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Regulatory Roles of *Chrysanthemum zawadskii* Roots in Nuclear Factor E2-related Factor 2/Antioxidant Response Element Pathway

Hye-Sook Kang, Min-Ji Park, Kyong-Suk Jin, Young-Hun Kim, Mira Jun[1], Ho-Jin Lim[2], Wan-Kuen Jo[2], Jong-Sang Kim[3], and Woo-Sik Jeong*

Food Science Institute, Institute of Biomedical Engineering, School of Food and Life Sciences, College of Biomedical Science and Engineering, Inje University, Gimhae, Gyeongnam 621-749, Korea

[1]*Division of Food Science, Dong-A University, Busan 604-714, Korea*

[2]*Department of Environmental Engineering, Kyungpook National University, Daegu 702-701, Korea*

[3]*Department of Animal Science and Biotechnology, Kyungpook National University, Daegu 702-701, Korea*

Abstract Cellular protection against carcinogens could be achieved by the induction of phase 2 detoxifying and antioxidant enzymes such as glutathione S-transferase (GST), NAD(P)H:quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO-1). Nuclear transcription factor E2-related factor 2 (Nrf2) binds to antioxidant response element (ARE) in the promoter region of these genes and the resulting transactivation occurs. In the present study the effect of *gujeolcho* (*Chrysanthemum zawadskii*) roots on the Nrf2-ARE pathway were investigated. *C. zawadskii* root extract was fractionated with a series of organic solvents and their ability to induce Nrf2-ARE pathway was examined. We separated the most potent dichloromethane (DCM) fraction into 12 sub-fractions and found several sub-fractions with strong effects on the Nrf2-ARE pathway. Fraction 4 strongly induced the ARE-reporter gene activity as well as Nrf2 expression. Sitosterol was isolated as a major compound in fraction 4 although its activity was not as potent as its mother fraction. These results indicate that *C. zawadskii* roots might be used as a potential natural chemopreventive source.

Keywords: nuclear factor E2-related factor 2 (Nrf2), antioxidant response element, chemoprevention, *Chrysanthemum zawadskii*, sitosterol

Introduction

Nuclear factor E2-related factor 2 (Nrf2) has been of great interest in the last few years because of its crucial status in cancer chemoprevention mechanisms by natural compounds. Nrf2 is known to play a key role in the induction of cellular defensive phase 2 detoxifying and antioxidant enzymes such as NAD(P)H:quinone oxidoreductase 1 (NQO1) and hemeoxygenase 1 (HO-1). These enzymes protect cells and/or tissues from carcinogens that can initiate carcinogenic process. Upon exposure of cells to inducers such as oxidative stress and chemopreventive agents, Nrf2 translocates from the cytosol to the nucleus and binds to the antioxidant response element (ARE) in the promoter region of the phase 2 and antioxidant genes (1,2). Several natural chemopreventive compounds have been reported to stimulate the Nrf2-ARE pathway and the resulting induction of phase 2 and antioxidant enzymes. These agents include isothiocyanates, catechins, diallyl sulfides, and curcuminoids, etc (3).

Chrysanthemum zawadskii var. *latilobum* K. is a perennial herb belonging to Compositae, called '*gujeolcho*' in Korea, and has been used as a traditional medicine to treat various diseases such as pneumonia, bronchitis, cough, common cold, pharyngitis, bladder-related disorders, women's diseases, gastroenteric disorders, and hypertension (4).

*Corresponding author: Tel: +82-55-320-3238; Fax: +82-55-321-0691
E-mail: jeongws@injc.ac.kr
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Chrysanthemum species are known to be a rich source of bioactive compounds such as flavonoids and terpenoids. For example, apigenin and acacetin derivatives isolated from this genus were found to possess inhibitory activities of human immuno-deficiency virus type 1 (5) and of acetylcholinesterase related with Alzheimer's disease (6). Triterpenes in *Chrysanthemum morifolium* flowers were shown to inhibit Epstein-Barr virus early antigen activation induced by a tumor promoter (7) and to have antitubercular activity (8). Essential oil from *C. boreale* exhibited antibacterial activity against 6 Gram-positive bacteria and 8 Gram-negative bacteria (9), and induced apoptosis on human oral epidermoid carcinoma KB cell line (10). Sesquiterpene lactone guaianolide from the same plant inhibited the etoposide-induced apoptosis in U937 cells (11). The extracts from *C. zawadskii* have been reported to have antiallergic activity (12). Linarin from this plant has been reported as a major component that inhibits nitric oxide production in the mouse macrophages RAW 246.7 cell line (13), and shows anticancer efficacy (14). Recently, our group has reported that the dichloromethane (DCM)-soluble fraction from *C. zawadskii* stimulates the enzymatic activity of phase 2 detoxifying enzymes such as NQO1 and glutathione S-transferase (GST) in Hepa-1c1c7 and BpRc1 cell lines (15).

In this study, we further fractionated the DCM fraction of *C. zawadskii* with open column chromatography, and examined the effect of column fractions on Nrf2 protein expression and ARE-reporter gene activity in HepG2 human hepatoma cells.

Materials and Methods

Materials Sulforaphane (SUL) was obtained from LKT Laboratories (St. Paul, MN, USA). Antibodies against Nrf2, NQO1, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Sitosterol which was used as an authentic sample for comparing with isolated compound was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other reagents were of analytical grade and were purchased from commercial sources.

Activity-guided fractionation *C. zawadskii* was kindly supplied by Euisung Medicinal Herb Experimental Station, Euisung, Gyeongbuk, Korea. A voucher specimen (no. 20050930) has been deposited in the author's laboratory. Dried powder of *C. zawadskii* roots (1 kg) was extracted 2 times with 95% ethanol at room temperature for 24 hr (2×10 L). The extract was suspended in water and partitioned with *n*-hexane (11.67 g), dichloromethane (DCM, 8.5 g), ethyl acetate (EtOAc, 2.53 g), *n*-butanol (BuOH, 8.14 g), in sequence. The DCM soluble fraction, which exhibited the most potent induction effect of Nrf2 gene expression among the organic solvent soluble fractions, was applied onto a silica gel (70-230 mesh, Merck, Darmstadt, Germany) column. The column was eluted using mixtures of *n*-hexane/EtOAc under gradient conditions (19 : 1-1 : 9) to yield the 12 fractions (F1–F12). The F4 (107 mg) was further purified with a Toyopearl HW-40F (Tosoh, Tokyo, Japan) column chromatography to yield the 11 subfractions (F4-1 to F4-11). TLC was carried out on a precoated Merck Kieselgel 60 F_{254} plate (0.25 mm) and the spots were detected under UV light using 50% H_2SO_4 reagent. All the solvent for column chromatography was of a reagent grade from commercial sources. Compound **1** (15 mg) was obtained from recrystallization of F4-2 with methanol (MeOH).

Instrumental analysis ^{1}H- and ^{13}C-NMR spectra were determined on an Avance digital 400 spectrometer (Bruker, Germany) using $CDCl_3$ with tetramethylsilane (TMS) as an internal standard. Column chromatography was done with silica gel (70-230 mesh, Merck) and Toyopearl HW-40F. LC-MS analysis of compound was performed on an Agilent 1100 HPLC system coupled to an in-line diode array detector (DAD) and an Agilent LC-MSD trap-SL ion trap mass spectrometer (Palo Alto, CA, USA). The separation was performed on a C18 column (4.6×250 mm, 5 mm, Phenomenex Inc., Torrance, CA, USA). Mobile phase was consisted with water (A) and acetonitrile (B) gradient from 0% B to100% B for 20 min with a flow rate of 1 mL/min. Detection was set at 210 nm at ambient temperature.

β-Sitosterol: White powder, LC-MS *m/z*: 415.7 $[M+H]^+$, $C_{29}H_{51}O$, ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 5.35 (1H, brd, *J*=5.6 Hz), 3.49 (1H, m), 1.01 (3H, s) 0.92 (3H, d, *J*=6.6 Hz), 0.84 (3H, s), 0.82 (3H, brd, *J*=2 Hz), 0.80 (3H, s), 0.68 (3H, s). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 140.73 (C-5), 121.74 (C-6), 71.80 (C-3), 56.74 (C-14), 56.01 (C-17), 50.09 (C-9), 45.79 (C-24), 42.30 (C-4), 42.27 (C-13), 39.74 (C-12), 37.23 (C-1), 36.49 (C-10), 36.14 (C-20), 33.91 (C-22), 31.88 (C-7), 31.88 (C-8), 31.64 (C-2), 29.08 (C-25), 28.26 (C-16), 25.99 (C-23), 24.29 (C-15), 23.03 (C-28), 21.12 (C-11), 19.83 (C-26), 19.41 (C-19), 19.01 (C-27), 18.77 (C-21), 11.98 (C-29), 11.85 (C-18).

Cell culture and treatments HepG2 human hepatoma cells were obtained from American Type Culture Collections (Rockville, MD, USA). Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1.17 g/L sodium bicarbonate, 100 units/mL penicillin, 100 μg/mL streptomycin, 1% essential amino acids, and 0.1% insulin, in a humidified atmosphere of 95%, 5% CO_2 at 37°C. Cells were seeded in 6-well plates and allowed to grow for 24 hr (about 80 to 90% confluency). Then, the cells were starved overnight with serum-free DMEM media prior to further treatments with either vehicle (DMSO, 0.1%) or extracts of *C. zawadskii* roots. For evaluation of Nrf2 expression, cells were treated with extracts for various time periods.

MTS assay The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay was performed with CellTiter 96 aqueous nonradioactive cell proliferation assay kit (Promega Corp., Madison, WI, USA) by the manufacturer's instructions. Briefly, the cells were plated on 96-well plates. After 24 hr of incubation, the cells were starved overnight with serum-free media prior to treatments with different doses of each sample for 48 hr. Then, media were removed, and culture media containing MTS and phenazine methosulfate solution were added. After 1-2 hr, the absorbance was measured at 490 nm with mQuant ELISA reader (Bio-Tek Instrument, Inc., Winooski, VT, USA).

ARE-luciferase activity assay HepG2-ARE-C8 (HepG2-C8) cell line was kindly donated by Dr. Ah-Ng Tony Kong (Rutgers University, Piscataway, NJ, USA), which was established by stable transfection of HepG2 cells with pARE-TI-luciferase reporter gene and previously described (16). The HepG2-C8 cells were treated with extracts of *C. zawadskii* roots for 12 hr. The ARE-luciferase activity was determined using a luciferase kit from Promega according to the manufacturer's instructions. Briefly, after treatments, the cells were washed twice with ice-cold phosphate buffered-saline (pH 7.4) and harvested in 1× Reporter lysis buffer. After centrifugation at 13,000×g for 10 min, a 10 μL aliquot of the supernatant was assayed for luciferase activity with a GloMax™ 20/20 luminometer (Promega). The luciferase activity was normalized against protein amount, determined by BCA protein assay (Pierce, Rockford, IL, USA), and expressed as fold of induction over the luciferase activity of control vehicle-treated cells. Data were statistically analyzed by ANOVA, followed by unpaired Student's *t*-test.

Western blot analysis After treatments, cells were washed with ice-cold phosphate buffered saline (PBS, pH 7.4) and harvested with 200 μL of a whole cell lysis buffer (pH 7.4) containing 10 mM Tris-HCl, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium orthovandate, 2 mM iodoacetic acid, 5 mM $ZnCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton-X 100. Cell lysates were vigorously vortexed, homogenized in an ultrasonicator for 10 sec and left on ice for 30 min. The homogenates were centrifuged at 13,000×g for 15 min at 4°C. The supernatants were collected and equal amounts of total protein of each sample, as

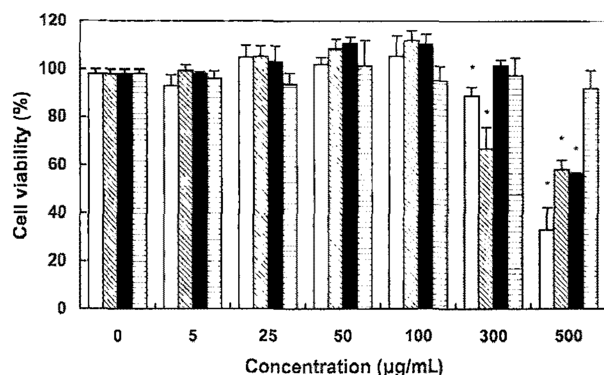

Fig. 1. Effects of solvent fractions from *C. zawadskii* roots on cell viability of HepG2 cells. HepG2 cells were treated with vehicle (DMSO, 0.1%) or each fraction (□, *n*-Hexane fr.; ▧, DCM fr.; ■, EtOAc fr.; ▤, *n*-BuOH fr.) for 48 hr with the indicated concentrations. Values are means±SD (n=3); *$p<0.05$ compared to control by unpaired Student's *t*-test.

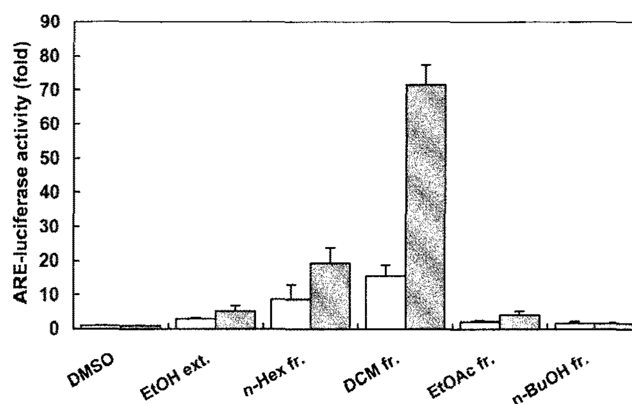

Fig. 2. ARE-luciferase activity by solvent fractions from *C. zawadskii* roots in HepG2-ARE-C8 cells. HepG2-C8 cells were treated with vehicle (DMSO, 0.1%) or each fraction (EtOH ext, *n*-hexane, DCM, and *n*-BuOH fr.) for 12 hr (□, 50 µg/mL; ▧, 100 µg/mL). Luciferase activity was normalized with protein content and expressed as fold induction against vehicle-treated control. Values are means±SD (n=3).

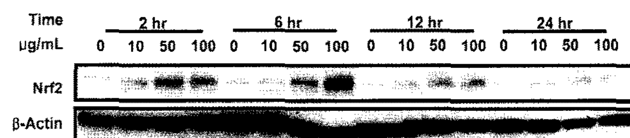

Fig. 3. Time dependent expression of Nrf2 by DCM fraction from *C. zawadskii* roots in HepG2 cells. HepG2 cells were treated with vehicle (DMSO, 0.1%) or different fractions and then equal amount of proteins from whole cell lysates were analyzed for Nrf2 and β-actin by Western blotting. The data shown are representative of 3 independent experiments with similar results.

determined by BCA protein assay (Pierce), were mixed with 4× loading buffer, and heated at 95°C for 5 min. The samples were then separated in a 10% criterion Tris-HCl precast (Bio-Rad, Hercules, CA, USA) gel at 200 V and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA, USA) for 1.5 hr using a semi-dry transfer system (Fisher). The membranes were blocked with 5% nonfat dry milk in 1× PBST buffer (0.1% Tween 20 in PBS) for 1 hr at room temperature and incubated with anti-Nrf2 antibodies in 3% nonfat dry milk (1 : 500 dilution) overnight at 4°C. After hybridization with primary antibody, membranes were washed 3 times with PBST, and then incubated with anti-rabbit (for Nrf2) antibodies with horseradish peroxidase (Santa Cruz Biotechnology Inc.) for 1 hr at room temperature and washed with PBST 3 times. Final detection was performed with enhanced chemiluminescence (ECL™) Western blotting reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Results and Discussion

Cytotoxicity on HepG2 cell line The effects of organic solvent soluble fractions from *C. zawadskii* roots on the viability of HepG2 cells were determined by a colorimetric MTS assay after 48 hr treatments. The data were expressed as percent cell viability compared to that of control (DMSO, 0.1%) (Fig. 1). The concentrations of the treatments of organic solvent soluble fractions from *C. zawadskii* roots varied from 5 to 500 mg/mL. Treatments with *n*-hexane, DCM, and EtOAc soluble fractions at the concentrations up to 100 mg/mL did not affect the cell viability while treatments with higher doses (300 and 500 mg/mL) significantly decreased the cell viability. Therefore, further biological assays were performed with concentrations below 100 mg/mL.

ARE-reporter gene activity To evaluate the effect of organic solvent soluble fractions from *C. zawadskii* roots on the ARE-mediated gene expression, ARE-luciferase activity by these fractions was determined in HepG2-C8 cell line that was stably transfected with pARE-TI-luciferase reporter gene. The ARE is located in the promoter region of the phase 2 and antioxidant genes and the activation and/or induction of these genes is believed as a crucial event for cellular defense against various carcinogens (3,17,18). Therefore, to examine the ARE-inducing activity might be a useful tool for screening unknown compounds and/or plant extracts with a potential chemopreventive property. The ARE-luciferase activity of treated groups was expressed as a fold-induction over that of vehicle (0.1% DMSO) - treated control. As shown in Fig. 2, the treatments with DCM fraction at 100 µg/mL tremendously amplified the ARE-luciferase activity (about 70-fold induction compared to control). The DCM fraction displayed almost 15-fold induction of the ARE-luciferase activity at 50 µg/mL. Beside the DCM fraction, the hexane fraction was a strong inducer to the reporter gene activity (up to 20-fold induction).

Induction of Nrf2 expression We further investigated the regulatory role of the DCM fraction in the protein expression of Nrf2, a transcription factor that is known to bind to the ARE sequence and thereby transactivates the genes containing the ARE. The DCM fraction dose-dependently induced Nrf2 protein expression in HepG2 cells within the dose range of 10-100 µg/mL (Fig 3). The protein expression was strongly induced after 2 hr treatment,

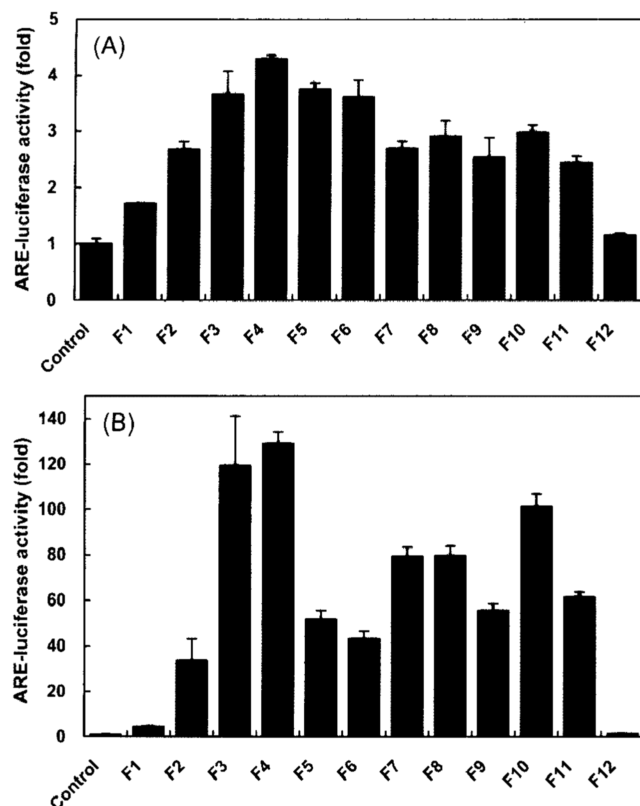

Fig. 4. ARE-luciferase activity by silica column fractions (F1-F12) of DCM fraction from *C. zawadskii* roots in HepG2-ARE-C8 cells. The concentrations were 10 (A) and 50 μg/mL (B). Values are means±SD (n=3).

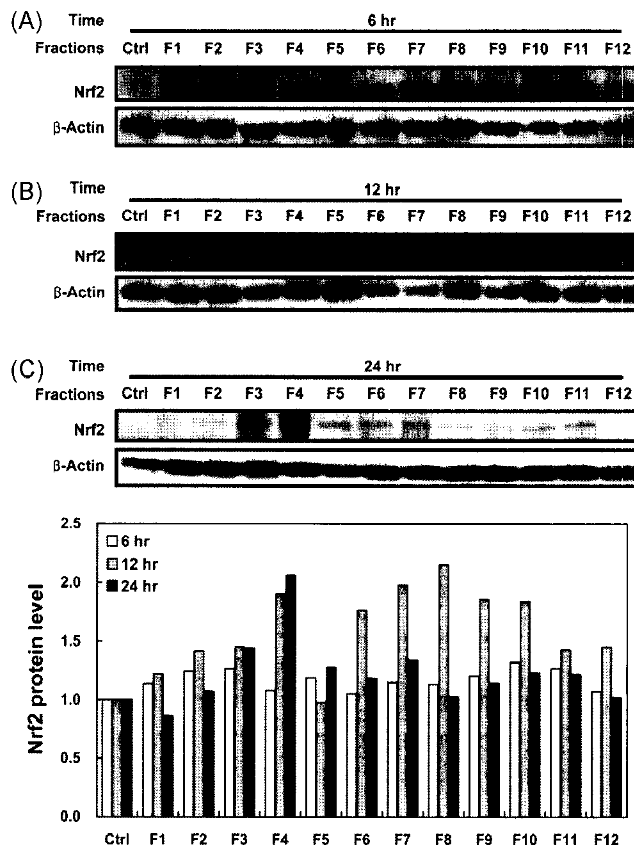

Fig. 5. Nrf2 protein expression by silica column fractions (F1-F12, 10 μg/mL) of DCM fraction from *C. zawadskii* roots in HepG2 cells. The results were quantified by densitometry and the Nrf2 protein levels were plotted after normalization with actin.

peaked at 6 hr, and diminished after 6 hr. The greatest induction was observed at 100 μg/mL treatment, which could support the data from ARE-luciferase assay.

Bioactive-guided fractionation To isolate active components from DCM fraction, we further fractionated the DCM fraction using an open-column chromatography packed with silica gel and obtained 12 column fractions (F1–F12). The ARE-luciferase activity by column fractions was evaluated with doses of 10 and 50 μg/mL (Fig. 4). Most of the column fractions had the ARE-inducing activity even at 10 μg/mL and several fractions at 50 μg/mL (fractions 3, 4, and 10) amplified the ARE-luciferase activity over 100-fold. The pattern of Nrf2 protein expression by treatments with the column fractions is slightly different from that of ARE data. Although most column fractions up-regulated the protein expression within 24 hr, only fractions 3 and 4 exhibited a strong induction on expression of Nrf2 until 24 hr (Fig. 5). The sustained Nrf2 protein expression might result from either inhibition of degradation or constant expression of the Nrf2 protein by F4 fraction. In the previous study, we observed a short half-life of Nrf2 protein (estimated to be about 45 min) and the natural chemopreventive agent sulforaphane was shown to inhibit the proteasomal degradation of Nrf2 and thereby increased the stability of the protein (19).

To purify the active compounds from the F4 silica column fraction, the F4 fraction was applied onto Toyopearl HW-40F column with MeOH as eluting solvent, and we obtained 11 subfractions (F4-1–11). Among the subfractions, F4-2 was precipitated as white amorphous powder. After recrystallization with MeOH, the precipitate was analyzed with NMR and LC-MS techniques for chemical structure elucidation. In the ^{1}H-NMR spectrum, an olefinic methine (δ 5.35), oxygenated methine (δ 3.49), a number of methylene, and methine proton signals (δ 2.29-1.02) was observed. In addition, there were 6 methyl proton signals (δ 1.01, 0.92, 0.84, 0.82, 0.80, and 0.68). In the ^{13}C-NMR spectrum, there were 29 carbon signals including a quaternary (δ 140.73), and olefinic methine (δ 121.74), oxygenated methine (δ 71.80), and 6 methyl (δ 19.83, 19.41, 19.01, 18.77, 11.98, and 11.85) carbon signals. Upon interpretation of NMR data, we tentatively assigned the compound as sitosterol. After comparison of the NMR and LC-MS data with those of authentic sample and references (20,21), we finally assigned the compound to sitosterol as the major component of the F4 fraction (Fig. 6).

Nrf2/ARE pathway by sitosterol Since sitosterol was the only compound identified from F4 fraction, we evaluated its effects on the ARE-luciferase activity and Nrf2 protein expression. Unexpectedly, there were only slight increases in ARE-luciferase activity and Nrf2 expression by sitosterol treatment (Fig. 7, 8). The reason why the isolated compound does not have a strong effect in the assay systems is not clear. However, it is possible that there might be other unidentified active compounds with

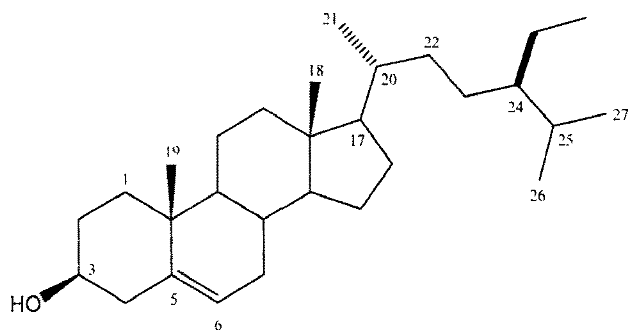

Fig. 6. The structure of sitosterol identified from *C. zawadskii* roots.

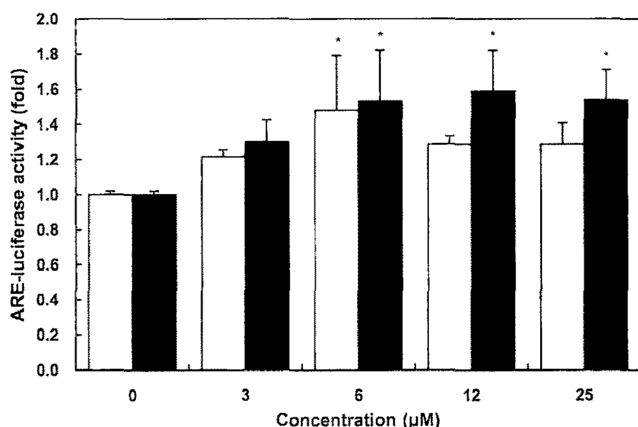

Fig. 7. ARE-luciferase activity by sitosterol from *C. zawadskii* roots in HepG2-ARE-C8 cells. HepG2-C8 cells were treated with vehicle (EtOH, 0.1%) or sitosterol (98% purity) for 12 (□) and 24 hr (■). Values are means±SD (n=3); *$p<0.05$ compared to control by unpaired Student's *t*-test.

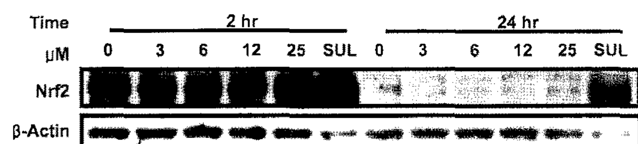

Fig. 8. Nrf2 protein expression by treatment of sitosterol from *C. zawadskii* roots in HepG2 cells. HepG2 cells were treated with sitosterol (98% purity) or controls. Vehicle (EtOH, 0.1%) and sulforaphane (SUL) were used as negative and positive controls, respectively. The data shown are representative of 3 independent experiments with similar results.

relatively low concentrations in its mother fraction (F4 fraction). It is also possible that other minor components in the F4 fraction might synergistically act with sitosterol for the induction of Nrf2/ARE cellular defense mechanism.

Phytosterols such as sitosterol, stigmasterol, and campesterol are present in almost all plants and sitosterol is present more frequently in plants than any other phytosterols (22). Sitosterol has been reported to have various biological activities and to affect several signaling pathways in chemoprevention mechanisms. It is known to induce apoptosis in hormone-related cancer cell lines such as breast and prostate cancer cell lines (23,24). In breast cancer cells, sitosterol increases Fas level and caspase-8 activity (23). Sitosterol has a modulatory effect on antioxidant enzyme response by reverting the phorbol ester-induced impairment of the glutathione/oxidized glutathione ratio in RAW 264.7 macrophages (25).

In the present study, we investigated the effects of *C. zawadskii* roots on cellular defense mechanism, in particular on Nrf2/ARE pathway. Although the isolated compound, sitosterol, was not as active as its mother fractions, we could see a possibility in *C. zawadskii* roots, especially the DCM fraction, as a candidate of a potent natural chemopreventive agent. Furthermore, the results of this study strongly support our previous findings that *C. zawadskii* root stimulates the enzymatic activities of NQO1 and GST in mouse hepatoma cell lines (15). Further studies on the regulatory roles of *C. zawadskii* roots in upstream signaling pathways as well as studies using *in vivo* models are needed.

Acknowledgment

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References

1. Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem. Bioph. Res. Co. 236: 313-322 (1997)
2. Kong AN, Yu R, Hebbar V, Chen C, Owuor E, Hu R, Ee R, Mandlekar S. Signal transduction events elicited by cancer prevention compounds. Mutat. Res. 480-481: 231-241 (2001)
3. Jeong WS, Jun M, Kong AN. Nrf2: A potential molecular target for cancer chemoprevention by natural compounds. Antioxid. Redox. Sign. 8: 99-106 (2006)
4. Kim YY, Lee SY, Yim DS. Biological activities of linarin from *Chrysanthemum zawadskii* var. latilobum. Yakhak Hoeji 45: 604-610 (2001)
5. Lee JS, Kim HJ, Lee YS. A new anti-HIV flavonoid glucuronide from *Chrysanthemum morifolium*. Planta Med. 69: 859-861 (2003)
6. Lim SS, Han S-M, Kim SY, Bae YS, Kang IJ. Isolation of acetylcholinesterase inhibitors from the flowers of *Chrysanthemum indicum* Linne. Food Sci. Biotechnol. 16: 265-269 (2007)
7. Ukiya M, Akihisa T, Tokuda H, Suzuki H, Mukainaka T, Ichiishi E, Yasukawa K, Kasahara Y, Nishino H. Constituents of Compositae plants III. Anti-tumor promoting effects and cytotoxic activity against human cancer cell lines of triterpene diols and triols from edible chrysanthemum flowers. Cancer Lett. 177: 7-12 (2002)
8. Akihisa T, Franzblau SG, Ukiya M, Okuda H, Zhang F, Yasukawa K, Suzuki T, Kimura Y. Antitubercular activity of triterpenoids from Asteraceae flowers. Biol. Pharm. Bull. 28: 158-160 (2005)
9. Kim KJ, Kim YH, Yu HH, Jeong SI, Cha JD, Kil BS, You YO. Antibacterial activity and chemical composition of essential oil of *Chrysanthemum boreale*. Planta Med. 69: 274-277 (2003)
10. Lee JR, Yang MS, Jang DS, Ha TJ, Park KM, Lee CH, Kho YH, Park KH. A new guaianolide as apoptosis inhibitor from *Chrysanthemum boreale*. Planta Med. 67: 585-587 (2001)
11. Cha JD, Jeong MR, Lee YE. Induction of apoptosis in human oral epidermoid carcinoma cells by essential oil of *Chrysanthemum boreale* Makino. Food Sci. Biotechnol. 14: 350-354 (2005)
12. Lee JH, Seo JY, Ko NY, Chang SH, Her E, Park T, Lee HY, Han JW, Kim YM, Choi WS. Inhibitory activity of *Chrysanthemi sibirici herba* extract on RBL-2H3 mast cells and compound 48/80-induced

anaphylaxis. J. Ethnopharmacol. 95: 425-430 (2004)
13. Han S, Sung KH, Yim D, Lee S, Lee CK, Ha NJ, Kim K. The effect of linarin on LPS-induced cytokine production and nitric oxide inhibition in murine macrophages cell line RAW264.7. Arch. Pharm. Res. 25: 170-177 (2002)
14. Singh RP, Agrawal P, Yim D, Agarwal C, Agarwal R. Acacetin inhibits cell growth and cell cycle progression, and induces apoptosis in human prostate cancer cells: Structure-activity relationship with linarin and linarin acetate. Carcinogenesis 26: 845-854 (2005)
15. Kim JR, Kim JH, Lim HA, Jang CH, Kim JH, Kwon CS, Kim YK, Kim JS. Induction of quinone reductase, an anticarcinogenic marker enzyme, by extract from *Chrysanthemum zawadskii* var. latilobum K. J. Food Sci. Nutr. 10: 340-343 (2005)
16. Yu R, Lei W, Mandlekar S, Weber MJ, Der CJ, Wu J, Kong AT. Role of a mitogen-activated protein kinase pathway in the induction of phase II detoxifying enzymes by chemicals. J. Biol. Chem. 274: 27545-27552 (1999)
17. Lim HA, Jang CH, Kim JH, Kim JR, Ha YR, Song YS, Kim YK, Kim J-S. Antiproliferative and anticarcinogenic enzyme-inducing activities of green tea seed extract in Hepatoma cells. Food Sci. Biotechnol. 15: 914-919 (2006)
18. Jang CH, Lee IA, Lim HA, Kim JR, Ha YR, Yu H, Sung MK, Kim JS. Anti-proliferative and anti-carcinogenic enzyme-inducing activities of delphinidin in Hepatoma cells. Food Sci. Biotechnol. 16: 641-645 (2007)
19. Jeong WS, Keum YS, Chen C, Jain MR, Shen G, Kim JH, Li W, Kong AN. Differential expression and stability of endogenous nuclear factor E2-related factor 2 (Nrf2) by natural chemopreventive compounds in HepG2 human hepatoma cells. J. Biochem. Mol. Biol. 38: 167-176 (2005)
20. Kim DH, Bang MH, Song MC, Kim SU, Chang YJ, Baek NI. Isolation of β-sitosterol, phytol, and zingerone 4-*O*-β-D-glucopyranoside from *Chrysanthmum Boreale* Makino. Korean J. Med. Corp Sci. 13: 284-287 (2005)
21. Goad LJ, Akihisa T. Analysis of Sterols. 1st ed. Chapman & Hall, London, UK. pp. 378-380 (1997)
22. Jeong WS, Lachance PA. Phytosterols and fatty acids in fig (*Ficus carica* var. *Mission*) fruit and tree components. J. Food Sci. 66: 278-281 (2001)
23. Awad AB, Chinnam M, Fink CS, Bradford PG. β-Sitosterol activates Fas signaling in human breast cancer cells. Phytomedicine 14: 747-754 (2007)
24. Von Holtz RL, Fink CS, Awad AB. β-Sitosterol activates the sphingomyelin cycle and induces apoptosis in LNCaP human prostate cancer cells. Nutr. Cancer 32: 8-12 (1998)
25. Vivancos M, Moreno JJ. β-Sitosterol modulates antioxidant enzyme response in RAW 264.7 macrophages. Free Radical Bio. Med. 39: 91-97 (2005)