Ethanol Extract of *Cirsium japonicum* var. *ussuriense* Kitamura Exhibits the Activation of Nuclear Factor Erythroid 2-Related Factor 2-dependent Antioxidant Response Element and Protects Human Keratinocyte HaCaT Cells Against Oxidative DNA Damage

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Keratinocytes are constantly exposed to extracellular insults, such as ultraviolet B, toxic chemicals and mechanical stress, all of which can facilitate the aging of keratinocytes via the generation of intracellular reactive oxygen species (ROS). Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that plays a critical role in protecting keratinocytes against oxidants and xenobiotics by binding to the antioxidant response element (ARE), a cis-acting element existing in the promoter of most phase II cytoprotective genes. In the present study, we have attempted to find novel ethanol extract(s) of indigenous plants of Jeju island, Korea that can activate the Nrf2/ARE-dependent gene expression in human keratinocyte HaCaT cells. As a result, we identified that ethanol extract of *Cirsium japonicum* var. *ussuriense* Kitamura (ECJUK) elicited strong stimulatory effect on the ARE-dependent gene expression. Supporting this observation, we found that ECJUK induced the expression of Nrf2, hemeoxygenase-1, and NAD(P)H:quinone oxidoreductase-1 and this event was correlated with Akt1 phosphorylation. We also found that ECJUK increased the intracellular reduced glutathione level and suppressed 12-O-tetradecanoylphorbol acetate-induced 8-hydroxyguanosine formation without affecting the overall viability. Collectively, our results provide evidence that ECJUK can protect against oxidative stress-mediated damages through the activation of Nrf2/ARE-dependent phase II cytoprotective gene expression.

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**Key Words:** Ethanol extract of *Cirsium japonicum* var. *ussuriense* Kitamura, Reactive oxygen species, Nuclear factor erythroid 2-related factor 2, Antioxidant response elements

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**INTRODUCTION**

Oxidative stress, caused by an imbalance between the production and destruction of reactive oxygen species (ROS), is responsible for various pathological disorders in human. Efficient ROS detoxification is particularly considered important in keratinocytes because they are constantly challenged by extracellular oxidants and electrophiles. To combat against these insults, keratinocytes possess diverse antioxidants, such as ascorbic acid (vitamin C), tocopherol (vitamin E), and reduced glutathione (GSH). In addition, keratinocytes are equipped with a number of phase II cytoprotective enzymes as well, such as hemoxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1) and glutamate- cysteine ligases (GCLs), all of which contribute to maintaining the redox balance in keratinocytes through diverse mechanisms of action.

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Transcription of phase II cytoprotective enzymes are under the control of a single transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2). Under normal condition, Kelch-like ECH-associated protein 1 (Keap1) retains Nrf2 in the cytoplasm and constantly targets it for poly-ubiquitination and proteosomal degradation. In response to oxidative or electrophilic stress, however, Nrf2 is released from Keap1 and translocates into the nucleus, where it binds to and activates the antioxidant response element (ARE), a cis-acting DNA element located in the promoter of most phase II cytoprotective enzymes. Follow-up mechanism-based studies have demonstrated that the Nrf2/ARE-dependent phase II cytoprotective gene activation can occur via two ways: (1) a direct conjugation and subsequent inactivation of Keap1 by oxidants or electrophiles or (2) phosphorylation of intracellular signaling pathways leading to Nrf2 transactivation.

Plants are the most utilized natural resources due to their abundance and accessibility. Therefore, exploring novel plant ingredients or extracts that can activate the Nrf2/ARE-dependent gene expression has been recently proposed as an efficient strategy to inhibit or delay the rate of aging and carcinogenesis progression. In the present study, we have acquired 100 ethanol extracts of indigenous plants of Jeju island, Korea and attempted to find new ethanol extract(s) that can stimulate the Nrf2/ARE-dependent gene expression.

**MATERIALS AND METHODS**

1. Cell culture, chemicals and reagents

Ethanol extracts of 100 indigenous plants of Jeju island (Table 1) were directly purchased from Jeju Technopark (Jeju, Korea). RPMI-1640 medium, heat-inactivated FBS, PBS, and 100× penicillin/streptomycin (Pen/Strep) were purchased from Welgene (Daegu, Korea). Human keratinocyte HaCaT cells were cultured in RPMI-1640 medium, containing 10% heat-inactivated FBS and 1× Pen/Strep at 37°C in humidified 5% CO2 incubator. Polyclonal antibodies against HO-1 and NQO1 were purchased from Enzo Life Sciences (Farmingdale, NY, USA) and Abcam (Cambridge, MA, USA), respectively. Primary antibody against Nrf2 and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bovine serum albumin (BSA), MTT and primary antibodies against 8′-hydroxyguanosine (8-OH-G) and actin were purchased from Sigma (St. Louis, MO, USA). Total and phospho-specific Akt1 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Fluorescein isothiocyanate (FITC)-conjugated secondary antibody was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Paraformaldehyde, bicinchoninic acid (BCA) protein assay kit, and polyvinylidene fluoride (PVDF) membranes were purchased from Millipore (Billerica, MA, USA). pGreenFire reporter plasmid was purchased from System Biosciences (Mountain View, CA, USA). pMD2.G and psPAX.2 lentiviral helper plasmids were acquired from Addgene (Cambridge, MA, USA).

2. Generation of HaCaT-antioxidant response element-luciferase cells and measurement of luciferase activity

In order to generate HaCaT-ARE-luciferase reporter cells, we have subcloned 3× tandem ARE oligonucleotides (CACCGTGACTCATGACTGACAATT CACCGTGACTCATGACTGACAATT CACCGTGACTCATGACTGACAATT with a core DNA sequence of ARE underlined) into pGreenFire reporter plasmid. 293T cells were then transfected with 3 μg pGreenFire-ARE plasmid together with 3 μg pMD2.G and 3 μg psPAX.2 plasmids, using JetPEI reagent (Polyplus-Transfection, New York, NY, USA). After 72 hours, lentiviral supernatant was collected and filtered, using a 0.45 μm syringe filter. HaCaT cells were transduced with lentiviral supernatant containing 10 μg/mL polybrenne for 12 hours at 37°C and further selected with 3 μg/mL puromycin for 48 hours. Established HaCaT-ARE-luciferase cells were seeded on 70% confluence in six-well plate and exposed to individual plant ethanol extracts at the concentration of 200 μg/mL. After 24 hours, cells were lysed with luciferase lysis buffer (0.1 M potassium phosphate buffer at pH 7.8, 1% Triton X-100, 1 mM dithiothreitol, 2 mM EDTA) and the resulting luciferase activity was measured by GLOMAX Multi-system (Promega, Madison, WI, USA). The data is depicted as a fold ratio of the firefly luciferase activity, compared with the control after normalization with protein concentration and the statistical analysis was conducted by Student t-test with n = 6.

3. Western blot analysis

After appropriate treatment, HaCaT cells were collected by centrifugation and resuspended with 200 μL RIPA buffer (50 mM Tris-HCl at pH 8.0. 150 mM NaCl. 1% NP-40. 0.5% sodium deoxycholate. protease inhibitors cocktail) and incubated on ice for 1 hour. After collection of cell lysates by centrifugation, protein concentration was measured by BCA Protein Assay Kit (Thermo Fisher, Pittsburgh, PA, USA). Equal amounts of cell lysates were resolved by 10% SDS PAGE and transferred to PVDF membrane. The membrane was incubated in blocking buffer (5% skim milk in 1× PBS-0.1% Tween-20, PBST) for 1 hour and hybridized with the appropriate primary antibodies in 1× PBS.
Table 1. List of ethanol extract of indigenous plants from Jeju island, Korea

| No. | Extract | No. | Extract |
|-----|---------|-----|---------|
| 1   | Euphorbia jolkini Boiss | 51  | Wisteria floribunda A.P. DC |
| 2   | Raphanus sativus var. hortensis f. raphanistroides Makino | 52  | Palmaria ramosissimus (Lour) |
| 3   | Korthalsella japonica Engl. | 53  | Hibiscus hamabo S. et Z |
| 4   | Neolitsea sericea (BL.) Koidz. | 54  | Callicarpa japonica Thunb |
| 5   | Cinnamomum japonicum Sieb. | 55  | Torreya nucifera S. et Z |
| 6   | Lycopodium clavatum var. nipponicum Nakai | 56  | Sapindus mukorossi Gaertner |
| 7   | Stauntonia hexaphylla (Thunb.) Decne. | 57  | Melosoma oolphamii Miq |
| 8   | Pyropyxis lingua (Thunb.) Fairwell | 58  | Rhus chinensis Mill. |
| 9   | Cyrtomium falcatum (Thunb.) Presl | 59  | Corylus sieboldiana Bl. |
| 10  | Hedera rhombea (Miq.) Bean | 60  | Albizia julibrissin Durazz |
| 11  | Gleichenia japonica Spreng | 61  | Xylosma congestum (Lour.) Merr. |
| 12  | Neolitsea aciculara (BL.) Koidz. | 62  | Ulmus davidiana var. japonica (Rehder) Nakai |
| 13  | Fatsia japonica (Thunb.) Decne. et Planch. | 63  | Zanthoxylum aethlantoides S. |
| 14  | Cyclosorus acuminatus (Houtt.) Nakai ex H.Ito | 64  | Actinodaphne lancifolia (S. et Z) Meinm |
| 15  | Eribotrya japonica Lindl. | 65  | Celtis sinensis Pers |
| 16  | Machilus thunbergii S. et Z. | 66  | Sapindus sebiferum (L.) ROX. |
| 17  | Actinodaphne lancifolia (S. et Z) Meinm | 67  | Securinega suffruticosa Rehder |
| 18  | Buxus microphylla var. koreana Nakai | 68  | Koylepanax pictus (Thunb.) Nakai |
| 19  | Ternstroemia japonica Thunb. | 69  | Caragana sinica (Bucho) Rehder |
| 20  | Citrus junos Sieb. ex Tanaka | 70  | Cnothera odorata Jacq. |
| 21  | Daphniphyllum macropodum D. glaucescens Blume | 71  | Platycodon grandiflorum (Jacq.) A. DC. |
| 22  | Ilex crenata Thunb. var. convexa Makino | 72  | Ampelopsis brevipedunculata var. heterophylla (Thunb.) Harada |
| 23  | Ligustrum lucidum Ait. | 73  | Cirsiium japonicum var. ussuriense Kitamura |
| 24  | Cinnamomum camphora Sieb. | 74  | Platanus orientalis L. |
| 25  | Pittosporum tobira Ait. | 75  | Cnothera erythrosepalas Borbas |
| 26  | Ilex crenata var. microphylla Max. | 76  | Euphorbia supina Rafin. |
| 27  | Citrus tangerina Hort.ex Tanaka | 77  | Plantago asiatica L. |
| 28  | Vicia anagostiola (var. segetis K. Koch. | 78  | Alarissis fordiI Hemsl. |
| 29  | Brassica campestris subsp. napus var. nippo-oleifer Makino | 79  | Euphorbia humifusa Willd. |
| 30  | Artemisia fukudo Makino | 80  | Vicia unijuga A. Br. |
| 31  | Lathyris japonica Wild. | 81  | Loranthus yadoviski Sieb. |
| 32  | Sonchus oleraceus L. | 82  | Solanum nirum L. |
| 33  | Rosa multiflora Thunb. | 83  | Brassica juncea var. integrifolia Sins. |
| 34  | Ateissa sp. | 84  | Daphniphyllum macropodum Miq. |
| 35  | Asparagus cochinichinsis Merr. | 85  | Ligustrum lucidum Ait. |
| 36  | Rumex acetocella L. | 86  | Caryata japonica (Thunb.) Gagnepain |
| 37  | Angelica japonica A. Gray | 87  | Broussonetia papyrifera (L.) L’ Heriter ex Ventenat |
| 38  | Lindera erythocarpa Makino | 88  | Sasa palmata (Bean) Makino |
| 39  | Acer mono Max. | 89  | Kadsura japonica (L.) Dunal |
| 40  | Akebia quinata DECNE. | 90  | Vaccinium bracteatum Thunb. |
| 41  | Saururus chinensis Baill. | 91  | Sedum bulbiferum Makino |
| 42  | Acanthophax koreanum Nakai | 92  | Persicaria sp. |
| 43  | Pterium aquilinum var. latissulum (Desv.) Underw. | 93  | Eupatorium lindleyanum DC. |
| 44  | Plantago lanceolata L. | 94  | Sapindus japonicum (Sieb. et Zucc.) Pax et Hoffmann |
| 45  | Cornus controversa Hemsl. | 95  | Maackia fauriei (Lev.) Takeda |
| 46  | Cudrania tricuspidata (Carr.) Bureau ex Lavalle | 96  | Dendropanax moriferum Leveille |
| 47  | Actinidia arguta Planch. | 97  | Glechisia japonica var. koreiensis (Nak.) Nakai |
| 48  | Clerodendron trichotomum Thunb. | 98  | Euphorbia esula L. |
| 49  | Boehmeria pannosa Nakai et Satake | 99  | Cnothera laciniata Hill. |
| 50  | Eribotrya japonica Lindl. | 100 | Litsea japonica (Thunb.) Juss. |

containing 3% BSA (in the case of phospho-specific Akt1) or 3% skim milk (in the case of total proteins) overnight at 4°C. After washing three times with 1× PBST for 30 minutes, the membrane was hybridized with appropriate HRP-conjugated secondary antibody for 1 hour at room temperature and washed three times with 1× PBST solution for 30 minutes. The membrane was
visualized by using an enhanced chemiluminescence detection system. Actin blot was used as control for an equal loading of samples.

4. Determination of intracellular reduced glutathione level

The intracellular GSH level was measured using reduced glutathione detection kit as recommended by the manufacturer (Enzo Life Sciences).

5. MTT assay

HaCaT cells (3 x 10⁴ cells/100 μL/well) were plated in 96-well culture plates in quadruplicate. After appropriate treatment, cells were exposed to 50 μL MTT stock solution (2 mg/mL) for 4 hours. HaCaT cells were then washed with 1x PBS and lysed with 50 μL DMSO. Measurement using spectrophotometer was conducted at the wavelength of 540 nm and the percentage of viable cells was plotted in comparison with the control group.

6. Detection of intracellular 8-hydroxyguanosine level

In order to measure the changes in the intracellular 8-OH-G level, HaCaT cells grown on a slice glass were incubated with blocking serum (1% BSA) for 30 minutes. After washing with 1x PBS three times, cells were hybridized with primary antibody against 8-OH-G overnight at 4°C. After washing with 1x PBS three times, the slides were probed with FITC-conjugated rabbit secondary antibody and the fluorescent images were obtained with a C2 confocal microscope (Nikon Korea, Seoul, Korea).

RESULTS

1. Identification of ethanol extract of Cirsium japonicum var. ussuriense Kitamura as a novel inducer of antioxidant response element-dependent gene expression

To find out novel plant extracts that stimulate the

![Figure 1](image-url)
ARE-dependent gene expression among ethanol extracts of 100 indigenous plants of Jeju island, Korea, we have exposed HaCaT ARE luciferase cells to individual natural extracts at the concentration 200 μg/mL for 24 hours and measured the resulting luciferase activity. While many extracts exhibited stimulatory or inhibitory effects on ARE-dependent luciferase activation, we observed that ethanol extract of *C. japonicum* var. *ussuriense* Kitamura (ECJUK; No. 73) exerted particularly strong stimulatory effect, whose ARE activation level was equivalent to that by 10 μM sulforaphane, a positive control in the experiment (Fig. 1A). Supporting this observation, an exposure to ethanol extract of ECJUK caused a concentration-dependent ARE-dependent gene activation in HaCaT ARE-luciferase cells (Fig. 1B). These results illustrate that ECJUK possesses strong stimulatory effect on ARE-dependent gene expression.

2. *Cirsium japonicum* var. *ussuriense* Kitamura induces the expression of phase II cytoprotective enzymes in HaCaT cells through nuclear factor erythroid 2-related factor 2-dependent transcriptional activation

We next examined whether ECJUK could induce the expression of Nr2 and phase II cytoprotective enzymes in HaCaT cells. To this end, HaCaT cells were exposed to ECJUK at various times and Western blotting was conducted. As a result, we observed that ECJUK induced the expression of Nrf2 and phase II cytoprotective enzymes (HO-1 and NQO1). The induction of Nrf2, HO-1, and NQO1 was closely associated with phosphorylation of Akt1 (Fig. 2A), a putative kinase that positively regulates the ARE-dependent gene expression.20 Real-time RT-PCR analysis illustrated that ECJUK stimulated transcription of HO-1 and NQO1 in HaCaT cells. Together, these results suggest that ECJUK induces Nrf2-dependent HO-1 and NQO1 expression, possibly via Akt1 phosphorylation.
3. *Cirsium japonicum* var. *ussuriense* Kitamura increases the amount of intracellular reduced glutathione and protects against ultraviolet B-mediated DNA damage

In addition to HO-1 and NQO1, Nrf2 is also responsible for transcriptional activation of GCL that boosts up the intracellular GSH level in response to oxidative stress. Therefore, we examined whether ECJUK could increase the intracellular GSH level in HaCaT cells. As a result, we found that ECJUK did not affect the overall viability of HaCaT cells (Fig. 3A). However, it significantly increased the intracellular GSH level (Fig. 3B) and inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced 8-OH-G formation (Fig. 3C). Overall, our results imply that ECJUK can activate ARE and increase the expression of Nrf2-dependent phase II cytoprotective enzymes, thereby contributing to maintaining the genome integrity against oxidative DNA damages.

**DISCUSSION**

Because Nrf2 activation plays a key role in the protection against oxidative stress, it was surmised that finding out novel compounds that can boost up the Nrf2 activity might be useful for treatment of pro-inflammatory diseases. In this sense, two recent clinical trials showed a simultaneous failure and success of Nrf2 activators as potential drug candidates. Because the methyl ester derivative of the synthetic triterpenoid, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO-Me) induces Nrf2 at low nanomolar concentrations, it underwent an initial development as a promising drug candidate under the generic name, bardoxolone methyl for treatment of advanced chronic kidney disease and type-2 diabetes mellitus. However, this clinical trial was terminated in the phase III phase for safety concerns.

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**Figure 3.** *Cirsium japonicum* var. *ussuriense* Kitamura (ECJUK) increases the intracellular reduced glutathione (GSH) level and protects HaCaT cells against oxidative DNA damage. After an exposure of ECJUK to HaCaT cells, (A) MTT assay was conducted to measure the cell viability and (B) the intracellular GSH level was measured as described. Symbols indicate a statistically significance with ***p < 0.001. (C) After an exposure of ECJUK to HaCaT cells, the intracellular 8-hydroxyguanosine (8-OH-G) level was visualized by immunofluorescence (IF) assay, using 8-OH-G antibody. TPA, 12-O-tetradecanoylphorbol-13-acetate. DAPI, 4’,6-diamidino-2-phenylindole.
concerns. On the other hand, dimethylfumarate, a synthetic Nrf2 activator, has been developed for treatment of multiple sclerosis (MS) and recently approved by the Food and Drug Administration under the trade name of Tecfidera.

In the present study, we have identified ECJUK possesses significant stimulatory effect on the Nrf2/ARE-dependent gene expression in HaCaT-ARE-luciferase reporter cells (Fig. 1). We also showed that ECJUK increased the expression of Nrf2 and phase II cytoprotective enzymes, e.g., HO-1 and NQO1 (Fig. 2) and that it protected against TPA-induced oxidative DNA damage in HaCaT cells (Fig. 3). Previous studies have demonstrated that C. japonicum induced adipocyte differentiation and exhibited pro-apoptotic effects in MCF-7 cells. Although detailed mechanisms of action of C. japonicum extract are currently unknown, it is possible that the above-mentioned biological effects could be mediated by Nrf2/ARE-dependent molecular mechanisms. A recent activity-guided fractionations by Lai et al. have identified phenylacrylic acid esters and new polyacetylenes existing in C. japonicum var. australe as major constituents. In another study, Zhang et al. have conducted LC-MS/MS determination and identified seven flavonoids, including pectolinarin, linarin, pectolinarigenin, hispidulin, diosmetin, acacetin, and apigenin in rat plasma after oral administration of C. japonicum DC. extract. At present, we are unaware which compounds primarily exist in ECJUK and the activity-guided fractionation is necessary to identify the lead compound(s) contributing to the Nrf2/ARE-dependent gene expression in ECJUK.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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