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RESEARCH NOTE

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Antioxidative Characteristics of Extracts from Aromatic Herb *Elsholtzia splendens*

Eun Jeong Choi, Yong Soo Lee, and Gun-Hee Kim*

Plant Resources Research Institute, Duksung Women's University, Seoul 132-714, Korea

Abstract We investigated the antioxidant activity of ethanol extracts obtained from the flowers of *Elsholtzia splendens* on Chinese hamster ovary CHO-K1 cells. When cells were treated with *E. splendens* extracts (ESEs), low concentrations (<12.5 ug/mL) of stimulated cell proliferation via radical generation. Relative mRNA expression of Cu/Zn-superoxide dismutase (SOD) and Mn-SOD in cells exposed to ESEs (1-20 ug/mL) was significantly induced in a dose-dependent manner ($p<0.05$). In the case of catalase, ESEs had opposing effects; that is, a low-level treatment caused a decrease, and a high-level treatment induced elevated levels ($p<0.05$). The results demonstrated that components of ESEs exhibit potential antioxidant properties. Also, further studies are required to clarify the active components of *Elsholtzia splendens* extracts responsible for such biological activities.

Key words: antioxidant, cell proliferation, *Elsholtzia splendens,* reactive oxygen species (ROS) generation

Introduction

Reactive oxygen species (ROS), such as superoxide ($O_2\cdot^-$, OOH·), hydroxyl (OH·), and peroxyl (ROOH·) radicals, reactive nitrogen species (RNS), and sulphur-centred radicals, play an important role in oxidative stress related to the pathogenesis of various common diseases. An imbalance of oxidative stress could cause a condition in which cellular antioxidant defenses are insufficient to maintain the levels of oxidants below a risk threshold.

Oxidative stress contributes to the initiation of lipid peroxidation, protein modification (oxidation of amino acid side chains, protein cross-link formation, and oxidation of polypeptide backbones resulting in protein fragmentation) and the formation of single-strand DNA breaks. These types of damage can lead to enzyme inactivation, mutation, and membrane disruption, increased atherogenicity of low-density lipoproteins, mitochondrial dysfunction, and cell death. They are implicated in aging and in the development of chronic, inflammatory, degenerative, and age-related diseases (1-3).

In order to protect tissues from these forms of oxidative damage, the human body has antioxidant defense systems, including both enzymatic and non-enzymatic mechanisms. The enzymatic defense system is composed of superoxide dismutase (SOD), catalase, and glutathione-related enzymes such as glutathione peroxidase, glutathione reductase, and glutathione-S-transferase.

In modern industrial societies, concerns regarding public health, especially in relation to food habits, are increasing daily. As a result of this trend, a great deal of attention has focused on the beneficial health effects of traditional herbs related to their biological properties, particularly their antioxidant activities (4-7). Increasing evidence indicates that their antioxidant capacity may contribute to a reduced incidence of various cancers and cardiovascular diseases, although overdosing may result in an undesirable pro-oxidative effect.

Elsholtzia splendens has been used as an ingredient in folk medicines in Northeast Asia (8, 9) and belongs to a subclass of the Labiatae family. Although it is well-known in Chinese traditional medicine, most such traditional herbs have been applied without studies of their biological and physiological effects. Previous studies have demonstrated that some of the herbs from the Labiatae family have physiological effects (10, 11); *Elsholtzia blanda* can reduce infarct size during acute myocardial infarction by inhibiting myocardial apoptosis *in vivo* (12, 13), and *E. splendens* Nakai extracts have been reported to have anti-inflammatory activity (14). In the present study, we assay that extracts from *E. splendens* possess antioxidative characteristics.

Materials and Methods

The cells culture and preparation of *E. splendens* extract Chinese hamster ovary CHO-K1 cells were purchased from the KCLB (Korean Cell Line Bank, Korea). Cells was routinely maintained in RPMI 1640 (Gibco; Invitrogen, Carlsbad, CA, USA), supplemented with 10% FBS and antibiotics (50 U/mL of penicillin and 50 ug/mL streptomycin, Gibco) at 37°C in a humidified atmosphere containing 5% CO_2. The flower of *E. splendensi* was collected from a home garden during efflorescence in the fall (from September to October). Ethanol extracts from *E. splendens* were prepared as followed; briefly, flowers of *E. splendensi* were freeze-dried and crushed. Then after, freeze-dried materials were extracted with 80% ethanol for 30 min at room temperature (5 g of dried materials per 500 mL solution). The yield (w/w) of the dehydrated powder among the primary net dry weight plant was about 1.6%. This dehydrated powder was diluted in dimethyl sulfoxide (DMSO) to 10 mg/mL just before use.

*Corresponding author: Tel: 82-2-901-8496; Fax: 82-2-901-8661
E-mail: ghkim@duksung.ac.kr
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Determination of reactive oxygen species (ROS) Production of intracellular ROS was measured by the fluorescence dyes, dichlorodihydro-fluorescein diacetate (H_2DCFDA) and dihydrorhodamine 123 (DHR 123; Molecular Probes, Eugene, OR, USA). H_2DCFDA is rapidly oxidized to the highly fluorescent dichloro-fluorescein (DCF) and DHR123 is oxidized intracellularly to form the fluorescent compound rhodamine123 (RH123) in the presence of intracellular ROS. Fluorescence was determined at 529/536 nm with the excitation and at 503/500 nm with emission for H_2DCFDA or DHR 123, respectively, on a spectrofluoro-meter (spectra max geminiXS; Molecular Devices, Sunnyvale, CA, USA). Also, for measurement of $O_2^{\cdot -}$ generation, NBT assay was used. The cells were treated with ESEs ranging from 6.25 to 100 ug/mL and incubated for 24 hr and added to nitroblue tetrazolium (NBT, Sigma, St. Louis, MO, USA). Four hr later, the cells were washed with PBS and then lysed in 200 μL of DMSO by repeated passage through the tip of a pipette. The formation of formazan from the reaction of NBT with $O_2^{\cdot -}$ was evaluated by measurement of absorbance at 540 nm in a microplate reader (SpectraMax Plus; Molecular Devices).

Determination of cell proliferation For measurement of cell proliferation, methyl thiazolyl (MTT) assay was used. The cells were treated with ESEs ranging from 6.25 to 100 ug/mL and incubated for 24 hr and added to MTT. Four hr later, DMSO was added to each well to dissolve the resulting formazan crystals and then absorbance was recorded at 490 nm in a microplate reader (SpectraMax Plus; Molecular Devices).

Reverse transcription and real-time polymerase chain reaction (RT-PCR) For measurement of quantified mRNA expression on antioxidant enzyme system, cells were treated with ESEs ranging from 1.25 to 20 ug/mL for 24 hr. Cells were directly sorted into tubes containing Trizol (Gibco-BRL; Invitrogen) and mRNA was extracted according to the manufacturer's protocol. First-strand cDNA was synthesized from 2 μg total RNA using an oligo(dT) primer and SuperScript first-strand synthesis system for RT-PCR (Invitrogen), according to the manufacturer's instructions. Each target mRNA expression was quantified by real-time PCR with the use of CFB-3120 MiniOpticon™ system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). CFB-3120 MiniOpticon™ system uses an array of 48-light-emmitting diodes (LED), which is efficiently excite fluorescent dyes with absorption spectra in the 470-505 nm range. PCR reactions were carried out in a total volume of 20 μL containing 10 μL of 2X SYBR® Green mix (Finnzymes Oy, Finland), 1 μL of diluted first-strand cDNA (produced from 2 ug of total RNA) and 1 μL of forward and reverse primers each (Catalase, CuZn-SOD, Mn-SOD, or GAPDH). All PCR reactions were performed in 0.1 mL tubes with caps in triplicates. Cycling conditions comprised an initial 5 min denaturing step at 95°C, and subsequently 35 cycling at 95°C for 20 sec, 53.5°C for 20 sec, and 72°C for 40 sec. These reactions were performed. For each sample, catalase mRNA levels were calculated by means of the comparative cycle threshold (C_t) method using $2^{-\Delta\Delta Ct}$ according to the manufacturer's instructions. GAPDH was used as an endogenous control (internal control). The fold change in target gene relative to the endogenous control was determined as Fold change = $2^{-\Delta\Delta Ct}$; where, $\Delta\Delta Ct = (Ct_{target} - Ct_{endogenous})_{treated\ group} - (Ct_{target} - Ct_{endogenous})_{control\ group}$. The untreated sample (control group) was defined as the calibrator in this experiment. Therefore, the amounts of Cu-ZnSOD, MnSOD, GPx, and CAT transcripts in the other samples were assigned dimensionless numbers relative to the levels in the calibrator sample.

Statistical analyses All values are expressed as means ± SD. Data were analyzed by unpaired Student's *t*-test or one-way analysis of variance followed by Dunnett's multiple comparison test (SigmaStat; Jandel, San Rafael, CA, USA). For all comparisons, differences were considered statistically significant at $p<0.05$.

Results and Discussion

Effect of ESEs on radical generation and cell proliferation The present study was designed to investigate the antioxidant characteristics of ethanol extracts from flowers of *E. splendens* (ESEs). First, we observed the ROS generation in CHO-K1 cells exposed to

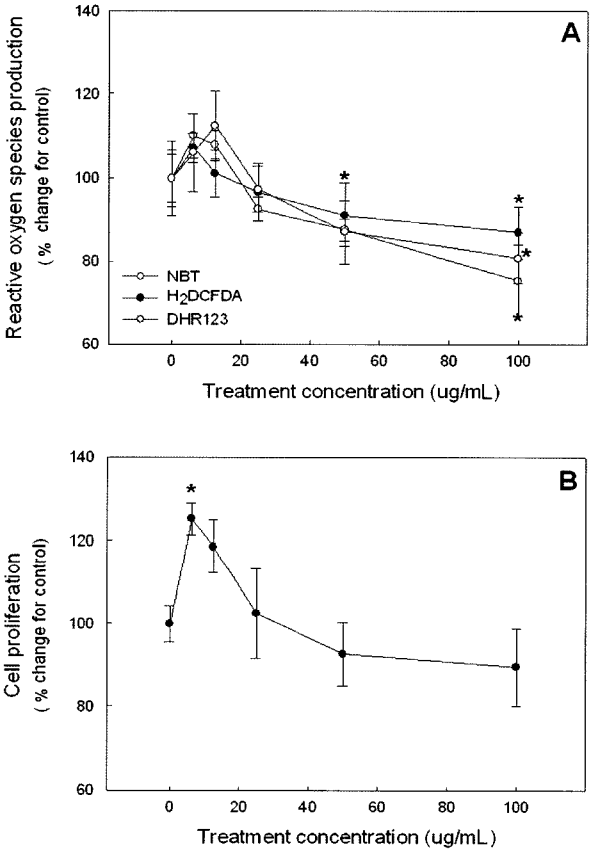

Fig. 1. Reactive oxygen species generation (A) and cell proliferation (B). The CHO-K1 cells were exposed to ESEs (1-100 μg/mL) for 24 hr. All data are reported as the percentage change in comparison with the vehicle-only group. *$p<0.05$, Significantly different from the vehicle-only group (ESEs concentration = '0').

ESEs at concentrations ranging from 1 to 100 ug/mL. To examine the radical scavenging capacity of ESEs, we used nitroblue tetrazolium (NBT) and fluorescence dyes such as dichlorodihydro-fluorescein diacetate (H_2DCFDA) and dihydrorhodamine 123 (DHR 123). ESEs showed a biphasic effect on superoxide anion generation of CHO-K1 cells (Fig. 1A). ESEs treatment increased superoxide anion generation at low concentrations (increases of 10.15 and 8.0% in the 6.25 and 12.5 ug/mL treatment groups, respectively). Beginning at 25 ug/mL concentration, superoxide anion generation decreased, and the reductions reached 12.4 and 24.8% at high concentrations of 50 and 100 ug/mL ESEs, respectively (p<0.05). Also, ROS generation measured by fluorescence dyes showed similar patterns. At the maximum concentration (100 ug/mL), ESEs decreased the generation of ROS, as compared with the control group (p<0.05, 13.1 and 19.3% for DHR123 and H_2DCFDA, respectively). Although there was no statistical difference, ROS generation increased with the ESEs treatment at 6.25 and 12.5 ug/mL, (Fig. 1A).

ESEs treatment at low concentrations (less than 12.5 ug/mL) had a tendency to slightly increase the level of reactive oxygen species (ROS), although the difference was not statistically significant. At the same concentration, ESEs significantly increased cell proliferation. This finding agrees with the results of previous studies, which found that ROS generation at low concentrations may act as a second messenger in the signal transduction process to stimulate cell proliferation (15, 16). However, ESEs at high concentration (25-100 ug/mL) significantly decreased ROS generation and did not change cell proliferation.

Cell proliferation in CHO-K1 cells was induced by ESEs treatment for 24 hr (increases of 25.28 and 18.68% at 6.25 and 12.5 ug/mL, respectively, compared to the control level, p<0.05; Fig. 1B). ESEs treatment at high concentra-tions, ranging from 50 to 100 ug/mL, showed a tendency towards a slight increase, but no statistically significant difference was observed.

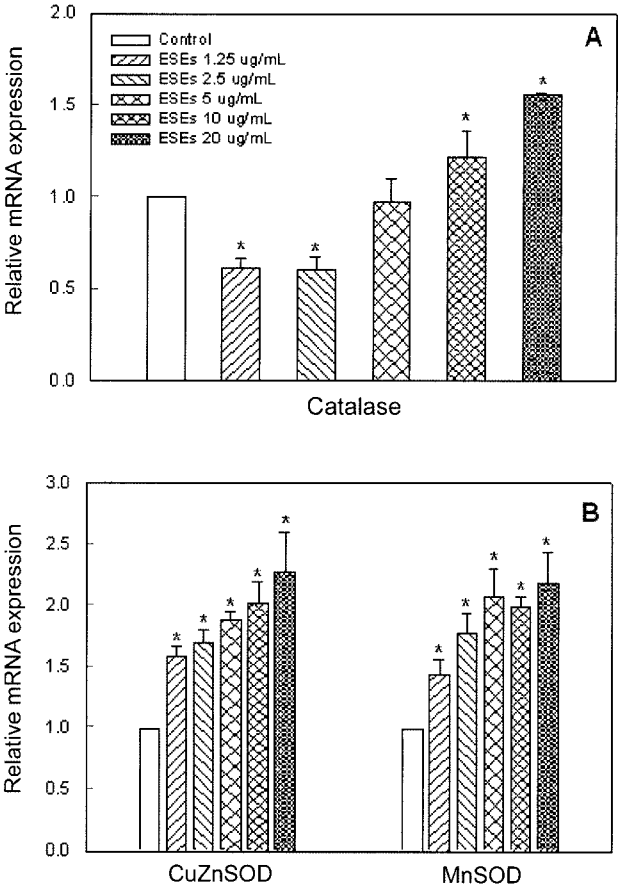

Fig. 2. Relative mRNA expression of antioxidant enzymes, *e.g.*, catalase (A) and CuZn-/Mn-SOD (B). The CHO-K1 cells were exposed to ESEs (1- 20 μg/mL) for 24 hr. Control group (vehicle-only group) was accepted to be '1.0'. *p<0.05, Significantly different from the vehicle-only group (ESEs concentration = '0').

Effect of ESEs on relative mRNA expression of the antioxidant defense system related enzymes Since we found that ESEs at low concentration were able to induce radical generation and proliferation, we next assessed the antioxidant defense enzymes by measuring the relative mRNA expression in cells that were exposed to ESEs at concentrations ranging from 1 to 20 ug/mL. Relative mRNA expression of the antioxidant enzymes catalase, CuZn-SOD, and Mn-SOD in CHO-K1 cells treated with ESEs ranging from 1 to 20 ug/mL was quantified by real-time PCR. Catalase mRNA expression significantly increased with the ESEs treatment at over 10 ug/mL, although at very low concentrations (<2.5 ug/mL), it significantly decreased (0.61- and 0.60-fold at 1.25 and 2.5 ug/mL ESEs treatment, respectively, compared to the control level, p<0.05; Fig. 2A). ESEs significantly increased the mRNA expression of CuZn-SOD and Mn-SOD in a dose-dependent manner (p<0.05; Fig. 2B). The more than twofold induction of CuZn-SOD and Mn-SOD mRNA expression began at 10 and 5 ug/mL, respectively. At 20 ug/mL ESEs treatment, CuZn-SOD and Mn-SOD mRNA expression increased 2.27- and 2.18-fold, respectively, compared to the control level.

Relative mRNA expression of Cu/Zn-SOD and Mn-SOD was significantly induced in a dose-dependent manner (p<0.05). However, catalase mRNA expression decreased about 40% with ESEs treatment at concentrations less than 2.5 ug/mL, compared to the control level (p<0.05), and significantly increased at 10 and 25 ug/mL. The decrease in catalase mRNA expression may be an action to protect cells from radical generation by ESEs. Induction of catalase mRNA expression has been reported to be elicited by oxidative stress, such as hydrogen peroxide or hypoxia, in various cells (17, 18). However, in the present study, despite the increase in radical generation together with the decrease in catalase mRNA expression, ESEs did not act as a prooxidant.

Interestingly, in the present study, ESEs were shown to have different effects depending on treatment concentration; that is, low concentrations of ESEs may stimulate cell proliferation via generation of radicals such as superoxide anion, and high concentrations may be more beneficial to human health via activation of antioxidant enzymes. However, further studies are needed to elucidate the exact mechanism of action and to isolate the active components.

Acknowledgments

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