

Neuroprotective Effect of Steamed and Fermented *Codonopsis lanceolata*

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Abstract

Codonopsis lanceolata has been used as an herbal medicine for several lung inflammatory diseases, such as asthma, tonsillitis, and pharyngitis. Previously, we showed the neuroprotective effect of steamed and fermented *C. lanceolata* (SFC) *in vitro* and *in vivo*. In the current study, the treatment of HT22 cells with SFC decreased glutamate-induced cell death, suggesting that SFC protected HT22 cells from glutamate-induced cytotoxicity. Based on these, we sought to elucidate the mechanisms of the neuroprotective effect of SFC by measuring the oxidative stress parameters and the expression of Bax and caspase-3 in HT22 cells. SFC reduced contents of ROS, Ca²⁺ and NO. Moreover, SFC restored contents of glutathione and glutathione reductase as well as inhibited Bax and caspase-3 activity in HT22 cells. These results indicate that steamed and fermented *C. lanceolata* (SFC) extract protected HT22 cells by anti-oxidative effect and inhibition of the expression of Bax and caspase-3.

Key Words: *Codonopsis lanceolata*, Steam and fermentation, Neuroprotective activity, Antioxidative activity, Bax, Caspase-3

INTRODUCTION

Alzheimer's disease (AD), the most common form of dementia, is neurodegenerative disorder characterized by memory and cognitive impairment and accounts for approximately 50 to 80% of dementia cases (Crapper and DeBoni, 1978).

There are several possible mechanisms for the onset of cognitive impairment including oxidative stress-induced neuronal cell death and induction of apoptosis by pro-apoptotic bcl-2 families. Oxidative stress in central nervous system (CNS) can lead to cell death and necrosis and contributes to various neurodegenerative disorders including AD, Parkinson's disease and Huntington's disease (Coyle and Puttfarcken, 1993; Satoh *et al.*, 1998). Glutamate is an endogenous excitatory neurotransmitter and high concentration of glutamate induced the cell death due to oxidative stress (Choi, 1988; Fukui *et al.*, 2009). Glutamate excitotoxicity results in calcium ion (Ca²⁺) influx, mitochondrial dysfunction, and elevation of ROS level and depletion of antioxidant defense system including glutathione (GSH) and glutathione reductase (GR) by inhibition of

cystine uptake (Choi, 1985; Murphy *et al.*, 1989; Tan *et al.*, 1998a). Expression of Bcl-2 family of protein and caspase is also associated with neuronal cell death. Bcl-2 family contains cell apoptosis regulator which exerts pro-apoptotic (e.g. Bax, Bak and Bok) as well as exerts anti-apoptotic (e.g. Bcl-2 and Bcl-xL) effect (Ola *et al.*, 2011). In addition, bcl-2 family is involved in regulation of Ca²⁺ and cytochrome c in the cytosol. Release of cytochrome c induced the activity of caspases, which plays a key role in apoptosis by cleaving proteins that are essential for cell survival (Antonsson, 2004).

Codonopsis lanceolata (*C. lanceolata*), a traditional medicinal plant, is belong to the Campaluceae family in East Asia including Korea, Japan and China. This plant is widely used to treat several inflammatory diseases, such as asthma, tonsillitis, and pharyngitis (Yongxu and Jicheng, 2008; Byeon *et al.*, 2009). The compounds from this plant are saponins, alkaloids, tannins, steroids, and polysaccharides (Ushijima *et al.*, 2008). Many studies have shown that *C. lanceolata* had an-

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tilipogenic, antiobesity and anti-inflammatory effect (Li *et al.*, 2007; Yongxu and Jicheng, 2008; Ryu, 2009).

Lancemaside A isolated from *Codonopsis lanceolata* improve scopolamine induced memory and learning deficits by inhibiting AChE activity and inducing BDNF and p-CREB expressions (Jung *et al.*, 2012).

In previous study, we confirmed that fermentation improve neuroprotective effect of traditional herbal medicine, Hwangryunhaedok-tang (Yang *et al.*, 2011).

The aim of this study was to investigate the mechanism of the neuroprotective effect of novel steamed and fermented *C. lanceolata* (SFC) using glutamate-induced cytotoxicity in HT22 cell based on our previous finding of the cognitive enhancement effect of SFC in animal model of scopolamine-induced memory deficit (Weon *et al.*, 2013).

MATERIALS AND METHODS

Plant materials, fermentation and extraction

The roots of *C. lanceolata* collected from Heongseong, Gangwon (Korea) was washed in tap water and shade-dried at 20–30°C for 2 days. Dried *C. lanceolata* was steamed using a steam device (Dechang Stainless, Seoul, Korea) at 90°C for 8 h and repeated five times.

Fermented microorganism, *Bifidobacterium longum* (KACC 20587), *Lactobacillus acidophilus* (KACC 12419), and *Leuconostoc mesenteroides* (KACC 12312) were provided from the Korean Agricultural Culture Collection (Suwon, Korea). The steamed *C. lanceolata* was aseptically inoculated with approximately 10^6 CFU/g of *Bifidobacterium longum*, *Lactobacillus acidophilus*, and *Leuconostoc mesenteroides* (1:1:1) in distilled water 8 times the weight of the *C. lanceolata* and fermented for 48 h at 30°C.

Fermented *C. lanceolata* was reflux extracted in 70% ethanol with 5 times the weight of the *C. lanceolata* at 80°C for 24 h. Then *C. lanceolata* extraction was filtered and concentrated using rotary evaporator. After evaporation, the fermented *C. lanceolata* sample was obtained by freeze dry.

Cell viability

The mouse hippocampal HT22 cells, a sub-line derived from parent HT4 cells is used to study glutamate-induced cell death mechanisms (Tan *et al.*, 1998b). Hippocampal HT22 cells derived from mouse were obtained from Seoul National University, Korea. HT22 cells in DMEM containing 10% (v/v) fetal bovine serum (FBS) with 1% penicillin/streptomycin, NaHCO₃ (2 mg/ml), and 15 mM HEPES were seeded at a density of 6.7×10^4 /well (48 wells plate), and incubated at 37°C at 5% CO₂. After incubation for 24 h, 10, 100 and 500 µg/ml of SFC and trolox (50 µM) were treated for 1 h before treatment of 2 mM glutamate. Cell viability was accessed using MTT assay as described in our previous study (Weon *et al.*, 2013). After 24 h, cells were treated with MTT solution (1 mg/ml) to each well and then incubated for 3 h. Dimethyl sulfoxide (DMSO) was added in each well and the optical density (OD) was measured 570 nm. Results are expressed as percentages of relative cell viability (%) of non-treated control group. Trolox was used as a positive control.

ROS and mitochondrial membrane potential ($\Delta\Psi$) measurement

ROS was determined using 2',7'-dichlorofluorescein diacetate (H₂-DCF-DA). HT22 cells were treated with 2 mM glutamate and 10, 100 and 500 µg/ml of SFC for 8 h. After incubation, the cells were washed with PBS and stained with 10 µM DCF-DA in Hanks' balanced salt solution in the dark room. After 30 min, cells washed with PBS and then suspend in 1% Triton X-100. Fluorescence was measured at an excitation wavelength of 490 nm and emission wavelength of 525 nm. Accumulation of fluorescent dye, rhodamine 123 (Rho123) was evaluated for the $\Delta\Psi$ change indirectly by assessing ROS level. HT22 cells stained for 15 min at 37°C with Rho123 and then washed. The Rho123 concentration was measured by spectrofluorometry at excitation wavelength of 488 nm and emission wavelength of 520 nm.

Ca²⁺ measurement

Cytosolic Ca²⁺ concentration in cultured HT22 cells was measured with the Fura-2AM. After treatment of 10, 100 and 500 µg/ml of SFC, 2 µM Fura-AM and glutamate was added to each well. The cells were then washed twice with PBS and extracted with 1% Triton X-100 in PBS for 10 min at 37°C. Fluorescence was analyzed for excitation wavelength of 400 nm and emission wavelength of 535 nm.

Analysis of NO

The production of NO was determined as nitrite assay using the Griess reaction with 4.25% phosphoric acid, 9.7 µM d-naphthylethylenediamine and 0.14 µM sulfanilic acid. Optical density was measured at 550 nm.

Estimation of antioxidant enzymes, glutathione and glutathione reductase

Cultured cells were washed with 0.2 M phosphate buffer (pH 7.4) and lysed with sulfosalicylic acid. Then, cells were centrifuged at 3000 g for 30 min at 4°C and supernatant was collected. Total glutathione (GSH) in the HT22 cell was investigated based on the reaction of reduced GSH with DTNB (5',5'-dithiobis (2-nitrobenzoic acid)). To supernatant, 5 unit/ml glutathione disulfide reductase, 0.3 mM NADPH and 0.5 mM DTNB were mixed together. The absorbance was measured at 412 nm within 15 min. Glutathione reductase (GR) was determined by the reduction of glutathione in the presence of NADPH. The reaction mixture contains supernatant, 1 mM glutathione, phosphate buffer containing 0.1 mM NADPH. The decrease in absorbance at 340 nm is immediately measured at an interval time of 15 seconds for a total time of 120 min.

Western blot analysis

After treatment with glutamate and SFC, HT22 cells were washed with PBS and lysed in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 mM NaF, 2 mM Na₃VO₄ and a protease inhibitor cocktail, pH 7.5) and centrifuged at 13,000×g for 20 min at 4°C. The supernatants were collected and stored at -80°C. The amount of protein was determined by Bradford method. 20 µg of sample protein was subjected on 13% SDS-PAGE gel. Then, the proteins were transferred to a PVDF membrane (Bio-Rad) and blocked in 5% skim milk in PBS (0.01% Tween 20) for 1 h at room temperature. The membrane was incubated with diluted primary antibodies, Bax and β-actin at 4°C overnight. The protein bands

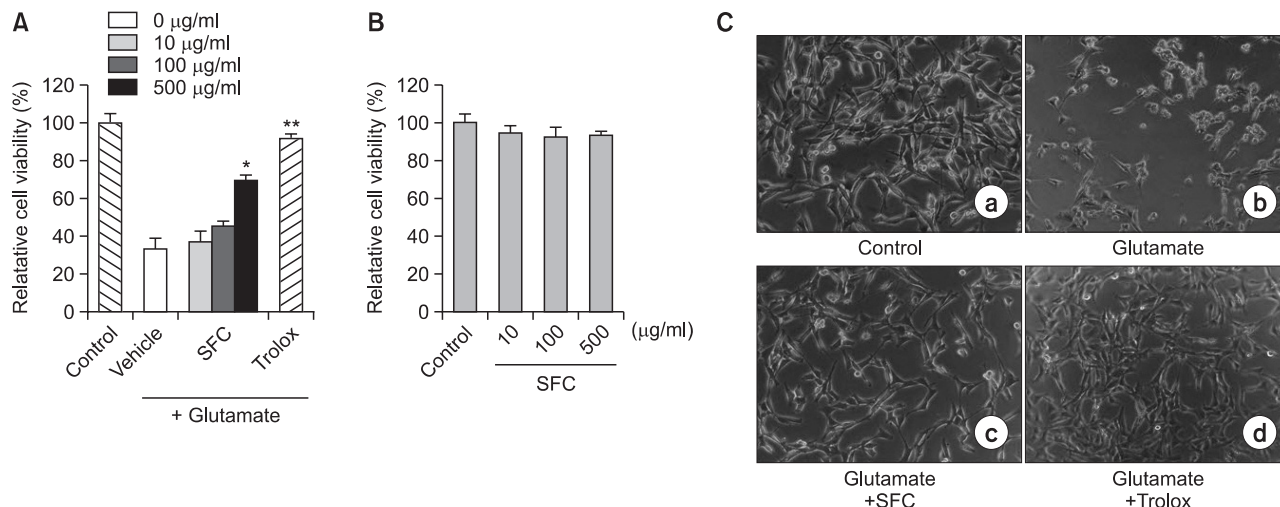


Fig. 1. (A) The neuroprotective effect of SFC on glutamate-induced cytotoxicity in neuronal HT22 cells. (B) The effect of SFC alone in HT22 cells. Each bar represents the mean relative cell viability (%) ± SD of three independent experiments. * $p < 0.05$ and ** $p < 0.01$ vs glutamate-injured cells. (C) Morphological phase change of HT 22 cells. a) Control cells; b) Glutamate-treated cells c) SFC (500 µg/ml)-treated cells d) Trolox (positive control)-treated cells.

were immunodetected using secondary antibodies conjugated to horse radish peroxidase. Signals were detected using western blotting detection reagents (Advansta, CA, USA).

DPPH radical scavenging assay

To investigate antioxidant activity of SFC, DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was measured. Different concentrations of SFC sample added to 150 µl of 0.4 mM of DPPH solution in 96 well plate. Absorbance of DPPH solution at 517 nm was determined using an ELISA reader and calculated.

HPLC analysis

We analyzed phenolic compounds, including gallic acid, 4-hydroxybenzoic acid, caffeic acid, vanillic acid, 4-coumaric acid, trans-ferulic acid, and caffeine, in SFC using HPLC. HPLC analysis was performed with an Agilent 1260 series (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a diode array detector (DAD). Separation was conducted using a ZORBAX Eclipse XDB-C₁₈ (250×4.60 mm i.d., 5 µm), maintained at 35°C. The mobile phase was composed of 10% acetonitrile with 0.1% formic acid (A) and 0.1% formic acid in 40% acetonitrile and 40% methanol (B). A gradient elution system of the mobile phase as follows: 95% A at 0-15 min; 60% A at 15-33 min; 0% A at 33-42 min; 95% A at 42-50 min. Flow rate was set of 1 ml/min. The UV wavelength was set at 280 nm and filtered sample injection volume was 20 µl.

Statistics

All results were expressed as mean ± SD. Statistical significance between two groups was analyzed using the *t*-student test. A value of $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered to be statistically significant.

RESULTS

Effect of SFC on glutamate-induced neurotoxicity

Previously, we investigated the effect of steamed and fermented *C. lanceolata* (SFC) on glutamate-induced cytotoxicity of HT22 cells by using an MTT assay (Weon *et al.*, 2013). Similarly, the treatment of cells with glutamate increased cell death and co-treatment with SFC (500 µg/ml) significantly reduced glutamate-induced cell death to $69.39 \pm 3.23\%$ of control ($p < 0.05$) (Fig. 1A). Trolox is an antioxidant derived from vitamine E, and used as a positive control. As expected, the treatment of glutamate-treated HT22 cells with Trolox significantly reduced cell death. When SFC alone was treated, there are no significant difference between control and 10, 100 and 500 µg/ml of SFC. It means that SFC not affected cell proliferation (Fig. 1B).

We investigated morphological character of HT22 cells. Glutamate induced morphological change as shrunken cells with rounded shape. In comparison, SFC inhibited morphological change and increased cell density (Fig. 1C).

These results indicate that glutamate-induced HT22 cell death may be mediate by oxidative stress and the treatment of cells with SFC showed a neuroprotective effect against glutamate-induced cell death.

Treatment with SFC reduced ROS accumulation by glutamate in HT22 cells

Glutamate was known to induce neurotoxicity by increasing intracellular ROS generation in CNS (Choi, 1988). ROS production mediated programmed cell death through oxidative stress (Suzuki *et al.*, 1997). ROS production was also increased in response to Ca²⁺ influx and mitochondrial dysfunction (Wang *et al.*, 1994). Thus, we assessed the intracellular ROS level using the H₂-DCF-DA to check whether SFC can inhibit glutamate-induced ROS production in HT22 cells. The treatment of HT22 cells with glutamate significantly increased the ROS production as compared with HT22 cells treated with vehicle only. Our data show that pretreatment of SFC (500 µg/

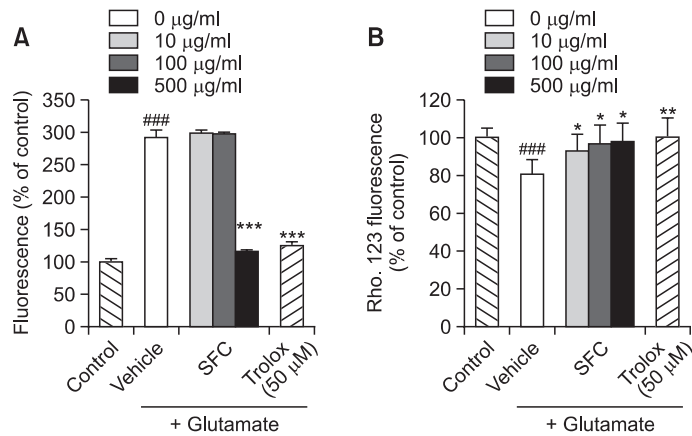


Fig. 2. (A) The effect of SFC on glutamate-induced ROS generation. (B) The effect of SFC on glutamate-induced disruption of mitochondrial membrane potential. Results are expressed as a percentage of values obtained for control cells. ###*p*<0.001 vs control group. **p*<0.05, ***p*<0.01 and ****p*<0.001 vs glutamate treated group.

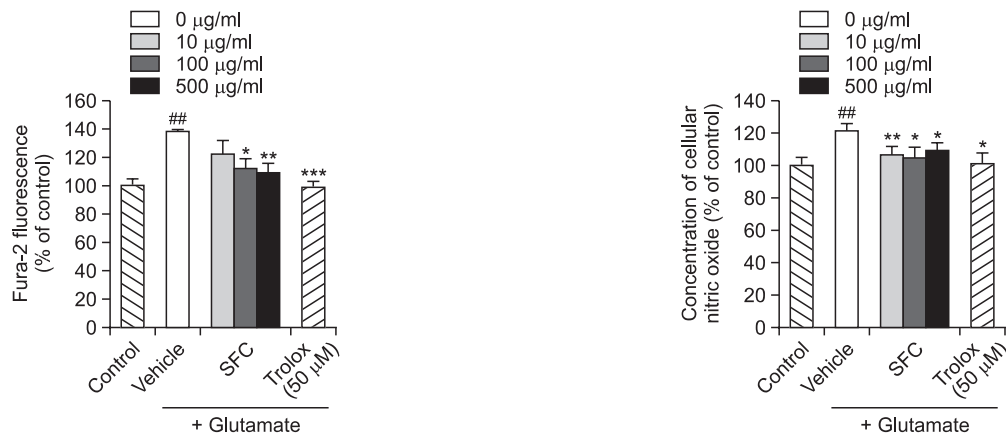


Fig. 3. Protective effect of SFC on glutamate-induced Ca²⁺ influx in HT22 cells. Results are expressed as a percentage of values obtained for control cells. ##*p*<0.01 vs control group. **p*<0.05, ***p*<0.01 and ****p*<0.001 vs glutamate treated group.

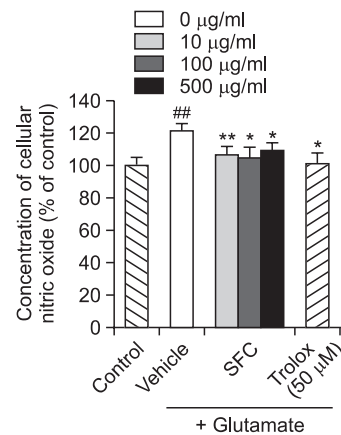


Fig. 4. The effect of SFC on glutamate-induced NO production. Results are expressed as a percentage of values obtained for control cells. ##*p*<0.01 vs control group. **p*<0.05 and ***p*<0.01 vs glutamate treated group.

ml) significantly inhibited the glutamate-induced elevation of ROS level to 115.91 ± 2.49% of control (*p*<0.01) (Fig. 2A). This result has confirmed that SFC protects neuronal cells from glutamate-induced cell death by inhibition of ROS generation.

In addition, based on the inhibition of ROS generation by SFC, we presumed that SFC exerted protective effect on mitochondrial dysfunction caused by glutamate. Glutamate was known to cause a decrease of mitochondrial membrane potential. Mitochondria have an essential role in life and death decision of neuronal cells. Mitochondria dysfunction induced cell apoptosis and was represented by the loss of mitochondrial membrane potential (Ly *et al.*, 2003). Although glutamate-induced Ca²⁺ influx decreased mitochondrial membrane potential (Duchen, 2000), SFC significantly recovered the mitochondrial membrane potential to 97.88 ± 9.60% of control (*p*<0.05) at a concentration of 500 μg/ml (Fig. 2B) as judged by Rho123 incorporation. Thus, our results indirectly indicated that SFC effectively restored mitochondrial membrane potential decreased by glutamate.

SFC inhibited on glutamate-mediated acceleration of Ca²⁺ influx

It was also well-known that glutamate increased intracellular Ca²⁺ concentration via N-methyl-D-aspartate (NMDA) receptors and neuronal cell death is associated with elevation of Ca²⁺ concentration (Butterfield and Pocernich, 2003). We examined the effect of SFC in glutamate-induced Ca²⁺ influx in HT22 cells. As shown in Fig. 3, exposure to glutamate increased Ca²⁺ concentration to 111.39 ± 0.73% of control, whereas SFC dose-dependently reduced the increased Ca²⁺ concentration compared to glutamate-treated cell. 100 and 500 μg/ml of SFC significantly reduced Ca²⁺ concentration to 96.14 ± 7.07 (*p*<0.05) and 84.90 ± 1.01% (*p*<0.001) of control, respectively. This suggests that SFC might decrease Ca²⁺ dependent neuronal cell death induced by glutamate.

SFC inhibited the glutamate-induced NO formation

Overproduction of NO in brain is mainly induced by neuronal nitric oxide synthase (nNOS) and implicated oxidative cell death. Influx of Ca²⁺ is also contributed to activate NO

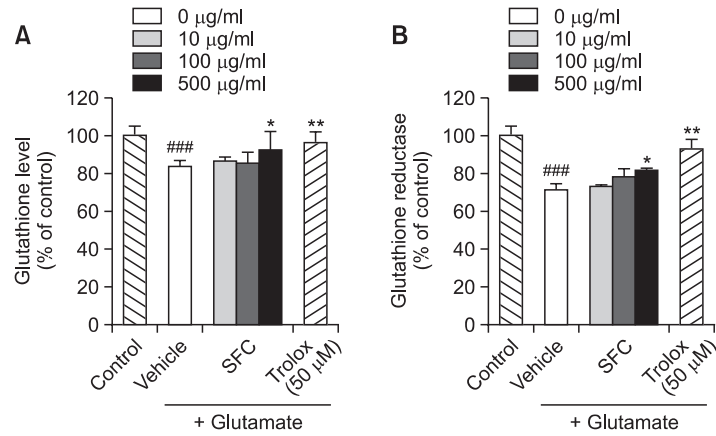


Fig. 5. Measurement of glutathione (GSH) (A) and glutathione reductase (GR) (B) in HT22 cells. Results are expressed as a percentage of values obtained for control cells. ###*p*<0.001 vs control group. **p*<0.05 and ***p*<0.01 vs glutamate treated group.

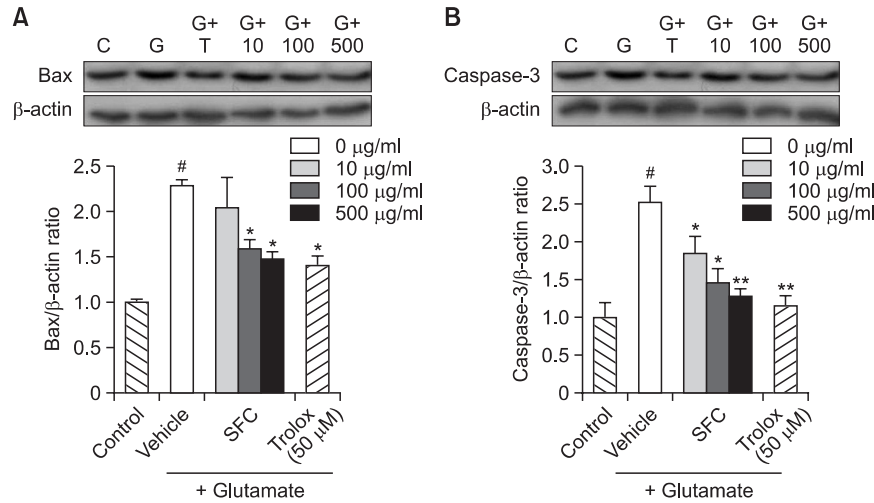


Fig. 6. Effect of SFC on Bax protein level (A) and caspase-3-like activity (B). Control group (C) glutamate group (G), Trolox group (G+T), and SFC group (G+10, G+100 and G+500; 10, 100 and 500 µg/ml treated 1 h before glutamate treated). #*p*<0.05 vs. control group. **p*<0.05 and ***p*<0.01 vs glutamate.

synthase (Knowles *et al.*, 1989). Glutamate mediated the enhancement of nNOS activity through NMDA receptors (Garthwaite, 1991). In this study, we investigated the effect of SFC on NO production in HT22 cell. The increased NO production (121.29 ± 4.69%) induced by glutamate was significantly decreased by the treatment of SFC. SFC was significantly reduced to 106.41 ± 5.43% (*p*<0.01), 104.65 ± 6.58% (*p*<0.05) and 109.19 ± 4.98% (*p*<0.05) of control by 10, 100 and 500 µg/ml (Fig. 4). These results demonstrated that SFC effectively decreased NO production induced by glutamate treatment.

SFC restored GSH and antioxidant enzyme, GR activity

We confirmed that glutamate-induced cell death in HT22 cells is related to oxidative stress and the treatment of cells with SFC recovered the oxidative stress condition. Since glutathione (GSH) is important antioxidant in CNS and GSH reductase (GR) is a critical enzyme for the production of GSH. High concentration of glutamate leads to deprivation of GSH by inhibiting of cysteine uptake into cells (Albrecht *et al.*,

2010). Depletion of GSH or antioxidant enzyme, such as GR causes neuronal cell death (Kane *et al.*, 1993). We investigated the effect of SFC associated with GSH and GR, and the results of GSH (Fig. 5A) and GR expression levels (Fig. 5B) showed similar pattern. The level of GSH and GR expression in glutamate-injured cells was decreased to 83.68 ± 3.18% and 71.32 ± 3.17%, respectively. However, SFC prevented glutamate-induced depletion of GSH (92.31 ± 9.77% (*p*<0.05) at 500 µg/ml) and GR (81.52 ± 1.36% (*p*<0.05) at 500 µg/ml). These results suggest that SFC exerted neuroprotective effect by antioxidant effect.

Effect of SFC on glutamate induced Bax and Caspase-3 levels in HT22 cells

Overexpression of pro-apoptotic regulators such as Bax and activity of caspase-3 is associated with neuronal cell death by mitochondrial dysfunction (Zhang and Bhavnani, 2005).

To elucidate possible mechanism of neuroprotective effect, we investigated the expression of some proteins related to

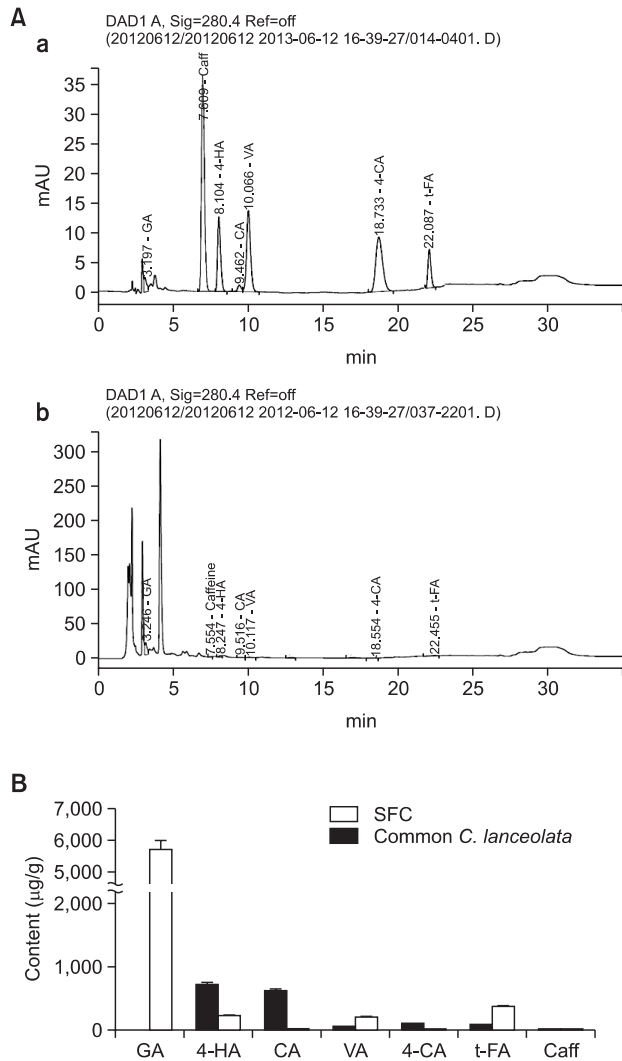


Fig. 7. (A) HPLC chromatogram of 7 phenolic acids (a) and SFC (b). (GA: gallic acid, Caff: caffeine, 4-HA: 4-hydroxybenzoic acid, CA: caffeic acid, VA: vanillic acid, 4-CA: 4-coumaric acid and t-FA: trans-ferulic acid). (B) Contents of 7 phenolic acid in SFC and common *C. lanceolata*.

neuronal cell death, including Bax and caspase-3 on HT22 cells by Western blot analysis. The level of Bax increased to 1.40 ± 0.07 -fold compared to control by glutamate, whereas that of SFC reduced to 0.90 ± 0.08 -fold ($p < 0.05$) at concentration of $500 \mu\text{g/ml}$ (Fig. 6A). In addition, glutamate increased caspase-3 levels by 1.17 ± 0.02 -fold compared to control (Fig. 6B). $500 \mu\text{g/ml}$ of SFC (0.59 ± 0.10 -fold ($p < 0.01$)) inhibited the up-regulation caspase-3 activity induced by glutamate. These results indicated that SFC reversed the level of Bax and caspase-3 activity increased by glutamate.

Antioxidant activity of SFC on DPPH radical scavenging assay

DPPH radical scavenging activity was investigated to determine antioxidant activity of SFC. SFC showed DPPH radical scavenging activity in this study. DPPH radical scavenging activity of SFC showed IC_{50} value as $7160.67 \mu\text{g/ml}$.

HPLC analysis of 7 phenolic compounds in SFC

Contents of 7 phenolic compounds, gallic acid, 4-hydroxybenzoic acid, caffeic acid, vanillic acid, 4-coumaric acid, trans-ferulic acid, and caffeine in SFC were evaluated by HPLC-DAD and HPLC chromatogram was shown in Fig. 7A. The result showed that the highest content $5702.21 \mu\text{g/g}$ of gallic acid existed in SFC. The contents of 4-hydroxybenzoic acid, caffeic acid, vanillic acid, 4-coumaric acid, trans-ferulic acid, and caffeine were $241.27 \mu\text{g/g}$, $20.86 \mu\text{g/g}$, $210 \mu\text{g/g}$, $1.5 \mu\text{g/g}$, $369.17 \mu\text{g/g}$ and $18.75 \mu\text{g/g}$, respectively. The data demonstrated that gallic acid was main phenolic compounds in SFC. Among 7 phenolic compounds, contents of gallic acid, vanillic acid and trans-ferulic acid were significantly increased compared to common *C. lanceolata* extraction (Fig. 7B).

DISCUSSION

Previous study have shown that SFC reduced cell death was induced by glutamate in HT22 cells (Weon *et al.*, 2013). We applied to *C. lanceolata* using new steamed and fermented process for systematic mass production and evaluated neuroprotective effect novel steamed and fermented *C. lanceolata*.

In MTT assay, novel steamed and fermented *C. lanceolata* have neuroprotective effect and are slightly higher than previous steamed and fermented *C. lanceolata* (not data).

This present study was also undertaken to elucidate possible mechanism of neuroprotective effect of SFC on HT22 cells.

Oxidative stress involved in loss of memory and associated with the pathogenesis of neurodegenerative diseases (Xu *et al.*, 2011).

Glutamate is an excitatory neurotransmitter, which causes two types of neurotoxic effects: receptor-induced excitotoxicity and production of non-receptor-mediated oxidative glutamate. Glutamate mediated neurotoxicity by NMDA receptors involves calcium (Ca^{2+}) entry into cells (Butterfield and Pocernich, 2003; Tanović and Alfaro, 2006). Intracellular Ca^{2+} influx affected activation of the neuronal nNOS and ROS formation (Lafon-Cazal *et al.*, 1993; Randall and Thayer, 1992). NO overproduction by nNOS activation and the increased formation of ROS induced neuronal cell death (Reynolds and Hastings, 1995).

Antioxidant, GSH and antioxidant enzyme, GR protected neuronal cell against oxidative stress. Cysteine is important for biosynthesis of proteins and the antioxidant GSH via system x_c^- (Conrad and Sato, 2012).

Glutamate-induced neuronal cell death was associated with the loss of GSH level and activity of GR by inhibition of cysteine uptake into cells via the cysteine/glutamate transport system (Lewerenz *et al.*, 2006). HT22 cells, immortalized cell lines derived from mouse hippocampus are model for studying oxidative glutamate toxicity caused by lack the ionotropic glutamate receptor. HT22 cells also essential properties of functional cholinergic neurons (Liu and Suo, 2009).

In this study, we investigated the neuroprotective effect of SFC in HT22 cells and SFC protected HT22 cells against glutamate-induced oxidative cytotoxicity. SFC inhibited ROS generation, Ca^{2+} influx, NO production, and restored activity of GSH and GR in HT22 cells. Moreover, SFC showed DPPH radical scavenging activity. Previously, study has reported that SFC exhibited an antioxidative activity (He *et al.*, 2011).

Therefore, the neuroprotective effect of SFC against glutamate-induced cell death was thought to be mediated through attenuation of oxidative stress.

Bcl-2-family proteins can affect the levels of releasable Ca^{2+} and lead to mitochondrial permeability transition (Nutt *et al.*, 2002). Bax, one of the proteins of the Bcl-2 family is proapoptotic protein and promote apoptosis of cells (Wyllie *et al.*, 1980; Deckwerth *et al.*, 1996). Bax activity appears to involve subcellular translocation and dimerization (Gross *et al.*, 1999). Overexpression of Bax induces the release of cytochrome c, which activates Apaf-1, which is associated caspase-independent activity (Epand *et al.*, 2002). Caspase-3 activity is an important signaling molecule in apoptotic process and affects the function of mitochondria by ROS and NO (Moncada and Erusalimsky *et al.*, 2002; Zhang and Bhavnani, 2005). Overexpression of Bax protein, followed by up-regulated activity of caspase-3 induces cell death (Xiang *et al.* 1996).

In present study, glutamate increases the Bax expression and caspase-3 activation and induces mitochondrial dysfunction. In contrast, SFC decreases the levels of Bax and caspase-3 and protects mitochondrial membrane potential.

We analyzed the contents of seven phenolic acids in SFC and content of gallic acid higher than other compounds. Contents of gallic acid, vanillic acid and trans-ferulic acid in *C. lanceolata* were increased by steaming and fermentation process. Previous study demonstrated that cognitive effect and neuroprotective effect of *C. lanceolata* was increased by fermentation process and was related with increased contents of gallic acid and vanillic acid (Weon *et al.*, 2013).

Gallic acid and vanillic acid showed neuroprotective effect, AchE inhibitory activity and antioxidant effect (Ban *et al.*, 2008; Ghayur *et al.*, 2011; Gan *et al.*, 2011; Weon *et al.*, 2012). Ferulic acid was also protected against memory and learning deficit induced β -amyloid peptide in mice and had antioxidant activity (Cho *et al.*; 2005). Thus, it seems that gallic acid, vanillic acid and ferulic acid may affect effect of SFC.

In conclusion, SFC effectively decreased glutamate-induced hippocampal HT22 cell death thorough inhibiting Ca^{2+} influx, ROS production, NO production and. In addition, up-regulation of GSH and GR, and amelioration of mitochondrial dysfunction by inhibiting of Bax and caspase-3 expression was associated with neuroprotective effect of SFC.

Therefore, SFC may have therapeutic potential for neurodegenerative diseases, such as Alzheimer's disease.

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