Neuroprotective Effect of Halophyte Salicornia herbacea L. Is Mediated by Activation of Heme Oxygenase-1 in Mouse Hippocampal HT22 Cells

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ABSTRACT  
Salicornia herbacea L. (glasswort, tungtungmadi in Korean), a halophyte that grows in salt marshes and muddy seashores along the western coast of Korea, has been used as a seasoning vegetable and a folk medicine for intestinal ailments, nephropathy, and hepatitis. As the salt-tolerant herb was reported to contain antioxidants, including tungtungmadi acid, quercetin, and chlorogenic acid, we hypothesized that the ethanolic extract of S. herbacea L. (SH extract) enriched with antioxidative compounds will have neuroprotective activity. The herbal extract and its methylene chloride (MC) fraction showed a strong protective effect against glutamate-induced cell death in murine hippocampal HT22 cells. In addition, SH extract and MC fraction not only scavenged reactive oxygen species efficiently but also caused nuclear translocation of the nuclear factor (erythroid-derived 2)-like 2 and subsequently significant induction of antioxidant enzymes such as NAD(P)H:quinone oxidoreductase, heme oxygenase 1 (HO-1), and glutathione reductase. Inhibition of the antioxidant enzyme HO-1 by tin protoporphyrin abolished the neuroprotective effect of the SH extract, suggesting an important role of HO-1 in protection against glutamate-induced neural damage. Metabolite profiling for ethanolic extract and solvent fractions of the herb suggested that diosmetin and a few unidentified compounds were responsible for the neuroprotective effect. Taken together, SH extract and its MC fraction exhibited a neuroprotective effect through Nrf2-mediated induction of antioxidant enzymes, such as HO-1, and warrant further in vivo and clinical studies to confirm its effects and potentially develop a neuroprotective salt substitute or dietary supplement.

KEYWORDS: • diosmetin • HO-1 • neuroprotection • Nrf2 • Salicornia herbacea L.

INTRODUCTION

Salicornia species, also called glasswort, is used as a food ingredient in North America, Europe, South Africa, and South Asia, and a medicinal herb for treating and/or alleviating constipation, obesity, diabetes, and cancer in Korea. The herb is also used as an alternative to pure salt because it contains a high content of salt (9.51 g NaCl/100 g). Salicornia herbacea L. was reported to exhibit antioxidative, anti-inflammatory, immunomodulatory, antihyperglycemic, and antihyperlipidemic activities. In particular, it has been reported to contain antioxidant compounds such as tungtungmadi acid, isorhamnetin 3-O-beta-D-glucoside, and quercetin 3-O-beta-D-glucoside. Oxidative stress is a prominent feature in some neurodegenerative diseases, including Alzheimer’s disease (AD), Parkinson’s disease, amyotrophic lateral sclerosis, and Huntington’s disease. Notably, mitochondrial dysfunction is a prominent feature in AD, which most likely is of critical importance in the genesis and amplification of reactive oxygen species (ROS) and the pathophysiology of the disease.

Several studies showed that antioxidants could protect against ROS-mediated neuronal damage. In particular, the altered expression of phase 2/antioxidant enzyme genes in postmortem tissues of patients with various neurological diseases was reported in a series of studies, suggesting an essential role for phase 2/antioxidant enzymes in the progression of such conditions. Furthermore, recent findings reveal that Nrf2-mediated overexpression of antioxidant enzyme genes, either by genetic or chemical approaches, confers neuroprotection in vitro and in vivo.

The phytochemicals present in S. herbacea such as tungtungmadi acid, quercetin, chlorogenic acid, and their glycosides contain multiple hydroxyl groups, making them highly electrophilic. The electrophilic compounds that usually show antioxidant activity are well defined for their mechanism to induce antioxidant enzymes through the Nrf2 signaling pathway and, thereby, exert protective effects against ROS-induced neuronal cell damage.

From the preliminary study we found that 80% ethanol extract (extract of S. herbacea L. [SH extract]) and its
methylene chloride (MC)-soluble fraction exhibited relative strong antioxidant activity, compared to the other fractions. Therefore, the present study was conducted to examine whether SH extract and its MC fraction, presumably enriched with the phytochemicals mentioned above, induces phase 2/antioxidant enzymes and, thereby, exerts neuroprotective effects, and to identify the compounds responsible for the effect.

MATERIALS AND METHODS

Materials

All reagents used were of ACS grade, and most of the reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). *S. herbacea* was harvested in Sinan-gun, Jeollanam-do (Republic of Korea) in June 2012, followed by air-drying in the shade. A mouse hippocampal cell line (HT22) was obtained from Professors Lee, Dong Seok and Song, Kyung Sik (Kyungpook National University, South Korea).

Preparation of the *S. herbacea* extract and its fractionation

The dried aerial part of *S. herbacea* was powdered using a Cyclotec mill (Tecator, Sweden) and was extracted with 20 volumes of 80% (v/v) ethanol for 12 h at 80°C, followed by concentration through rotary evaporation at 40°C and freeze-drying to yield a powder (SH extract). Subsequently, the ethanolic extract was subjected to sequential fractionation with n-hexane (Hex), MC, ethyl acetate (EtOAc), *n*-butanol, and water according to the polarity of the compound(s) of interest. The 80% ethanol extract (SH extract) and MC fraction showed the strongest antioxidant activity and were, hence, used as test agents.

Cell viability assay

HT22 cells were seeded into a 96-well culture dish at a density of 2×10^3 cells per well in Dulbecco’s modified Eagle’s medium (DMEM) (Welgene, Gyeongbuk, South Korea) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA). The next day 50–200 µg/mL for SH extract and 10–50 µg/mL for solvent fractions in the absence or presence of 5 mM glutamate were added to cell culture media. After an additional incubation for 20 h, the cells were stained with either 4’,6-diamidino-2-phenylindole (DAPI) or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to assess apoptosis and cell viability, respectively. To examine the effect of tin protoporphyrin (SnPP), a competitive inhibitor of heme oxygenase 1 (HO-1), one of the major antioxidant enzymes, on glutamate-induced cytotoxicity, HT22 cells (2×10^3 cells) were plated into a 96-well plate and were cultured for 24 h, treated with SnPP (10–40 µM), and dissolved in serum-free media. After a subsequent 2-h incubation, the SH extract (200 µg/mL) or its MC fraction (50 µg/mL) was added to the cells. After incubating for 10 h, the MTT solution was added to each well and after reacting for 2 h, the absorbance was measured at 570 nm/600 nm ref. Cell viability was calculated as the percentage of the controls.

Mitochondrial membrane potential assay

Mitochondrial membrane potential (MMP) was determined by staining with JC-1 dye as described previously. Briefly, HT22 cells were plated onto a 24-well culture plate containing 0.1% gelatin-coated cover glass (12 mm diameter; Fisher Scientific, Hampton, NH, USA) at a density of 3×10^4 cells per well. After culturing the cells for 18 h in DMEM (+10% FBS) at 37°C in CO₂ incubator, the medium was changed into serum-free medium containing the designated sample in the presence or absence of 5 mM glutamate, followed by incubation for another 12 h. The cells were stained with 2 µg/mL JC-1 for 20 min and fixed with 3.7% formalin solution. The mounted cells were visualized by a fluorescence microscope (Eclipse 80i, Nikon, Tokyo, Japan).

ROS scavenging assay

Oxidative stress was quantified in cells by the 2,7-dichlorofluorescin (DCF) assay according to Wang and Joseph, with slight modifications. For routine maintenance, HT22 cells were grown in DMEM (Gibco) supplemented with 10% heat-inactivated FBS at 37°C under an atmosphere of 5% CO₂/95% air and saturating humidity. The cells were passaged every other day (1:4 split ratio) by trypsinization with 0.25% trypsin/0.02% EDTA sodium salt solution (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

The cells (2×10^3 cells per well) were seeded into black-bottom 96-well plates and were cultured for 24 h. After cell attachment, the plates were washed with phosphate-buffered saline (PBS), and the cells were incubated with glutamate (5 mM) for 2 h before treatment with increasing concentrations of the SH extract or its MC fraction prepared in 10% FBS containing media for 12 h. The stimulated cells were washed with PBS and were incubated for 30 min with dihydroethidium (DHE) dye followed by a published staining protocol with modifications. All procedures were performed the same as described in a DCF staining method before DHE treatment. Stimulated cells were treated with 2 µM DHE dye dissolved in DMSO. Cells were washed with warm PBS once. Fluorescence was detected at an excitation of 540 nm and an emission of 590 nm. Results are expressed as relative intensity of fluorescence (as percentage of negative control). For visualization, cell images were detected using fluorescence microscope (Eclipse 80i Nikon).
**Assessment of the NAD(P)H:quinone oxidoreductase 1 activity**

Mouse hippocampal HT22 cells were treated with various concentrations of the SH extract for 24 h after which the activity of the antioxidant enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1) was measured. The enzyme activity of cell homogenates was measured using a spectrophotometric assay in which the rate of reduction of 2,6-dichlorophenolindophenol was monitored at 600 nm.19 The specific activities of the enzymes were normalized to the protein concentration, which was determined in duplicate using the Lowry method.20 All values were expressed as the mean ± standard deviation (SD).

**Western blotting**

SH extract- or MC fraction-treated HT22 cells were washed with precooled PBS twice and were lysed in precooled lysis buffer (TBS/T; Tris-HCl 20 mM, NaCl 145 mM, glycerol 10%, EDTA 5 mM, Triton-100 1%, and Nonidet P40 0.05%). After 30 min of incubation on ice, the cell extracts were cleared by centrifugation at 12,000g for 10 min at 4°C, and the supernatants were denatured in sample buffer for 5 min at 95°C. Proteins were separated by electrophoresis on a 10–12% sodium dodecyl sulfate–polyacrylamide gel for 1 h at 200 V and were transferred onto PVDF membranes (Millipore, Bedford, MA, USA) for 50 min at 110 V. The membranes were incubated with anti-NQO1, anti-HO-1, anti-glutathione reductase (GR), anti-nuclear factor (erythroid-derived 2)-like 2 (Nrf2), anti-lamin B, and anti-ß-actin antibodies at a dilution of 1:1000 in TBS/T overnight at 4°C. The protein bands were detected using a Chemiluminesence Kit (Pierce, Cheshire, UK). Densitometry analysis was performed using the Multi Gauge V3.0 software (Fujifilm, Tokyo, Japan).

**Measurement of the antioxidant responsive element-reporter gene transcriptional activity**

HT22-ARE cells were stably established by transfection with pGL4.37 (luc2P/ARE/hygromycin) vector containing four copies of an antioxidant responsive element (ARE) that drives the transcription of the luciferase reporter gene (Promega, Fitchburg, WI, USA). The HT22-ARE cells were grown in DMEM containing 10% FBS, 100 μM hygromycin, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. The luciferase activity was determined as per the manufacturer’s instructions (Promega Corp., Madison, WI, USA). Briefly, the cells were incubated with different concentrations of the SH extract and its MC fraction in the absence and presence of 5 mM glutamate for 12 h after which the ARE-luciferase reporter gene activity was determined. The luminescence intensity was detected using a luminometer (GloMax 96; Promega GmbH, Mannheim, Germany). The luciferase activity was normalized against the protein concentration as determined by the Bradford protein quantification analysis (Bio-Rad, Richmond, CA, USA).

**GC-TOF-MS analysis**

For GC-TOF-MS analysis, each 1 μL aliquot sample was injected into Agilent 7890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA), coupled with an Agilent 7693 auto sampler and equipped with a Pegasus high-throughput-Time of flight-Mass Spectrometry (LECO, St. Joseph, MI, USA) system. Metabolites were separated by Rtx-5MS column (30 m inner diameter × 0.25 mm length, with 0.25 μm particle size, Restek Corp., Bellefonte, PA, USA), and helium was used as the carrier gas at a constant flow rate of 1.5 mL/min. The split ratio of each sample was 10:1. The front inlet, transfer line, and ion source temperatures were set at 250, 240, and 230°C, respectively. The oven temperature program for metabolome analysis was maintained at 75°C for 2 min, then increased to 300°C at 15°C/min, and which was maintained at the final temperature for 3 min. Electron ionization was performed at 70 eV, and mass data were collected by full scanning over a mass-to-charge ratio (m/z) range of m/z 45–1000.

**UPLC-Q-TOF-MS analysis**

Ultra-performance liquid chromatography-quadrupletime of flight-mass spectrometry was used to analyze secondary metabolites. Each dried sample was redissolved with DMSO and methanol. Waters UPLC Acuity system (Waters, Milford, MA, USA) coupled with a binary solvent delivery system, a UV detector, and an autosampler. Metabolites were separated on an Acquity UPLC BEH C18 column (100 × 2.1 mm; Waters; 1.7 μm particle size). The injection volume was 5 μL, and the flow rate was maintained at 0.3 mL/min. The mobile phase consisted of solvent A (0.1% [v/v] formic acid in water) and solvent B (0.1% [v/v] formic acid in acetonitrile) and the gradient conditions were increased from 5% to 100% of solvent B over 10 min and then decreased back to 5% over 2 min. In case of MS experiments, the waters Q-TOF Premier (Micromass MS Technologies, Manchester, United Kingdom) were performed in negative (−) and positive (+) ion modes within a range of 100–1000 m/z. The operating parameters were as follows: ion source temperature, 100°C; cone gas flow, 0.0 L/h; desolvation gas flow, 650 L/h; capillary voltage, 2.5 kV; and cone voltage, up to 50 V. As reference lock mass, leucine enkephalin was used (m/z [−] 554.2615, [+] 556.2771) by independent LockSpray interference.

**Data processing and multivariate statistical analysis**

Raw data files of GC-TOF-MS were converted to NetCDF (*.cdf) format using LECO ChromaTOF software. The UPLC-Q-TOF-MS data were acquired with MassLynx software (version 4.1; Waters Corp.) and converted into NetCDF format (*.cdf) using the MassLynx DataBridge (version 4.1; Waters Corp.). After conversion, the NetCDF files were subjected to preprocessing, correction of retention time and baseline, and peak extraction using the MetAlign software package (www.metalign.nl). The resulting data were exported to a Microsoft Excel (Microsoft, Redmond,
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WA, USA). Multivariate statistical analysis was processed using SIMCA-P+ 12.0 software (Umetrics, Umea, Sweden). Principal component analysis and partial least squares discriminant analysis (PLS-DA) were performed to analyze metabolite differences between samples. The variables were selected based on variable importance in the projection (VIP) value, and significance was tested by analysis of variance (ANOVA). After multivariate statistical analysis, variables were identified using authentic standard compounds by comparing both mass spectra and retention time. When standard compounds were not available, a tentative identification was performed based on the MS spectra using the national institute of standards and technology (NIST MS Search Program, version 2.0, Gaithersburg, MD), combined chemical dictionary version 7.2 (Chapman & Hall/CRC), references, and an in-house library. Box and whisker plot analysis was performed using STATISTICA7 (version 7.0; StatSoft, Inc., Tulsa, OK, USA).

Statistical analysis

Statistical significance of the data was tested by ANOVA, followed by Duncan’s multiple range test using the SPSS Statistics 22 software (SPSS, Inc., Chicago, IL, USA). The data were presented as the mean ± standard error. P < 0.05 was considered statistically significant. The mean values sharing a common letter were not statistically significant.

RESULTS

The protective effects of the S. herbacea extract and its MC fraction against glutamate-induced cytotoxicity

Treatment of HT22 cells with 5 mM glutamate caused significant cell death, whereas the MC fraction of the SH extract attenuated glutamate-mediated cell death (Fig. 1) as shown by DAPI staining of the DNA of the cells. That is, the MC fraction partially rescued cells from glutamate-induced cell death as assessed by chromatin condensation (Fig. 1). Furthermore, the inhibition of the antioxidant enzyme HO-I by SnPP dramatically attenuated the cytoprotective effect of the SH extract or its MC fraction, suggesting protective effects of antioxidant enzymes, including HO-I, against glutamate-induced cell death (Fig. 2).

Protective effects of S. herbacea extract and its MC fraction against glutamate-induced MMP dysfunction

Glutamate-induced neuronal cell death was associated with increased intracellular ROS generation, loss of MMP (ΔΨm), and release and translocation of apoptosis inducing factor from mitochondria into the cytosol as a result of abnormal levels of mitochondrial proteins.21 In this study, the change of glutamate-induced MMP was monitored using JC-1 dye staining. Under stable cell condition, JC-1 exists as red JC-1 aggregates in mitochondria, whereas it is converted to green fluorescent JC-1 monomer in mitochondria of neuronal cells treated with glutamate. As shown in Figure 3, the treatment of HT22 cells with glutamate caused a dramatic increase in the JC-1 monomer as shown by increased green fluorescence. However, simultaneous treatment with either SH extract or MC fraction significantly attenuated glutamate-induced MMP alteration, suggesting a protective effect of SH extract and MC fraction against glutamate-induced mitochondrial damage.

ROS scavenging activity of the S. herbacea extract and its MC fraction

The SH extract and its MC fraction significantly reduced glutamate-induced oxidative stress in HT22 cells as measured by the H2DCFDA fluorescent probe (Fig. 4). The glutamate-induced decline in the level of reduced glutathione (GSH) was partially restored by the concomitant treatment with the SH extract or its MC fraction (Fig. 5).

Effects of SH extract and its MC fraction on the Nrf2-signaling pathway and antioxidant enzyme expression

The enzyme activity of NQO1, an antioxidant and anti-carcinogenic biomarker enzyme, was dose dependently induced by the SH extract and its MC fraction in mouse hippocampal HT22 cells in the range of 50 to 200 μg/mL and 10 to 50 μg/mL, respectively (Fig. 6). The NQO1 induction by SH extract was synergistically increased by the presence of glutamate in the cell culture media.

The SH extract and its MC fraction caused a significant accumulation of nuclear Nrf2. In addition, both agents significantly enhanced ARE-luciferase activity, which is a transcriptional activity marker for antioxidant enzymes downstream of Nrf2, in a dose-dependent manner (Fig. 7). The expressions of NQO1, HO-1, and GR were also induced dose dependently by both the SH extract and its MC fraction (Fig. 8). The accumulation of nuclear Nrf2, ARE transcriptional activity, and the expression of NQO1, HO-1, and GR were slightly enhanced by the presence of glutamate in the cell culture media.

Primary Metabolite Profiling of solvent fractions by GC-TOF-MS

To investigate metabolite differences between aqueous ethanol (80%, v/v) extract of SH and other solvent fractions such as n-hexane, MC, ethyl acetate, n-butanol, and water, multivariate analysis was conducted. In Supplementary Figure S1 (Supplementary Data are available online at www.liebertpub.com/jmf), clear separation was shown between MC fraction and the other solvent fractions as shown by PC1 (35.52%) (Supplementary Fig. S1A). In addition, PLS-DA score plots showed similar separation pattern (Supplementary Fig. S1B). Significantly different metabolites among solvent fractions were selected based on VIP > 0.7 and P value < 0.05. A total of 25 metabolites, including 3 amino acids, 7 organic acids, 4 fatty acid, and 11 sugars and sugar alcohols, were identified with their mass fragment patterns and retention time with standard compounds and NIST (Supplementary Fig. S1). The relative levels of these metabolites were visualized with the box and whisker plots (Supplementary Fig. S2). As shown in Supplementary Figure S2, the levels of pyroglutamatic acid,
propanoic acid, malic acid, gluconic acid, some sugars, and sugar alcohols, including xylitol, fructose, glucose, glucitol, ribonic acid, myo-inositol, and turanose, were significantly different among samples.

Secondary Metabolite Profiling of solvent fractions by UPLC-Q-TOF-MS

To investigate metabolite differences between aqueous ethanol (80%, v/v) extract of SH and other solvent fractions, including n-hexane, MC, ethyl acetate, n-butanol, and water, multivariate analysis was conducted. The chromatograms for 80% ethanol extract and its five solvent fractions are shown in Supplementary Figure S3. In Supplementary Figure S4, clear separation was shown between MC and Hex fraction and the other solvent fractions by PC1 (21.21%) (Supplementary Fig. S1A). In addition, PLS-DA score plots showed similar separation pattern (Supplementary Fig. S1B). Significantly different secondary metabolites among solvent fractions were selected based on VIP >0.7 and \( P < .05 \) of PLS1 and PLS2. A total of 17 metabolites, including 5 flavonoids, 5 lysophospholipids, and 7 unidentified compounds, were selected (Supplementary Fig. S4).20–24 These secondary metabolites were tentatively identified by retention time and
molecular weight in comparison to standard compounds and references.25,26 Relative metabolite levels were visualized by box and whisker plots (Supplementary Fig. S5). Among 17 metabolites, diosmetin and four unidentified compounds showed relatively high levels in MC fraction than other fractions (Supplementary Fig. S5).

DISCUSSION

Our study found that the ethanolic extract and its MC fraction of S. herbacea L. exhibited protective effects against glutamate-induced cell death and mitochondrial dysfunction in mouse hippocampal HT22 cells. In addition,
the neuroprotective action by the samples appeared to be mediated by the induction of antioxidant enzymes, in particular, HO-1, which is again mediated by increased nuclear translocation of Nrf2. That is, the Nrf2 signaling pathway and its downstream antioxidant enzyme NQO1 were upregulated by the SH extract and its MC fraction, suggesting that the observed neuroprotective action of the SH extract was probably mediated by the induction of antioxidant enzymes.

Nrf2, a transcription factor that regulates the expression of numerous cellular antioxidant enzymes, such as NQO1, HO-1, glutathione reductase, γ-glutamyl cysteine ligase, and glutathione S-transferase, is present as a heterodimer with Keap1 in the unstressed cellular state and is degraded through ubiquitination in the proteosomal pathway. However, once separated from Keap1 by various modifiers, including certain phytochemicals, Nrf2 migrates into the nucleus, binds to the ARE in the promoter region of the relevant antioxidant enzymes’ genes, and promotes their expression. Since HO-1 inhibition by SnPP abolished the neuroprotective effect of both the SH extract and its MC fraction, the antioxidant action of HO-1 that was upregulated by the herbal extract or its MC fraction is most likely to play an essential role in its neuroprotective activity.

FIG. 4. The reduction of intracellular reactive oxygen species level by S. herbacea extract and its MC fraction. (A) Mouse hippocampal HT22 cells were treated with either the 80% ethanol S. herbacea (SH) extract or its MC fraction in the absence or presence of glutamate (5 mM), followed by measurement of the fluorescence generated by the addition of DCFDA or DHE. (B) The cells were stimulated with various concentrations of the SH extract and its hexane (Hex) and MC fraction for 6 h. Subsequently, 50 μM of DCFDA was added. The generated fluorescence was measured using a fluorescence microplate reader. The values are expressed as the mean ± SD (n = 10). Means without a common letter are significantly different (P < .05). DCFDA, dichlorofluorescein diacetate; DHE, dihydroethidium; SD, standard deviation; SH extract, extract of S. herbacea L.
Although the compounds responsible for neuroprotective activity or antioxidant enzyme induction in *S. herbacea* were not revealed from this study, several phytochemicals present in the herb might have contributed to the protective effect in combination. In particular, diosmetin, which was present at higher concentrations in the MC fraction than in the other solvent fractions, is one of the candidates that conferred relatively high neuroprotective effects of the MC fraction compared to the other fractions. Diosmetin was reported to have strong intracellular antioxidant,31 anti-inflammatory,32 anticancer, antimicrobial, and estrogenic activities. It is converted to luteolin in HepG2 cells by cytochrome P450 enzymes, as well as in breast adenocarcinoma cells that express CYP1 enzymes.33 Therefore, the possibility that luteolin converted from diosmetin could exert neuroprotective effects cannot be excluded, since luteolin was previously reported to provide neuroprotection in models of traumatic brain injury through the Nrf2-ARE pathway.34 In addition, the herb is also known to contain various other antioxidant compounds such as tungtungmadic acid (3-caffeoyl-4-dihydrocaffeoylquinic acid), quercetin 3-O-glucoside, and isorhamnetin 3-O-glucoside.10,35 These compounds are also major candidates for neuroprotection and antioxidant enzyme induction. In fact, the antioxidant tungtungmadic acid isolated from *S. herbacea* showed protective effects against tert-butyl hydroperoxide-induced hepatotoxicity in hepa1c1c7 cells.36 And the isolated antioxidant flavonol glucosides isorhamnetin 3-O-beta-d-glucopyranoside and quercetin 3-O-beta-d-glucopyranoside inhibited certain enzymes, such as rat lens aldose reductase and matrix metalloproteinase, in HT1080 cells, and prevented oxidation-induced cellular damage.37,38

Glutamate, which was used as a ROS generator in this study, is also an important endogenous excitatory neurotransmitter that plays a principal role in neural activation. It is neurotoxic at high levels in cells and is reported to be involved in the etiology of certain neurodegenerative disease such as dementia and Parkinson’s disease.39 Glutamate is also thought to be involved in the inhibition of *N*-acetylcysteine uptake, which results in decreased intracellular
glutathione levels and subsequently oxidative stress and cell death.\textsuperscript{40,41} Interestingly enough, glutamate synergistically activated Nrf2 signaling pathways in the presence of SH extract or MC fraction. That is, the herbal extract caused synergistic induction of Nrf2 signaling pathway and its downstream enzymes such as NQO1 (Figs. 7 and 8), HO-1, and GR, while glutamate itself did not stimulate the Nrf2 signaling pathway or its downstream gene expression; Nrf2 activator(s) in the sample appeared to act synergistically in the presence of glutamate by an unknown mechanism. For instance, there is a possibility that glutamate promotes intracellular transport of active compound(s) directly or indirectly, increasing cellular levels of the compound(s).\textsuperscript{42} Glutamate might also facilitate the separation of Nrf2 from Keap1 synergistically in combination with active component(s) in the herbal extract.

To further examine the precise mechanism underlying the observed neuroprotective effect, the isolation of active compound(s) from the MC fraction of the SH extract is required. In addition, cell culture systems have limitations in terms that the neuroprotective action of natural compounds or herbal extracts do not provide any information on the compounds’ transport across the blood–brain barrier, gastrointestinal absorption, and pharmacokinetics. Accordingly, our observations based on hippocampal HT22 cells

![FIG. 6. The effect of S. herbacea extract and its MC fraction on the nuclear Nrf2 level and the ARE transcriptional activity. (A) Mouse hippocampal HT22 cells were cultured for 24 h and were treated with the 80% ethanol S. herbacea (SH) extract and its MC fraction for 6 h, followed by western blot detection of the nuclear Nrf2 level. (B) The relative Nrf2 levels are expressed as densitometric values. (C) The cells were pretreated with the SH extract or its MC fraction in the presence or absence of glutamate for 12 h. ARE-luciferase reporter gene activity was determined as described in the Materials and Methods section (“Measurement of the ARE-reporter gene transcriptional activity”). ARE, antioxidant responsive element.]}
FIG. 7. The effects of the S. herbacea extract and its MC fraction on the intracellular expression levels of the antioxidant enzymes NQO1, HO-1, and GR. Mouse hippocampal HT22 cells were cultured for 12 h and were treated with the 80% ethanol S. herbacea (SH) extract and its MC fraction in the absence and presence of glutamate. The intracellular level of NQO1, HO-1, and GR was subsequently measured using western blotting (A). The relative protein levels are expressed as densitometric values (B). GR, glutathione reductase; NQO1, NAD(P)H:quinone oxidoreductase 1.

FIG. 8. NQO1 activity induction by S. herbacea extract and its MC fraction in the absence or presence of glutamate. Mouse hippocampal HT22 cells were treated with different doses of the 80% ethanol S. herbacea (SH) extract and its MC fraction for 12 h. The NQO1 activity was measured using a specific assay as described in the Materials and Methods section (“Assessment of the NQO1 activity”). The values represent the mean±SD (n=10). Means without a common letter are significantly different (P<.05).
need to be confirmed in a proper in vivo disease model. In conclusion, the neuroprotective potential of *S. herbacea* warrants further studies as the herb could be utilized as a functional food ingredient and a salt substitute for ameliorating neurodegenerative diseases.

**ACKNOWLEDGMENT**

This research was supported by Kyungpook National University Research Fund, 2014.

**AUTHOR DISCLOSURE STATEMENT**

No competing financial interests exist.

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