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Quantitative Analysis of Compounds in Fermented Insampaedok-san and Their Neuroprotective Activity in HT22 Cells

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Abstract – Insampaedok-san is a traditional medicine used for the treatment of colds. We investigated several compounds in Insampeadok-san, and tested their neuroprotective and anti-oxidative activities after fermentation with *Lactobacillus*. The amounts of four marker compounds (ferulic acid, hesperidin, 6-gingerol and glycyrrhizin) and unidentified compounds in Insampaedok-san (IS) and fermented Insampaedok-san (FIS) were measured and compared by an established HPLC-DAD method. Neuroprotective activity of IS and FIS extracts was evaluated and compared after glutamate-induced neurotoxicity in HT22 cells. Anti-oxidative activity of IS and FIS was also compared in DPPH free radical, hydroxyl radical and hydrogen peroxide scavenging activity assays. Contents of two compounds, ferulic acid and glycyrrhizin were decreased while 6-gingerol was increased by fermentation. FIS showed more potent neuroprotective activity than IS. DPPH, hydroxyl radical and hydrogen peroxide scavenging was slightly increased by FIS when compared to IS. In conclusion, fermentation with *Lactobacillus* can vary the amounts of the marker compounds in IS and improve neuroprotective and anti-oxidative activities of IS.
Keywords – Insampaedok-san, fermentation, marker compound, neuroprotective activity, anti-oxidative activity

Introduction

As life expectancy increases in humans, neurodegenerative disorders such as Alzheimer's disease, Parkison's disease and Huntington's disease affect more elderly people worldwide. The formation of reactive oxygen species (ROS) or oxidative stress causes neurodegenerative disorders (Coyle and Puttfarcken, 1993; Satoh *et al*., 1998; Smith *et al*., 1991). Glutamate is the excitatory neurotransmitter but can also induce neuronal cell death. The pathogenesis of neurodegenerative disorders is varied and complicated (Choi, 1998; Davis *et al*., 1994; Fukui *et al*., 2009). Mouse hippocampal HT22 cells have been used as a model system for studying the mechanism of glutamate-induced neuron cell death (Braun *et al*., 2000; Liu *et al*., 2009). Traditional medicine includes many compounds from herb components. These medicines appear to have numerous effects and have been used in various diseases (Jiang, 2005). Therefore traditional medicines may be used either as remedies or for the prevention of neurodegenerative disorders.

Insampaedok-san has been used as a Korean traditional oriental medicine for the treatment of cold-related symptoms. Effects of Insampaedok-san including analgesia and antipyresis were reported in the past (Shim and Kim, 1984). Insampaedok-san is composed of 12 herbs, *Lonicera japonica* Thunberg, *Forsythia viridissima* Lindley, *Schizonepeta tenuifolia* Briquet, *Saposhnikovia divaricata* Schiskin, *Panax ginseng* C. A. Meyer, *Bupleurum falcatum* Linne, *Angelica decursiva* Franchet et Savatier, *Ostericum koreanum* Maximowicz, *Aralia continentalis* Kitagawa, *Citrus aurantum* Linne, *Playtcodon grandiflorum*, *Cnidium officinale* Makino, *Poria cocos* Wolf, *Glycyrrhiza uralensis* Fischer, *Zingiber officinale* Roscoe and *Mentha arvensis* Linne var.

Bio-conversion such as fermentation can maximize absorption of the effective components in herbs as well as increase the bioactivity of those herbs. Research on the quality and effective improvement of medical herbs by fermentation with microorganisms has been recently conducted (Kim et al., 2009; Doh *et al*., 2010; Hyon *et al*., 2009).

In this study, we fermented Insampaedok-san by *Lactobacillus*. *Lactobacillus* has been widely used as a functional food material. *Lactobacillus* inhibited the

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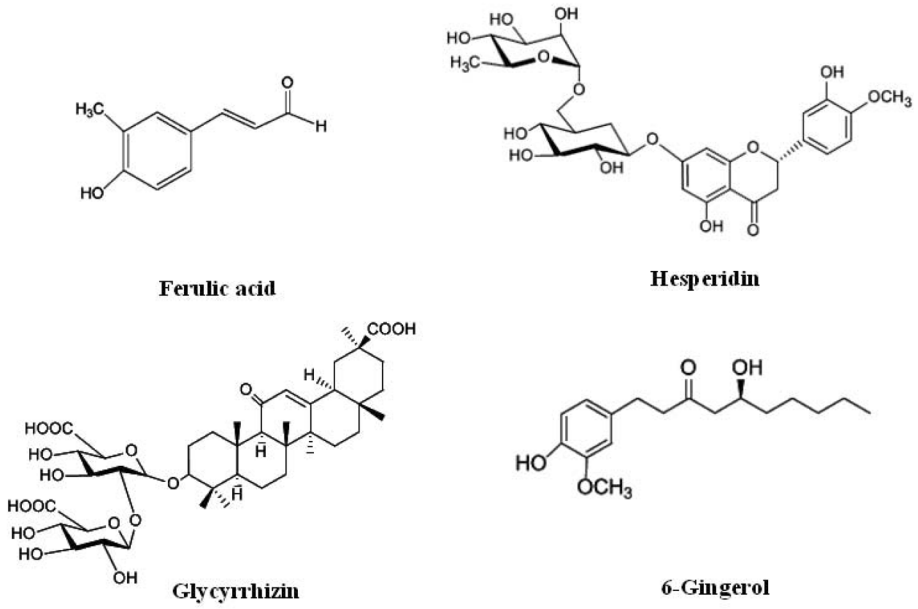

Fig. 1. Chemical structures of four marker constituents in Insampaedok-san.

growth of some harmful bacteria by the production of lactic acid and had therapeutic effects including anti-inflammatory and anti-cancer activities (Chen *et al*., 2009; Goldin, 1998). To determine the change of compounds in insampaedok-san after fermentation, four marker compounds, ferulic acid (*Cnidium officinale* Makino), hesperidin (*Citrus aurantum* Linne), 6-gingerol (*Zingiber officinale* Roscoe) and glycyrrhizin (*Glycyrrhiza uralensis* Fischer) were selected (Fig. 1.). Amounts of the four marker compounds in Insampaedok-san (IS) and fermented Insampaedok-san (FIS) were measured by an established HPLC-DAD method. Further, the neuroprotective activities against glutamate-induced cytotoxicity of IS was evaluated in HT22 cells by the MTT assay and was compared to the effects of FIS. We also investigated the anti-oxidative activity of IS as compared to FIS in DPPH, hydroxyl radical and hydrogen peroxide scavenging activity assays and confirmed their neuroprotective and anti-oxidative activities.

Experimental

Materials and reagents – The powder of a Insampaedok-san sample (3.0 g) was obtained from the Korea Institute of Oriental Medicine. HPLC grade solvents (water and methanol) were purchased from J.T. Baker (USA). Analytical grade trifluoroacetic acid (TFA) was obtained from DAE JUNG (Korea). Marker compounds including ferulic acid was purchased from Sigma (USA), while 6-gingerol, glycyrrhizin and hesperidin were purchased from the Korea Food & Drug Administration. The purities of the four standard compounds were greater than 98%. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) was obtained from Gibco BRL. Co. Glutamate and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carbboxylic acid (trolox), 3-(4,5 –dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) and 2,2-azinobis(3-ethylbenthiazolin)-6-sulfonic -acid (ABTS) were purchased from Sigma (USA).

Fermentation of Insampaedok-san – Bacterial strains, *Lactobacillus casei* KFRI 129 was derived from Korea Food Research Institute (KFRI, Korea). After successful transfer of the test organisms in MRS broth for *Lactobacillus* spp. at 37 °C for 24 h, the activated culture was again inoculated into each broth under the same conditions. It was properly diluted to obtain an initial population of $1 - 5 \times 10^7$ CFU/ml and served as the inoculum. The Insampaedok-san water extract was used as the culture media for fermentation after adjusting the pH to 7.0 using 1 M NaOH and autoclaving for 15 min at 121 °C. After cooling, 750 ml of IS was inoculated with 7.5 ml inoculums as described above. This was incubated at 37 °C for a period of 48 h. Fermented insampaedok-san was prepared into a powder by freeze-drying.

Preparation of samples – The powers of IS (100 mg) and FIS (100 mg) were weighed accurately and dissolved in 10 ml of 60% methanol. This sample solution was stored at 4 °C and filtered through a 0.45 μm membrane filter before HPLC analysis and analysis in the bioassays.

Analysis of compounds in IS and FIS – The HPLC system was a Dionex Ultimate 3000 system (Germany) equipped with a pump (LPG 3X00), an auto sampler (ACC-3000), a column oven (TCC-3000SD) and diode array UV/VIS detector (DAD-3000(RS)). System control and data analyses were executed by Dionex ChromelonTM Chromatography Data System. The analysis of compounds in the IS and FIS samples was conducted using a SHISEIDO C_{18} column (5 μm, 4.60 mm I.D. × 250 mm) at 35 °C. The mobile phase consisted of methanol (A) and water with 0.1% TFA (B) at a flow rate of 1 ml/min. The mobile phase system used was a gradient of solvent A and solvent B as follows; 0 - 10 min, 20% A; 10 - 15 min, 30% A; 15 - 40 min, 30 - 40% A; 40 - 45 min, 40 - 60% A; 45 - 55 min, 60 - 70% A; 55 - 60 min, 70% A. According to the maximal UV absorption of each four compounds, UV wavelength of the DAD detector was set. The UV wavelength was set at 250 nm for glycyrrhizin, 280 nm for hesperidin and 6-gingerol and 320 nm for ferulic acid. The sample injection volume was 20 μl.

Cell culture and cell viability assay – The mouse hippocampal HT22 cells were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 1% penicillin/streptomycin, $NaHCO_3$ (2 mg/ml) and 15 mM HEPES. They were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. HT22 cells were obtained from Seoul National University, Korea. Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay according to the modified procedure described previously by Mosmann (1983). HT22 cells were seeded in 48-well plates at a density of 6.7×10^4 cells / 300 μl. After one day, different concentrations of sample was added to wells that were then treated with 2 mM glutamate for 24 hours. Media with a concentration 1 mg/ml of MTT solution was then added into each well and was incubated for 3 h at 37 °C. Culture medium was removed and the dark blue formazan crystal was dissolved in 300 μl of DMSO. Optical density (OD) was measured at 570 nm using an ELISA plate reader. The neuroprotective activity of samples was performed by a relative protection ratio (%). Relative protection (%) was calculated using the following formula: (OD of glutamate-treated with sample – OD of glutamate-treated) / (OD of control – OD of glutamate-treated).

DPPH radical scavenging assay – The DPPH assay was used for the determination of free radical-scavenging activity of IS and FIS. 150 μl of varying concentrations of each sample was added to 150 μl of 0.4 mM DPPH-methanol solution in a 96-well plate. After 30 min in a darkroom, reduction of the DPPH free radical was measured by recording the absorbance at 517 nm. The assay was repeated three times. % Inhibition was calculated by the equation (1 – sample absorbance / DPPH absorbance) × 100.

Hydrogen peroxide scavenging assay – The hydrogen peroxide scavenging activity was measured according to the method previously described by Muller (1985). 80 μl sample at various concentrations and 100 μl of phosphate buffer (pH 0.5, 0.1 M) were mixed with 20 μl of 10 mM H_2O_2 in a 96-well microplate and incubated at 35 °C for 5 min. After incubation, 30 μl of 1.25 mM ABTS (2,2-azinobis(3-ethylbenzthiazolin)-6-sulfonicacid) and 30 μl of 1 unit/ml peroxide were added and incubated at 35 °C for 10 min. The absorbance was then measured at 405 nm. Hydrogen peroxide scavenging activity (%) was calculated in the same way as in the DPPH assay.

Hydroxyl radical scavenging assay – Hydroxyl radical scavenging activity was performed using a method previously described by Chung *et al*. (1997). The reaction mixture contained 200 μl of 10 mM $FeSO_4 \cdot 7H_2O$, 200 μl of 10 mM EDTA and 200 μl of 10 mM 2-deoxyribose in a conical tube. A 200 μl of each concentration of sample and 1 ml of phosphate buffer solution (0.1 M, pH 7.4) were added together. Then 200 μl of 10 mM H_2O_2 was added. After incubation at 37 °C for 4 h, 1 ml of TCA (2.8%) and 1 ml of TBA (0.1%) was added to the reaction mixture and maintained in a boiling water bath for 10 min. The absorbance was measured at 532 nm. Hydroxyl radical scavenging activity (%) was calculated the same way as in the DPPH assay.

Results and discussion

Analysis of compounds in IS and FIS – To determine the change of compounds in IS by fermentation, the four marker compounds, ferulic acid, hesperidin, glycyrrhizin and 6-gingerol were analyzed by an established HPLC-DAD method and compared with and without fermentation. Ferulic acid and glycyrrhizin in IS were decreased by 16.23% and 8.93%, respectively after fermentation. 6-Gingerol was increased by 40.81% by fermentation (Table 1). As shown in Table 2, peak areas of unknown compounds (1) and (2) were also decreased by fermentation.

Neuroprotective activity assay – The neuroprotective effects of IS and FIS on glutamate-induced cytotoxicity in HT22 cells were evaluated at concentrations of 10 μg/ml and 100 μg/ml using the MTT assay. The relative protection ratio (%) of FIS (32.34% at 10 μg/ml and 41.56% at 100 μg/ml) was much higher than the relative

Table 1. Comparison of four marker compounds in the IS and FIS

Sample	Content (μg/mg)			
	Ferulic acid	Hesperidin	6-Gingerol	Glycyrrhizin
IS	0.404 ± 0.005	0.516 ± 0.004	0.010 ± 0.001	4.195 ± 0.226
FIS	0.339 ± 0.013**	0.515 ± 0.001	0.014 ± 0.002*	3.821 ± 0.018*

*p < 0.05, **p < 0.01, ***p < 0.001 vs. IS (ANOVA)

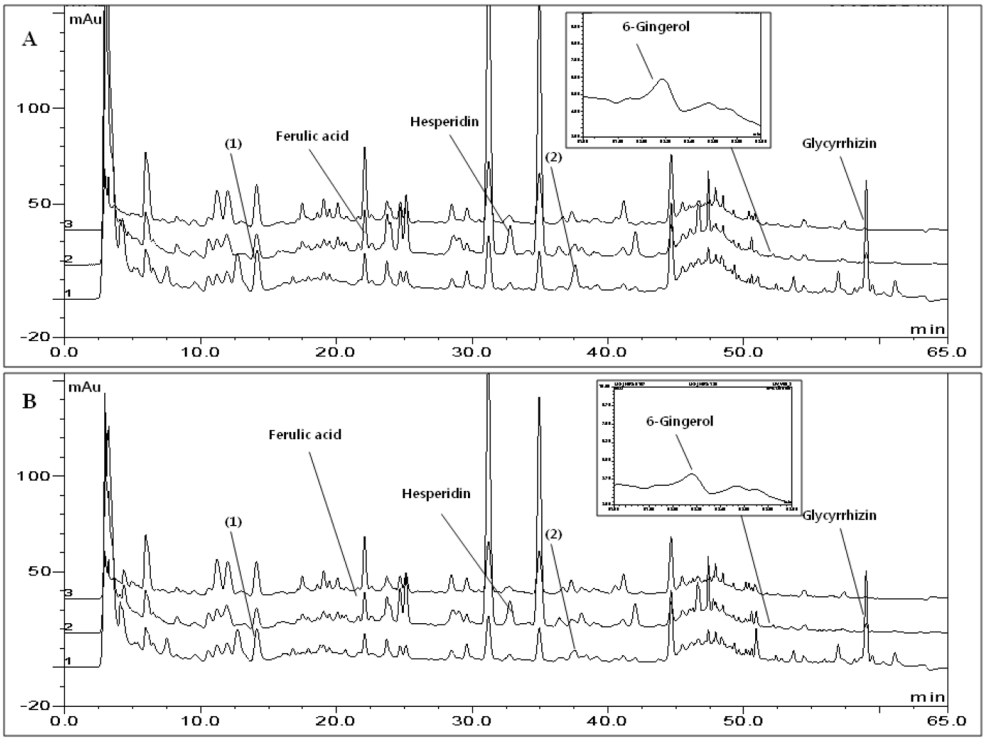

Fig. 2. HPLC chromatograms of IS (A) and FIS (B). (1 = 250 nm, 2 = 280 nm, 3 = 320 nm).

Table 2. Comparison of peak areas of (1) and (2) unknown compounds in the IS and FIS

Sample	Peak area value (mAU*min)	
	Peak (1)	Peak (2)
IS	8.468 ± 0.066	4.926 ± 0.105
FIS	6.823 ± 0.012***	1.963 ± 0.0368

*p < 0.05, **p < 0.01, ***p < 0.001 vs. IS (ANOVA)

protection ratio (%) for IS (3.21% at 100 μg/ml) (Fig. 2). The neuroprotective activity was increased by 373.83% (10 μg/ml) and 1194.647% (100 μg/ml) after fermentation.

Anti-oxidative activity assay – The anti-oxidative activity of IS and FIS was investigated by using DPPH, hydroxyl radical and hydrogen peroxide scavenging assays. The DPPH radical and hydrogen peroxide scavenging activities of FIS were slightly higher than IS. Hydroxyl radical scavenging activity of FIS was higher

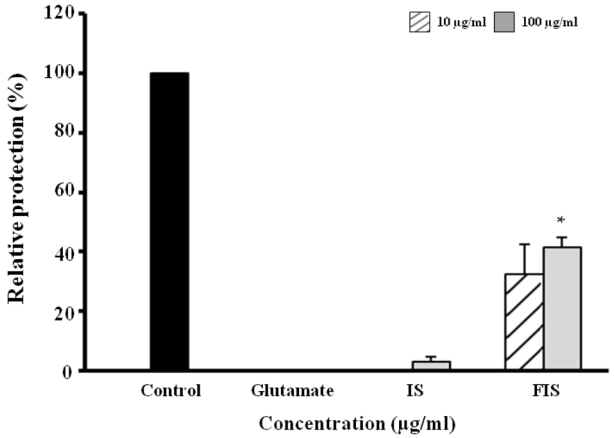

Fig. 3. Neuroprotective effects of IS and FIS on glutamate-induced cytotoxicity in HT22 cells. HT22 cells were treated with 10 and 100 μg/ml of IS and FIS and incubated for 24 h with glutamate (2 mM). The positive control, trolox (50 μM) exhibited a relative protective activity (82.04 ± 0.87%). Each bar represents the mean ± SD of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 vs. IS (ANOVA).

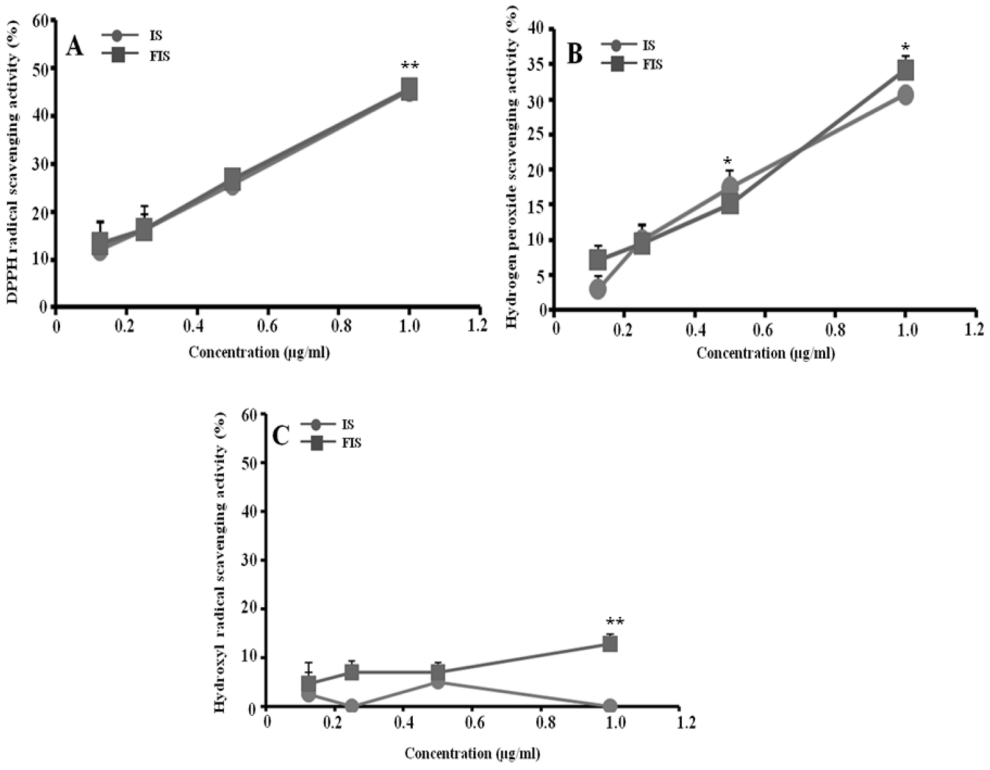

Fig. 4. Antioxidant effects of IS and FIS. (A) DPPH radical scavenging activity, (B) hydrogen peroxide scavenging activity, (C) hydroxyl radical scavenging activity. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. IS (ANOVA).

than IS. Therefore, improvement of anti-oxidative activity by fermentation was noted (Fig. 3).

Conclusion

In this study, alteration of the amounts of ferulic acid, hesperidin, glycyrrhizin and 6-gingerol in Insampaedok-san after fermentation by *Lactobacillus casei* KFRI 129 was analyzed. Neuroprotective activity and anti-oxidative activity of fermented Insampaedok-san was evaluated and compared with Insampaedok-san. We determined that the concentrations of three marker compounds in Insampaedok-san and other compounds such as the unknown compounds (1) and (2) were altered and the neuroprotective and anti-oxidative activities of Insampaedok-san was improved through fermentation. In conclusion, we demonstrate the enhancement of neuroprotective and anti-oxidative activities by fermentation of Insampaedok-san and demonstrated that neuroprotective activity of IS and FIS is associated with oxidative stress-induced cell death. Further research on converted compounds and newly identified compounds by fermentation and bioactivity of fermented herbal medicines is required.

Acknowledgments

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