Rat C6 glioma as experimental model system for the study of glioblastoma growth and invasion. *Cell Tissue Res.* **310**, 257-270.

Gu, L., Wu, T. and Wang, Z. (2009) TLC bioautography-guided isolation of antioxidants from fruit of *Perilla frutescens* var. acuta. *LWT-Food Sci. Technol.* **42**, 131-136.

Guix, F. X., Uribesalgo, I., Coma, M. and Munoz, F. J. (2005) The physiology and pathophysiology of nitric oxide in the brain. *Prog. Neurobiol.* **76**, 126-152.

Gutteridge. J. M. (1995) Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clin. Chem.* **41**, 1819-1828.

Hanisch, U. K. (2002) Microglia as a source and target of cytokines. *Glia* **40**, 140-155.

Ho, L., Pieroni, C., Winger, D., Purohit, D. P., Aisen P. S. and Pasinetti, G. M. (1999) Regional distribution of cyclooxygenase-2 in the hippocampal formation in Alzheimer's disease. *J. Neurosci. Res.* **57**, 295-303.

Hocman, G. (1989) Prevention of cancer: Vegetables and plants. *Comp. Biochem. Physiol. B* **93**, 201-212.

Huang, N., Hauck, C., Yum, Y. M., Rizshsky, L., Widrlechner, M. P., McCoy, J. A., Murphy, P. A., Nikolau, B. J. and Birt, D. F. (2009) Rosmarinic acid in *Prunella vulgaris* ethanol extract inhibits lipopolysaccharide-induced prostaglandin E2 and nitric oxide in RAW 264.7 mouse macrophages. *J. Agric. Food Chem.* **57**, 10579-10589.

Hull, M., Lieb, K. and Fiebich, B. L. (2000) Anti-inflammatory drugs: a hope a Alzheimer's disease?. *Expert Opin. Investig. Drugs* **9**, 671-683.

Ishikura, N. (1981) Anthocyanins and flavones in leaves and seeds of *Perilla* plant. *Agric. Biol. Chem.* **45**, 1855-1860.

Iuvone, T., De Filippis, D., Esposito, G., D'Amico, A. and Izzo, A. A. (2006) The spice sage and its active ingredient rosmarinic acid protect PC12 cells from amyloid-β peptide-induced neurotoxicity. *J. Pharmacol. Exp. Ther.* **317**, 1143-1149.

Kim, E. K., Lee, S. J., Lim, B. O., Jeon, Y. J., Song, M. D., Park, T. K., Lee, K. H., Kim, B., Lee, S. R., Moon, S. H., Jeon, B. T. and Park, P. J. (2008) Antioxidative and neuroprotective effects enzymatic extracts from leaves of *Perilla frutescens* var. *japonica*. *Food Sci. Biotechnol.* **17**, 279-286.

Kim, H., Kim, Y. S., Kim, S. Y. and Suk, K. (2001) The plant flavonoid wogonin suppresses death of activated C6 rat glial cells by inhibiting nitric oxide production. *Neurosci. Lett.* **309**, 67-71.

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

Lee, K. I., Rhee, S. H., Kim, J. O., Chung, H. Y. and Park, K. Y. (1993) Antimutagenic and antioxidative effects of perilla leaf extracts. *J. Korean Soc. Food Nutr.* **22**, 175-180.

Makino, T., Furuta, Y., Fujii, H., Nakagawa, T., Wakushima, H., Saito, K. and Kano, Y. (2001) Effect of oral treatment of *Perilla frutescens* and its constituents on type-I allergy in mice. *Biol. Pharm. Bull.* **24**, 1206-1209.

Makino, T., Ono, T., Muso, E. and Honda, G. (1998) Inhibitory effect of *Perilla frutescens* and its phenolic constituents on cultured murine mesangial cell proliferation. *Planta Med.* **64**, 541-545.

Masferrer, J. L., Zweifel, B. S., Manning, P. T., Hauser, S. D., Leahy, K. M., Smith, W. G., Isakson, P. C. and Seibert, K. (1994) Selective inhibition of inducible cyclooxygenase 2 *in vivo* is antiinflammatory and nonulcerogenic. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3228-3232.

Masahiro, T., Risa, M., harutaka, Y. and Kazuhiro, C. (1996) Novel antioxidants isolated from *Perilla frutescens* Britton var. Crispa (Thunb.). *Biosci. Biotech. Biochem.* **60**, 1093-1095.

Meng, L., Lozano, Y. F. Gaydou, E. M. and Li, B. (2009) Antioxidant activities of polyphenols extracted from *Perilla frutescens* varieties. *Molecules* **14**, 133-140.

Minagar, A., Shapshak, P., Fujimura, R., Ownby, R., Heyes, M. and Eisdorfer, C. (2002) The role of macrophage/microglia and astrocytes in the pathogenesis of three neurologic disorders: HIV-associated dementia, Alzheimer disease, and multiple sclerosis. *J.*

Neurol. Sci. **202**, 13-23.
Minghetti, L. and Levi, G. (1998) Microglia as effector cells in brain damage and repair: focus on prostanoids and nitric oxide. *Prog. Neurobiol.* **54**, 99-125.
Mosley, R. L., Benner, E. J., Kadiu, I., Thomas, M., Boska, M. D., Hasan, K., Laurie, C. and Gendelman, H. E. (2006) Neuroinflammation, oxidative stress, and the pathogenesis of Parkinson's disease. *Clin. Neurosci. Res.* **6**, 261-281.
Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55-63.
Nakamura, Y., Ohto, Y., Murakami, A. and Ohigashi, H. (1998) Superoxide scavenging activity of rosmarinic acid from *Perilla frutescens* Britton Var. *acuta* f. *viridis*. *J. Agric. Food Chem.* **46**, 4545-4550.
Niranjan, R., Kamat, P. K., Nath, C. and Shukla. (2010) Evaluation of guggulipid and nimesulide on production of inflammatory mediators and GFAP expression in LPS stimulated rat astrocytoma, cell line (C6). *J. Ethnopharmacol.* **127**, 625-630.
Nogawa, S., Zhang, F., Ross M. E. and Iadecola C. (1997) Cyclo-oxygenase-2 gene expression in neurons contributes to ischemic brain damage. *J. Neurosci.* **17**, 2746-2755.
Osakabe, N., Yasuda, A., Natsume, M., Sanbongi, C., Kato, Y., Osawa T. and Yoshikawa, T. (2002) Rosmarinic acid, a major polyphenolic component of *Perilla frutescens*, reduces lipopolysaccharide (LPS)-induced liver injury in D-galactosamine (D-GalN)-sensitized mice. *Free Radic. Biol. Med.* **33**, 798-806.
Quincozes-Santos, A., Andreazza, A. C., Goncalves, C. A. and Gottfried, C. (2010) Actions of redox-active compound resveratrol under hydrogen peroxide insult in C6 astroglial cells. *Toxicol. In Vitro* **24**, 916-920.
Quincozes-Santos, A., Bobermin, L. D., Latini, A., Wajner, M., Souza, D. O., Gonçalves, C. A. and Gottfried, C. (2013) Resveratrol protects C6 astrocyte cell line against hydrogen peroxide-induced oxidative stress through heme oxygenase 1. *PLoS ONE* **8**, e64372.
Rampe, D., Wang, L. and Ringeim, G. E. (2004) P2X7 receptor modulation of beta-amyloid- and LPS-induced cytokine secretion from human macrophages and microglia. *J. Neuroimmunol.* **147**, 56-61.
Sagara, J. I., Miura, K. and Bannai, S. (1993) Maintenance of neuronal glutathione by glial cells. *J. Neurochem.* **61**, 1672-1676.
Smith, W. L., Gavavito, R. M. and Dewitt, D. L. (1996) Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. *J. Biol. Chem.* **271**, 33157-33160.
Soliman, K. F. and Mazzio, E. A. (1998) In vitro attenuation of nitric oxide production in C6 astrocyte cell culture by various dietary compounds. *Proc. Soc. Exp. Biol. Med.* **218**, 390-397.
Surh, Y. J., Chun, K. S., Cha, H. H., Han, S. S., Keum, Y. S., Park, K. K. and Lee, S. S. (2001) Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-κB activation. *Mutat. Res.-Fund. Mol. M.* **480-481**, 243-268.
Tocco, G., Freire-Moar, O., Schreiber, S. S., Sakhi, S. H., Aisen, P. S. and Pasinetti, G. M. (1997) Maturational regulation and regional induction of cyclooxygenase-2 in rat brain: implications for Alzheimer's disease. *Exp. Neurol.* **144**, 339-349.
Ueda, H., Yamazaki, C. and Yamazaki, M. (2002) Luteolin as an anti-inflammatory and anti-allergic constituent of Perilla frutescens. *Biol. Pharm. Bull.* **25**, 1197-1202.
Ullian, E. M., Sapperstain, S. K., Christopherson, K. S. and Barres, B. A. (2001) Control of synapse number by glia. *Science* **291**, 657-661.
Whittemore, E. R., Loo, D. T. and Corman, C. W. (1994) Exposure to hydrogen peroxide induces cell death via apoptosis in cultured rat cortical neurons. *Neuroreport,* **5**, 1485-1488.
Yang, S. Y., Hong, C. O., Lee, G. P., Kim, C. T. and Lee, K. W. (2013) The hepatoprotection of caffeic acid and rosmarinic acid, major compounds of *Perilla frutescens*, against t-BHP-induced oxidative liver damage. *Food Chem. Toxicol.* **55**, 92-99.
Yu, H., Liu, Z., Zhou, H., Dai, W., Chen, S., Shu, Y. and Feng, J. (2012) JAK-STAT pathway modulates the roles of iNOS and COX-2 in the cytoprotection of early phase of hydrogen peroxide preconditioning against apoptosis induced by oxidative stress. *Neurosci. Lett.* **529**, 166-171.
Zhang, H. Y., Yang, D. P. and Tang, G. Y. (2006) Multipotent antioxidants: from screening to design. *Drug Discov. Today* **11**, 749-754.