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RESEARCH NOTE

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In vivo Antioxidative Characteristics of Extracts from the Aromatic Herb *Elsholtzia splendens*

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Abstract The antioxidative properties of an *Elsholtzia splendens* ethanol extract (ESE) were examined *in vivo*. Oral administration of 10 or 50 mg ESE/kg BW in mice for 50 days resulted in a dose-dependent decrease in several biomarkers of oxidative stress including thiobarbituric acid reactive substance (TBARS), protein carbonyls, and serum 8-hydroxy-2'-deoxy guanosine (8-OH-dG). Moreover, the level of activity and mRNA expression of catalase and superoxide dismutase (SOD) were significantly increased by ESE treatment. Taken together, these results indicate that ESE may be beneficial to human health via its antioxidative properties.

Keywords: antioxidant, *Elsholtzia splendens, in vivo*, oxidative stress

Introduction

Because the natural antioxidants found in plants protect against oxidative stress and play an important role in disease prevention, it has been recommended to increase consumption of antioxidants (1,2).

Elsholtzia splendens (Labiatae family) has traditionally been used in northeast Asia as a treatment for diarrhea, as an expectorant, and as a diuretic (3-5). Although the use of herbs is well established in traditional Korean and Chinese medicine, the biological and physiological effects of most medicinal herbs, including those of *E. splendens*, have not been reported. Our previous work focused on the antioxidative, anti-inflammatory, and antitumor effects of *E. splendens in vitro* (6,7). In the present study, the *in vivo* antioxidative effects of an *E. splendens* ethanol extract (ESE) were investigated.

Materials and Methods

Preparation of *Elsholtzia splendens* extract The flowers of *E. splendens* were collected from a home garden during efflorescence in the fall (from September to October). The flowers were freeze-dried, crushed, and extracted with 80% ethanol for 30 min at room temperature (5 g/500 mL). The ethanol supernatant was evaporated in a rotary evaporator (Eyela NYC-2000; Tokyo Rikakikai Co., Ltd., Tokyo, Japan) under reduced pressure, and the remaining ethanol was dried in a desiccator with a high vacuum pump (W2v40; Woosung Automa Co., Ltd., Seoul, Korea). The yield (w/w) of the dehydrated powder from the primary net dry weight of the plant material was about 1.6%.

Animal care and serum analysis Female ICR mice (23-25 g; Central Lab. Animal Inc., Seoul, Korea) were housed 5 to a polypropylene cage (24±2°C, 40-50% relative humidity) under controlled lighting (12L:12D). The mice were fed an AIN 93M diet (Dyets, Bethlehem, PA, USA) and allowed free access to water. After an adaptation period, the mice were randomly divided into 3 treatment groups. Extracts of *E. splendens* (ESE) were suspended in water and administered orally to 2 of the 3 groups at 10 and 50 mg/kg BW for 50 days. Mice in the remaining (control) group were given the vehicle (water) alone. Animal care in this study conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (8). At the end of experiment, the mice were rapidly anesthetized with ether 6 hr after the final dose of ESE. The livers were isolated, blotted, weighed, frozen in liquid nitrogen, and stored at 70°C until assayed. Our preliminary study determined that ESE did not affect serum antioxidant molecules such as α-tocopherol (9).

Determination of oxidative stress biomarkers As a biomarker of oxidative stress to lipid, protein, and DNA, we measured thiobarituric acid reactive substances (TBARS), liver carbonyl content, and serum 8-hydroxy-2'-deoxy guanosine (8-OH-dG). TBARS was determined by measuring the malondialdehyde concentration (10), and its calculation was based on the molar absorption coefficient of malondialdehyde (MDA), $\varepsilon=1.56\times10^{-5}$/M/cm at 535 nm. The formation of protein carbonyl in the liver was determined using 2,4-dinitrophenylhydrazine (DNPH) (11) at an absorbance of 365 nm. The results were expressed as moles of DNPH incorporated/100 mg protein using a molar extinction coefficient of 2.1 m/M/cm. Serum concentrations of 8-OH-dG were measured by enzyme-linked immunosorbent assay (ELISA, StressXpress® DNA Damage ELISA kit; Stressgen Bioreagents, Ann Arbor, MI, USA).

Analysis of antioxidant enzymes Superoxide dismutase (SOD) activity was assayed according to the pyrogallol autoxidation method (12). Each unit of SOD activity was defined as the quantity of enzyme that resulted in a 50%

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Table 1. Biomarkers of oxidative stress

Group[1)]	8-OH-dG (ng/mL serum)	Carbonyls (nmole/mg protein)	TBARS value (nmole MDA/mg protein)
Vehicle (control)	10.04±0.97[a]	0.94±0.07[a]	0.63±0.04[a]
10 mg/kg BW/day	8.68±0.98[ab]	0.74±0.04[ab]	0.47±0.04[ab]
50 mg/kg BW/day	7.42±0.44[b]	0.64±0.04[b]	0.38±0.03[b]

[1)]Two groups of mice were orally administered 10 or 50 mg/kg BW for 50 days. A third group was administered vehicle (water) alone. Values are mean±SD (n=6). Numbers with different letter superscripts are significantly different ($p<0.05$).

Table 2. Antioxidant enzymes

Group[1)]	Catalase		Total SOD	CuZn-SOD	Mn-SOD
	nmole H_2O_2 decomposed/ min/mg protein	mRNA level	unit/mg protein	mRNA level	
Vehicle (control)	1.15±0.08[a]	1.0[a]	0.22±0.01[a]	1.0[a]	1.0[a]
10 mg/kg BW/day	1.37±0.07[ab]	1.21±0.07[ab]	0.29±0.02[ab]	1.17±0.21[a]	1.14±0.11[a]
50 mg/kg BW/day	1.55±0.04[b]	1.44±0.05[b]	0.38±0.02[b]	1.34±0.12[b]	1.52±0.18[b]

[1)]Two groups of mice were orally administered 10 or 50 mg/kg BW for 50 days. A third group was administered vehicle (water) alone. Values are mean±SD (n=6). For relative mRNA expression, the value for the control group was considered to be 1.0. Numbers with different letter superscripts are significantly different ($p<0.05$).

inhibition of pyrogallol auto oxidation under experimental conditions. Catalase activity was assayed by the method of Aebi (9), and calculated as nmol of H_2O_2 decomposed/min/mg protein. Real-time polymerase chain reaction (PCR) was used to measure the relative mRNA expression. Samples were homogenized with Trizol (TRIzol® Reagent; Gibco-BRL, Invitrogen, Carlsbad, CA, USA) and mRNA was extracted according to the manufacturer's protocol. First-strand cDNA was synthesized using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Each target mRNA transcript was quantified by real-time PCR with a CFB-3120 MiniOpticon™ system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). This system uses an array of 48 light-emitting diodes (LED) that efficiently excites fluorescent dyes with absorption spectra in the 470-505 nm range. PCR reactions were carried out with 2X SYBR® Green mix (Finnzymes, Espoo, Finland). The 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. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The fold-change in target gene relative to the endogenous control was determined as: $2^{-\Delta\Delta Ct}$; where $DDCt=(Ct_{target}-Ct_{endogenous})_{treated\ group}-(Ct_{target}-Ct_{endogenous})_{control\ group}$. The untreated sample (control group) was defined as the calibrator in this experiment. Therefore, the catalase, copper and zinc-containing SOD (CuZn-SOD), and manganese SOD (Mn-SOD) transcripts were assigned dimensionless numbers relative to the levels in the calibrator sample.

Statistical analysis All values are expressed as means±SD. Data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range comparison test (SigmaStat; Jandel; San Rafael, CA, USA). For all comparisons, differences were considered statistically significant at $p<0.05$.

Results and Discussion

To evaluate the antioxidant effect of ESE, we measured levels of serum 8-OHdG (oxidized DNA bases), carbonyls (protein oxidation), and TBARS (lipid peroxidation). Mice given ESE orally at 10 or 50 mg/kg BW for 50 days had a significant dose-dependant decrease in TBARS, serum 8-OH-dG content, and the level of liver protein carbonyls ($p<0.05$; Table 1). Cellular oxidative stress has been implicated in the etiology and pathology of many diseases (14,15). Thus, ESE, which reduces oxidative stress, may be very beneficial to human health as an antioxidant.

Because we found that ESE efficiently inhibited oxidative stress, we were interested in assessing the antioxidant defense system in the mouse liver (Table 2). SOD is the first and most important mechanism of enzymatic defense against oxidative stress. SOD scavenges superoxide by converting it to peroxide, which is destroyed by catalase (16). That is, SOD and catalase act together as antioxidant enzymes to provide a protective defense against reactive oxygen species (ROS). The activity and mRNA expression of catalase increased significantly in a dose-dependent manner by ESE ($p<0.05$). Similar changes were observed in the level of SOD activity. In addition, at 50 mg/kg BW, ESE significantly increased the mRNA expression of CuZn- and Mn-SOD. This is consistent with previously reported *in vitro* data showing the capacity of ESE to stimulate catalase and SOD activity (7).

Although the active antioxidants in our ESE extract have yet to be identified, these results suggest that ESE may benefit human health by activating antioxidative enzymes and inhibiting oxidative stress. Further studies are needed to elucidate the exact ESE mechanism of action and to isolate the active antioxidant components.

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