

Taraxacum coreanum protects against glutamate-induced neurotoxicity through heme oxygenase-1 expression in mouse hippocampal HT22 cells

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Abstract. *Taraxacum coreanum* Nakai is a dandelion that is native to Korea, and is widely used as an edible and medicinal herb. The present study revealed the neuroprotective effect of this plant against glutamate-induced oxidative stress in HT22 murine hippocampal neuronal cells. Ethanolic extracts from the aerial (TCAE) and the root parts (TCRE) of *T. coreanum* were prepared. Both extracts were demonstrated, by high performance liquid chromatography, to contain caffeic acid and ferulic acid as representative constituents. TCAE and TCRE significantly increased cell viability against glutamate-induced oxidative stress in mouse hippocampal HT22 cells. Western blot analysis revealed that treatment of HT22 cells with the extracts induced increased expression of the enzyme heme oxygenase-1 (HO-1), compared with untreated cells, in a concentration-dependent manner. Increased HO-1 enzymatic activity, compared with untreated cells, was also demonstrated following treatment with TCAE and TCRE. In addition, western blot analysis of the nuclear fractions of both TCAE and TCRE-treated HT22 cells revealed increased levels of nuclear factor erythroid 2 like 2 (Nrf2) compared with untreated cells, and decreased Nrf2 levels in the cytoplasmic fraction compared with untreated cells. The present study suggested that the neuroprotective effect of *T. coreanum* is associated with induction of HO-1 expression and Nrf2 translocation to the nucleus. Therefore, *T. coreanum* exhibits a promising

function in prevention of neurodegeneration. Further studies will be required for the isolation and the full characterization of its active substances.

Introduction

Oxidative stress is involved in the progress of neuronal degenerative disorders, such as multiple sclerosis (1). Glutamate is a crucial neurotransmitter for neural activation. However, when released as a result of neural injury, high concentrations of extracellular glutamate are toxic to neurons. Glutamate cytotoxicity is a result of non-receptor-mediated oxidative stress and receptor-initiated excitotoxicity (2). Mouse hippocampal neuronal HT22 cells have previously been used to examine the mechanism of glutamate-induced oxidative stress (3); because HT22 cells express low levels of glutamate receptors, glutamate-mediated cell damage in these cells is primarily due to non-receptor-mediated oxidative stress (4,5).

The rate limiting enzyme in heme catabolism is heme oxygenase-1 (HO-1; official gene symbol HMOX1), which is thus a crucial component in the cellular antioxidant system. HO-1 gene expression is inducible (6) and HO-1 has been demonstrated to be involved in cytoprotection against glutamate-induced oxidative damage in HT22 cells (7). Induction of HO-1 expression is performed at the transcriptional level, and its expression is regulated by nuclear factor erythroid 2 like 2 (Nrf2; official gene symbol Nfe2l2) (8). Nrf2 has been reported to induce expression of various antioxidant stress-related proteins, including glutathione (GSH) and HO-1 (9).

Taraxacum coreanum Nakai (Asteraceae family) is a dandelion native to Korea, and it is extensively consumed as a vegetable and used as a traditional therapeutic agent for inflammatory diseases in Korea. Previous studies of this species have revealed the presence of taraxinic acid-1'-O- β -D-glucopyranoside, of which the hydrolysate, taraxinic acid, possesses an anti-leukemic effect (10). The anti-inflammatory effect of a methanolic extract of the aerial part of *T. coreanum* has also been reported (11). In the present study, which aimed to investigate the neuroprotective potential of natural Korean

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medicinal sources, the cytoprotective effect of *T. coreanum* against oxidative stress was evaluated *in vitro* in HT22 cells.

Materials and methods

Preparation of plant extract. The whole plant of *Taraxacum coreanum* Nakai was collected from the Botanical Garden of Wonkwang University (Iksan, Korea) in May 2014. This species was identified by Dr Kyu-Kwan Chang, and the voucher specimen (WK-2014-028) was deposited in Wonkwang University. Each fresh aerial and root part (50 g) was soaked in 500 ml ethanol and left for 7 days at room temperature. Following filtration with filter paper, the solvent was dried using rotary evaporator to yield 7.5 g of aerial ethanolic extract (TCAE) and 12.8 g of root ethanolic extract (TCRE).

High performance liquid chromatography (HPLC). Chromatography was performed using a HPLC instrument of the YL-9100 series (YoungLin Instrument Co., Ltd., Anyang, Korea). In all experiments, a Capcell Pak C18 column (4.6x250 mm, 5 μ m; Shiseido Co., Ltd, Tokyo, Japan) was used as the stationary phase, and the injection volume was 20 μ l. Samples containing 2 mg/ml of TCAE or TCRE were prepared. The mobile phase consisted of water containing 0.1% formic acid (A) and acetonitrile (B) in a gradient system: 0-50 min linearly changed 10 to 50% B, 50-55 min linearly changed 50 to 100% B, 55-60 min remained at 100% B. The detection wavelength was adjusted to 254 nm, and the flow rate was 0.7 ml/min.

Chemicals and reagents. All cell culture reagents were obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Tin protoporphyrin (SnPP) and cobalt protoporphyrin IX (CoPP) were obtained from Frontier Scientific, Inc. (Logan, UT, USA). Caffeic acid, chlorogenic acid, ferulic acid, and all other chemicals, unless indicated otherwise, were obtained from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany).

Cell culture and viability assay. Mouse hippocampal HT22 cells were obtained from Professor Hyun Park (Wonkwang University). Cell culture and MTT assay were conducted as described previously (12). Briefly, a total of 2×10^4 cells/well were seeded in 96-well plates. They were pre-treated with the indicated concentration of TCAE or TCRE for 3 h, and this was followed by treatment with 5 mM glutamate. For measurement of cell viability, cells were maintained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at a final concentration of 0.5 mg/ml for 4 h, and the formazan formed was dissolved in acidic 2-propanol.

Western blot analysis. HT22 cells were harvested and pelleted by centrifugation at $200 \times g$ for 3 min. Subsequently, the cells were rinsed with PBS and lysed using radioimmunoprecipitation assay (RIPA) lysis buffer containing 25 mmol/l Tris-HCl buffer (pH 7.6), 150 mmol/l NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS for 15 min at 4°C, and then underwent centrifugation at $15,000 \times g$ for 10 min at 4°C. The protein concentration was determined using Bradford Assay Reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A total of 30 μ g protein samples were resolved using SDS-polyacrylamide gel electrophoresis

and electrophoretically transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk, washed with TBST buffer, and then incubated with the following primary antibodies: Anti-HO-1 (catalog no. sc-10789; 1:1,000), anti-Nrf2, (catalog no. sc-722; 1:1,000) anti-Lamin B, (catalog no. sc-6216; 1:1,000), anti-Actin (catalog no. sc-1616; 1:1,000) all from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). They were then incubated with horseradish peroxidase-conjugated goat (catalog no. ap106p; 1:1,000) and rabbit (catalog no. ap132p; 1:1,000) secondary antibodies, obtained from EMD Millipore (Billerica, MA, USA), followed by ECL detection. Primary and secondary antibodies were diluted with 3% skimmed milk in TBST buffer. The bands were visualized with enhanced chemiluminescence (GE Healthcare Life Sciences, Chalfont, UK) and quantified by densitometry (Image J, National Institutes of Health, USA). Nuclear and cytoplasmic extracts of cells were prepared using NE-PER reagents, as per the manufacturer's instructions (Thermo Fisher Scientific, Inc.).

HO-1 activity. Determination of HO activity occurred as previously described by Motterlini *et al* (13). Briefly, the HT22 cells were scraped off the dish, and centrifuged ($1,000 \times g$ for 10 min at 4°C). The pellet of HT22 cell was suspended in MgCl₂ phosphate buffer (20 mM, pH 7.4), frozen at -70°C, and finally sonicated on ice prior to centrifugation at $18,000 \times g$ for 10 min at 4°C. The supernatant was added to a nicotinamide adenine dinucleotide phosphate (NADPH)-generating system containing 0.8 mM NADPH, 2 mM glucose-6-phosphate, 0.2 U glucose-6-phosphate-L-dehydrogenase, and 2 mg protein of rat liver cytosol prepared from the $15,000 \times g$ supernatant fraction as a source of biliverdin reductase, potassium phosphate buffer (100 mM, pH 7.4), and hemin (10 μ M) in a final volume of 200 μ l. The reaction was conducted for 1 h at 37°C in the dark and terminated by addition of 1 ml chloroform. The extracted bilirubin was calculated by the difference in absorption between wavelengths of 464 and 530 nm using a quartz cuvette (extinction coefficient, 40 mM⁻¹ cm⁻¹ for bilirubin).

Preparation of cytosolic and nuclear fractions. HT22 Cells were homogenized (1:20, w:v) in PER-Mammalian Protein Extraction buffer (Pierce; Thermo Fisher Scientific, Inc.) including freshly added 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail I (EMD Millipore). The cytosolic fraction of the cell was prepared by centrifugation at $15,000 \times g$ for 10 min at 4°C. Cytoplasmic and nuclear extracts of the cells were prepared using NE-PER cytoplasmic and nuclear extraction reagents (Pierce; Thermo Fisher Scientific Inc.), respectively. These methods were conducted as previously described (12).

Statistical analysis. Data were expressed as the mean \pm standard deviation of at least 3 independent experiments. To compare 3 or more groups, one-way analysis of variance followed by the Newman-Keuls post hoc test was used. Statistical analysis was performed using GraphPad Prism software version 3.03 (GraphPad Software Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

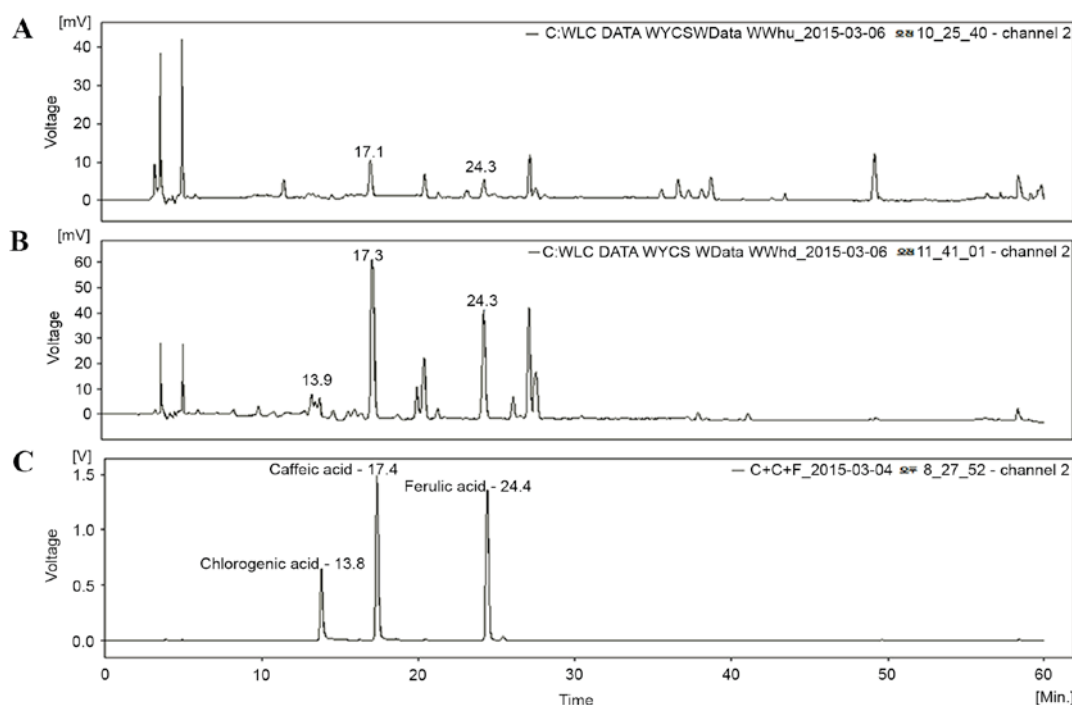


Figure 1. High performance liquid chromatography chromatograms of (A) TCAE, (B) TCRE and (C) internal standard compounds. TCAE, *Taraxacum coreanum* aerial extract; TCRE, *T. coreanum* root extract.

Results

HPLC analysis of TCAE and TCRE. Three phenolic compounds, ferulic acid, caffeic acid and chlorogenic acid, have been isolated from plants of the *Taraxacum* genus (14,15). Therefore, TCAE and TCRE were analyzed by HPLC in order to assess the presence of these compounds. As demonstrated in Fig. 1, the peaks of ferulic acid and caffeic acid appeared clearly in the HPLC chromatograms of both TCAE (Fig. 1A) and TCRE (Fig. 1B), however, chlorogenic acid was only detected in TCRE (Fig. 1B).

Effects of TCAE and TCRE on oxidative toxicity. To investigate the protective effects of TCAE and TCRE against glutamate-induced cytotoxicity, HT22 cell viability was measured by MTT assay. Non-cytotoxic effects were obtained with concentrations of up to 400 $\mu\text{g/ml}$ for both TCAE and TCRE (data not shown). The viability of glutamate-treated HT22 cells (5 mM for 24 h) was tested following treatment with 0, 50, 100, 200 and 400 $\mu\text{g/ml}$ of each extract (Fig. 2). The results demonstrated that both TCAE and TCRE significantly restored the cell viability following glutamate-mediated damage in a concentration-dependent manner compared with cells treated with glutamate only (Fig. 2). Trolox, a well-known antioxidant, was used as a positive control in this assay and was confirmed to exhibit a significant protective effect (Fig. 2). The present results suggested that both aerial and root ethanolic extracts of *T. coreanum* could protect HT22 cells against oxidative stress.

Effects of TCAE and TCRE on HO-1 expression and activity. The effect of TCAE and TCRE on HO-1 expression was examined in HT22 cells. When cells were treated with

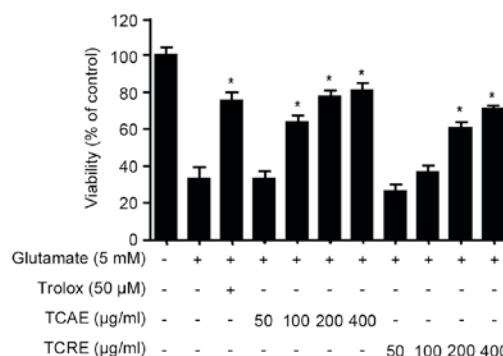


Figure 2. Effects of TCAE and TCRE on glutamate-induced oxidative toxicity in HT22 cells. The cells were incubated with either 50 μM Trolox (as a positive control) or 50, 100, 200 or 400 $\mu\text{g/ml}$ TCAE or TCRE (for 3 h). Cell damage was then induced by treatment with 5 mM glutamate in HT22 cells for 12 h. Viability was then measured by MTT assay. * $P < 0.05$ vs. glutamate-treated cells. TCAE, *Taraxacum coreanum* aerial extract; TCRE, *T. coreanum* root extract.

50, 100, 200 or 400 $\mu\text{g/ml}$ TCAE or TCRE for 12 h, both extracts visibly increased the expression of HO-1 protein in a concentration-dependent manner, compared with untreated cells (Fig. 3A). In agreement with the concentration-dependent expression of HO-1, both TCAE and TCRE treatments also significantly increased HO-1 activity in HT22 cells compared with untreated cells (Fig. 3B). CoPP, used as a positive control, exhibited a prominent induction of both HO-1 protein expression and activity (Fig. 3).

Effects of HO-1 inhibitor on the cytoprotection activity of TCAE and TCRE. The hypothesis that the protective effect of TCAE and TCRE in HT22 cells arose from HO-1 expression was further tested. Pretreatment of cells with SnPP, a

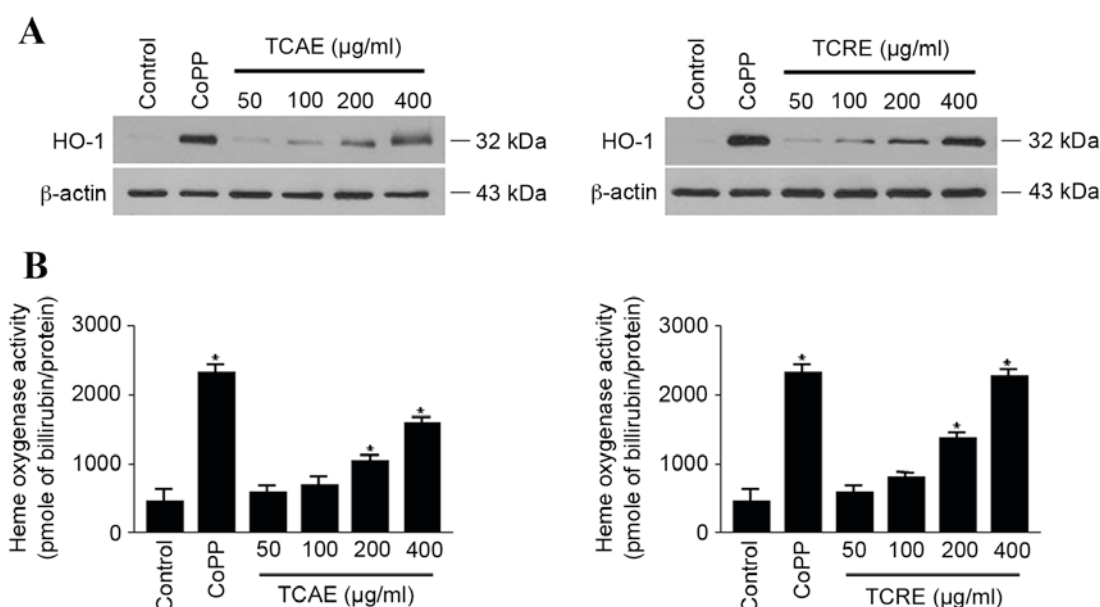


Figure 3. Effects of TCAE and TCRE on HO-1 expression and activity. HT22 cells were treated with 0, 50, 100, 200 or 400 μg/ml TCAE or TCRE extract for 12 h. CoPP (20 μM) treatment was used as a positive control. (A) HO-1 protein expression was analyzed by western blot (representative images). (B) HO-1 activity. * $P < 0.05$ vs. control (untreated cells). TCAE, *Taraxacum coreanum* aerial extract; TCRE, *T. coreanum* root extract; HO-1, heme oxygenase-1; CoPP, cobalt protoporphyrin IX.

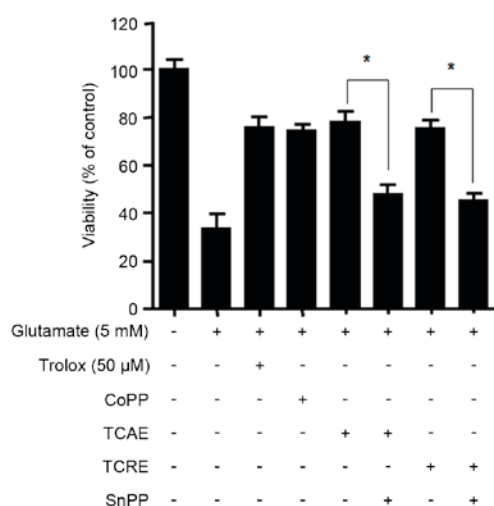


Figure 4. Effects of HO-1 inhibitor SnPP on TCAE- and TCRE-mediated cytoprotection. To test the effect of HO-1 inhibition, cells were pretreated with SnPP (50 μM) prior to TCAE/TCRE-treatment. Cells were then incubated with either trolox (50 μM, positive control), CoPP (20 μM, positive control), TCAE (400 μg/ml) or TCRE (400 μg/ml). Cell damage was induced by treatment with 5 mM glutamate in HT22 cells for 12 h. Viability was measured by MTT assay. * $P < 0.05$, with comparisons indicated by brackets. HO-1, heme oxygenase-1; SnPP, tin protoporphyrin; TCAE, *Taraxacum coreanum* aerial extract; TCRE, *T. coreanum* root extract; CoPP, cobalt protoporphyrin IX.

competitive inhibitor of HO-1, significantly reduced the cytoprotective effects of TCAE and TCRE on glutamate-induced cell damage ($P < 0.05$; Fig. 4). The present data, therefore, suggested that the neuroprotective effects of TCAE and TCRE against oxidative stress are a result of their ability to induce HO-1 expression.

Effects of TCAE and TCRE on Nrf2 nuclear translocation. In order to examine whether Nrf2 is involved in the

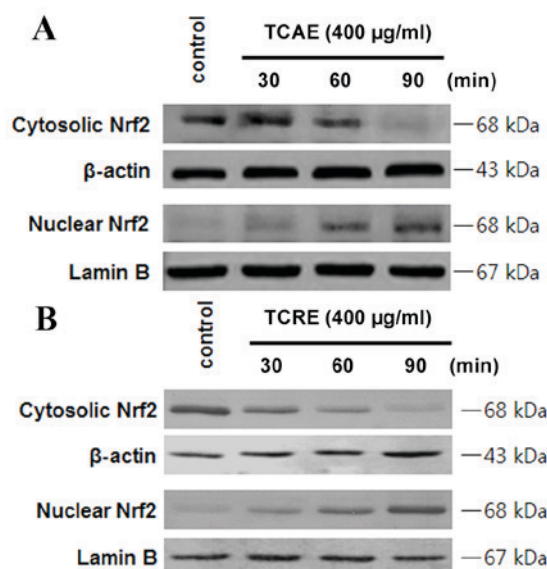


Figure 5. Effects of (A) TCAE and (B) TCRE on Nrf2 nuclear translocation. HT22 cells were either untreated (control) or incubated with 400 μg/ml TCAE or TCRE for 30, 60 or 90 min. The nuclear and cytosolic fractions were then separated and assayed for Nrf2 expression by western blot. β-actin was used as an internal control for the cytoplasmic fraction, and lamin B for the nuclear fraction. TCAE, *Taraxacum coreanum* aerial extract; TCRE, *T. coreanum* root extract; Nrf2, nuclear factor erythroid 2 like 2.

extract-mediated HO-1 induction, the effect on Nrf2 nuclear translocation in HT22 cells treated with TCAE and TCRE was tested (Fig. 5). HT22 cells were incubated with 400 μg/ml TCAE (Fig. 5A) or TCRE (Fig. 5B) for 30, 60 and 90 min, then Nrf2 protein levels in the nuclear and cytosolic fractions were examined by western blot analysis. The nuclear extracts of both TCAE and TCRE-treated cells exhibited a time-dependent increase in Nrf2 levels compared with untreated

control, while Nrf2 levels decreased in the cytoplasmic fraction of HT22 cells (Fig. 5).

Discussion

Taraxacum is a taxonomically complex genus of the family Asteraceae. Some botanists have classified the genus into ~34 macrospecies, and ~2000 microspecies, while other botanists only accept a total of ~60 species (16). *Taraxacum coreanum* Nakai is a dandelion native to Korea and is widely consumed as a vegetable and as a traditional medicine for inflammatory disorders in Korea. Although the whole plant of *T. coreanum* has been used in Korean traditional medicine, the present study aimed to evaluate the neuroprotective effects of its aerial and root parts separately. TCAE and TCRE ethanolic extracts were subjected to HPLC for evaluation of their chemical profile. As demonstrated in Fig. 1, the peaks of caffeic acid and ferulic acid were apparent in both extracts from their HPLC chromatograms. These results, therefore, provide insights into the chemical composition in the aerial and the root parts of *T. coreanum*.

Glutamate is one of the most important transmitters for brain function and is an important neural activator, however, excess glutamate can trigger neurodegenerative diseases (2). It is generally acknowledged that glutamate neurotoxicity is mediated by non-receptor-mediated oxidative stress and receptor-initiated excitotoxicity (2). To examine the neuroprotective effects of TCAE and TCRE, these extracts were added at non-toxic concentrations (50–400 µg/ml) in mouse hippocampal HT22 cells treated with glutamate. Both extracts significantly increased the survival of glutamate-treated HT22 cells (Fig. 2). HT22 cells lack glutamate receptors (5), suggesting that the protective effects of TCAE and TCRE are likely derived from their anti-oxidative properties rather than receptor-mediated signaling. In addition, caffeic acid and ferulic acid, which were used as internal standards in this study, did not exhibit any cytoprotective effects at their non-toxic concentrations against glutamate-induced HT22 cells (data not shown), suggesting that other components exist in TCAE and TCRE that are responsible for their neuroprotective activity.

HO-1 is induced as a protective enzyme in response to diverse stimuli, therefore it may be suitable for the therapy of oxidative tissue damage (17). Therefore, the hypothesis that TCAE and TCRE could affect HO-1 expression was tested. The present study demonstrated that both extracts induced expression and activity of HO-1 in a concentration-dependent manner (Fig. 3). Furthermore, the neuroprotective activity of TCAE and TCRE were reversed by the HO-1 inhibitor SnPP (Fig. 4), suggesting that the protective properties of these extracts are mediated by HO-1.

HO-1 expression is associated with Nrf2 translocation (18). Under normal conditions, Nrf2 interacts with Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm, but signals from electrophilic stimuli or increased reactive oxygen species production result in separation of the Nrf2-Keap1 complex, and Nrf2 translocation to the nucleus. Following nuclear translocation, Nrf2 interacts with antioxidant response element sites in the promoter regions of specific target genes, initiating transcription of antioxidative genes, including GSH and HO-1 (19). The hypothesis that TCAE and TCRE may cause Nrf2 to translocate

into the nucleus was, therefore, examined in HT22 cells. The results confirmed that Nrf2 levels were increased in the nuclear fractions of HT22 cells following TCAE and TCRE treatments, while Nrf2 levels decreased in the cytoplasm (Fig. 5), indicating increased translocation as a result of TCAE and TCRE treatment.

The present study revealed that *T. coreanum*, a native plant of Korea, confers a protective effect against glutamate-induced oxidative stress in HT22 cells. This cytoprotective effect of *T. coreanum* is mediated by induction of HO-1 expression, via Nrf2 nuclear translocation. Therefore, *T. coreanum* might serve as a potential therapeutic agent for preventing neurodegeneration and further studies are warranted for the isolation and characterization of its active substances.

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