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RESEARCH NOTE

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Antioxidant Effects of *Elsholtzia splendens* Extract on DMBA-induced Oxidative Stress in Mice

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Abstract The present study was conducted to investigate the effects of flowers ethanol extract of *Elsholtzia splendens* (ESE) on the antioxidant defence system in mice with 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced oxidative stress. The ESE was pre-administered orally to 2 groups of mice at 10 and 50 mg/kg body weight (BW) for 5 weeks. Subsequently, mice with pretreatment of ESE received DMBA intragastrically at a dose of 34 mg/kg BW twice a week for 2 weeks. In DMBA alone group, biomarkers of oxidative stress (TBARS value, carbonyl content, and serum 8-OH-dG) were significantly increased. Also, the antioxidant enzymes were down-regulated. ESE significantly restored the TBARS value and carbonyl content at both doses, while a decrease in the elevated serum 8-OH-dG content was observed only at the higher dose. The DMBA-induced decreases in catalase and superoxide dismutase (SOD) activities were restored to nearly control levels by ESE. Glutathione peroxidase (GSH-px) and glutathione reductase (GR) activities, as well as the reduced glutathione (GSH)/oxidized GSH (GSSG) ratio, were significantly affected by ESE at the higher dose. These results suggest that ESE possesses antioxidant activity, which plays a protective role against DMBA-induced oxidative stress.

Keywords: *Elsholtzia splendens*, 7,12-dimethylbenz(*a*)anthracene (DMBA), antioxidant, oxidative stress

Introduction

A great deal of research has shown that oxidative stress induces biochemical changes, and these changes are crucial etiological factors in several chronic human diseases, including diabetes mellitus, cancer, atherosclerosis, arthritis, and neurodegenerative diseases, in addition to the aging process (1,2). Many compounds in plants possess strong antioxidant activities; thus, to protect against the various diseases induced by oxidative stress in humans, the consumption of plant derived from natural antioxidants has been strongly recommended (3).

Elsholtzia splendens is an ingredient which is used in traditional medicines in northeast Asia and belongs to a subclass of the family Labiatae (4,5). Besides, *Elsholtzia* genus includes *E. ciliate, E. saxatilis, E. angustifolia,* and *E. splendens* Nakai etc. *E. splendens* and *E. ciliate* are used mainly in folk remedies for diarrhea, as expectorants and for their diuretic effects (6,7). Several researchers have demonstrated that some species of *Elsholtzia* have physiological effects to benefit human health *in vitro* (5,8,9). Moreover, our research group has also identified useful biological activities of *E. splendens*, such as its antioxidant, anti-inflammatory, and antitumor actions *in vitro* (10,11), suggesting *E. splendens* might be used as a food material and has a potential application to relieve and prevent disease.

Until recently, no data were available to show whether the repeated intake of high doses of *E. splendens* produced antioxidantative effects. Therefore, this study was designed to evaluate the protective effects of extracts obtained from the flowers of *E. splendens* on 7,12-dimethylbenz(a) anthracene (DMBA)-induced oxidative stress in mice.

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Materials and Methods

***E. splendens* extract** The flower of *E. splendens* was collected from a home garden during efflorescence in the fall (from September to October). Ethanol extracts from *E. splendens* (ESE) were prepared as follows; briefly, flowers of *E. splendens* were freeze-dried, crushed, and extracted with 80% ethanol for 30 min at room temperature (5 g of dried materials/500 mL solution). The yield (w/w) of the dehydrated powder based on the primary net dry weight plant was about 1.6%.

Animal care and sample preparation 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 (12-hr light/dark cycle). Mice were fed an AIN 93M diet (Dyets, Bethlehem, PA, USA) and allowed free access to water. After an adaptation period, mice were divided randomly into 4 groups. ESE was suspended in water and was administered orally to 2 of the 3 groups at 10 and 50 mg/kg body weight (BW) for 50 days. Subsequently, DMBA were intragastrically administered a dose of 34 mg/kg BW in corn oil vehicle twice a week for 2 weeks. Mice in the remaining (control) group were given the vehicle alone as oral administration. 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 (NIH) (12). At the end of experiment, mice were rapidly anesthetized using ether at 6 hr after final administration of ESE. Then after, their livers were isolated, blotted, weighed, frozen in liquid nitrogen and stored at −70°C until assayed. For assay, each livers were homogenized for

approximetely 45 sec in 9 volume of ice-cold 10 mM phosphate buffered saline (PBS, pH 7.4) containing 1.15% KCl. Homogenates were centrifuged at 800×g to remove cell debris and nuclei; the supernatants were centrifuged at 10,000×g for 10 min and transferred the portions of the post-mitochondrial fraction. To obtain the cytosolic fractions for the measurement of superoxide dismutase (SOD) activity, the remaining supernatant was centrifuged further at 105,000×g for 45 min using a 50 Ti rotor in a Beckman model L90 ultracentrifuge (Fullerton, CA, USA). Post-mitochondrial and cytosolic fractions were stored at 80°C in aliquots until analysis within 1 week.

Oxidative stress measurement Thiobarbituric acid reactive substances (TBARS) value was determined by measuring the concentration of malondialdehyde according to the method of Ohkawa *et al.* (13) and its calculated according to the molar absorption coefficient of malondialdehyde (MDA), $\varepsilon=1.56\times10^{-5}$ mM/cm at 535 nm. The formation of protein carbonyl in the liver was determined using 2,4-dinitrophenylhydrazine (DNPH) according to the method of Reznick and Packer (14). The absorbance was read at 365 nm and the results were expressed as moles of DNPH incorporated/100 mg protein using a molar extinction coefficient of 2.1 mM/cm. Serum concentrations of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) were measured by enzyme-linked immunosorbent assay (ELISA, StressXpress® DNA Damage ELISA kit; Stressgen Bioreagents, Ann Arbor, MI, USA).

Antioxidant enzymes activities Total SOD activity was assayed according to the pyrogallol autoxidation method of Marklund and Marklund (15). Each unit of SOD activity was defined as the quantity of enzyme that inhibited auto-oxidation of pyrogallol by 50% under experimental conditions. Catalase activity was assayed by the method of Aebi (16). Catalase activity was calculated as nmol of H_2O_2 decomposed/min/mg protein. In addition, protein expression levels were investigated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoblotting (11). Antibodies against catalase, Mn-, CuZn-SOD and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The specific protein bands were detected by Opti-4CN Substrate kit (Bio-Rad, Laboratories, Hercules, CA, USA).

Activities of glutathione peroxidase (GSH-px) and glutathione reductase (GR) were determined with a spectrophotometer by measuring the disappearance of nicotinamide adenine dinucleotide phosphate hydrogenase (NADPH) at 340 nm, based on the methods of Flohé and Günzler (17) and Carlberg and Mannervik (18), respectively. The enzyme activities of glutathione peroxidase and reductase were defined as nmol NADPH oxidized/min/mg protein using molar extinction coefficient of 6.22×10^3 mM/cm.

Determination of ratio of GSH and GSSG The concentration of reduced glutathione (GSH) was measured with use of *O*-phthalaldehyde as a fluorescent reagent according to method of Hissin and Hilf (19). For oxidized GSH (GSSG) measurement, each sample was incubated with *N*-ethyl maleimide to interact with GSH present in the tissue homogenate. The GSH and GSSG content (μg/mg protein) were obtained from a standard curve and then after the GSH/GSSG ratio was calculated. Protein concentration was determined by Bradford protein assay kit II (Bio-Rad).

Statistical analyses Data were analyzed by unpaired Student's *t*-test or one-way analysis of variance (ANOVA) 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 ESE on oxidative stress The present study was investigated the effects of ESE pretreatment on oxidative stress induced by DMBA, a polycyclic aromatic hydrocarbon that is widely distributed in the environment and has been implicated in various types of cancer. The DMBA acts as a potent carcinogen by generating various reactive metabolic intermediates leading to oxidative stress in such organs as the liver and mammary glands (20,21). In the present study, the TBARS value and protein carbonyl content in DMBA-treated mice were significantly greater (1.5 and 1.74 times, respectively, Fig. 1A) than in untreated control mice. This is consistent with previous reports

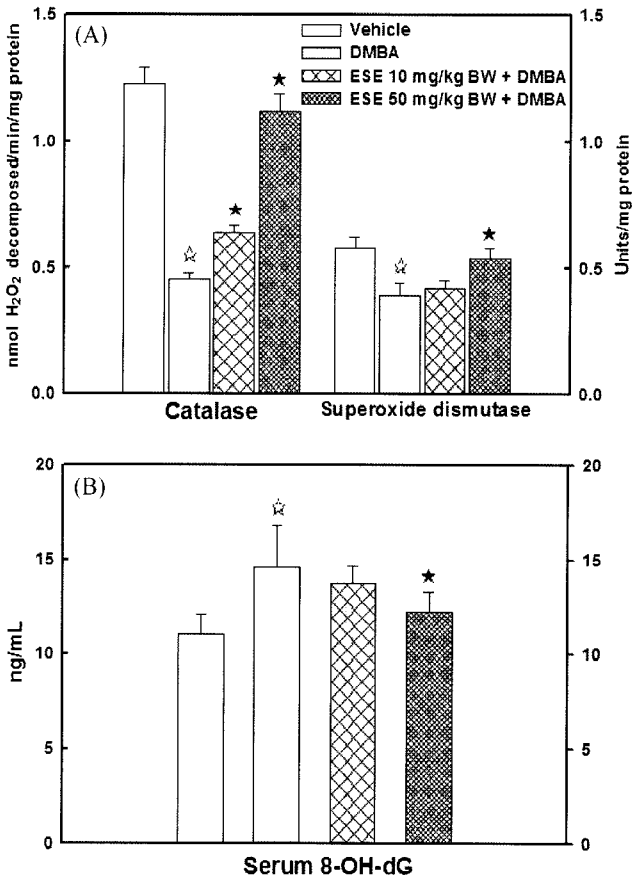

Fig. 1. Carbonyls content, TBARS value (A) and serum 8-OH-dG content (B) as biomarkers of oxidative stress. Values are means±SD (n=6). ☆,★$p<0.05$, significantly different between DMBA-treated control and untreated control group (☆) and from DMBA-treated control group (★), respectively.

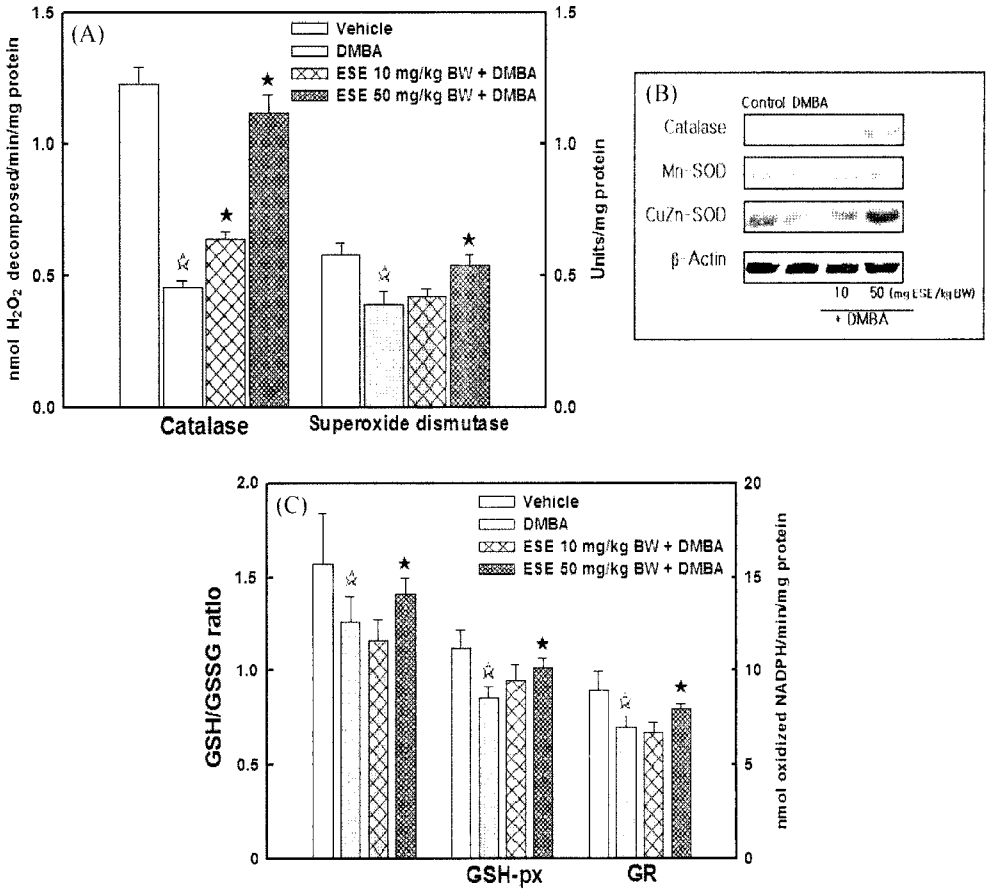

Fig. 2 Catalase, total superoxide dismutase (A, their activities; B, their expression) and GSH-related system (C). Values are means±SD (n=6). ☆,★$p<0.05$, significantly different between DMBA-treated control and untreated control group (☆) and from DMBA-treated control group (★), respectively.

showing that DMBA induces critical oxidative damage in the liver *in vivo* (22,23). The ESE significantly decreased both the TBARS value and protein carbonyl content in the DMBA-treated group in a dose-dependent manner ($p<0.05$), although the effect on the TBARS value was more pronounced (29.7 and 44.1% decrease at 10 and 50 mg/kg BW compared to the DMBA-treated group, respectively). In addition, a significant decrease in the elevated serum 8-OH-dG content of the DMBA-treated mice resulting from ESE treatment was observed only at the higher dose (50 mg/kg BW, Fig. 1B). Thus, ESE, which reduces oxidative stress, may be very useful for human health as an antioxidant.

Effect of ESE on antioxidant defense system SOD, the first and most important line of antioxidant enzyme defense against oxidative stress, deals with oxyradicals by accelerating the dismutation of superoxide (O_2) to hydrogen peroxide (H_2O_2). Catalase, in turn, detoxifies a significant amount of the H_2O_2 produced by the electron transport chain and SOD. In the DMBA-treated mice, total SOD and catalase activity were significantly decreased, suggesting the induction of oxidative stress by DMBA. In the DMBA-treated mice, the catalase and total SOD activities were significantly decreased to less than half of the values in the control group (a 62.6 and 32.8% decrease compared to the controls, respectively, Fig. 2A). At the higher dose of ESE (50 mg/kg BW), however, the levels of catalase and total SOD activity were restored to normal. Catalase activity was increased 39.7% by pre-treatment with ESE, even at the lower dose. Similar changes were observed in the expression of catalase, Mn-, and CuZn-SOD (Fig. 2B).

GSH-px catalyzes the reduction of H_2O_2 and alkyl hydroperoxides by oxidizing GSH. GR is responsible for recycling the GSSG that is formed during oxidation events by reducing it back to GSH. GSH is a major nonenzymatic antioxidant that plays a critical role in enzymatic reactions as a co-factor or in the cellular redox status as a major thiol-disulfide (24).

The basal activity of GSH-px and GR in the DMBA-treated mice was significantly lower than that in the control group (23.6 and 22.2% decrease compared with control, respectively, Fig. 2C). Although ESE had no effect on GSH-px and GR activity at the lower dose, a significant decrease resulting from ESE at the higher dose was observed (approximately 15%). The dysfunction of GSH and its related antioxidant enzymes, such as GSH-px and GR, is involved in the initiation of cancer (25,26). In addition, the GSH/GSSG ratio was significantly decreased (by approximately 20%) in the DMBA-treated control mice compared to the untreated control mice (Fig. 2C). ESE pre-treatment caused a slight but insignificant increase of about 10% in the GSH/GSSG ratio at the higher dose The GSH/GSSG ratio is a very sensitive marker of the body's antioxidant defense system against oxidative stress. Glutathione deficiency contributes to oxidative stress, and

may therefore play a key role in aging and in the pathogenesis of many diseases (27). Thus, up-regulation of a glutathione-related mechanism by ESE may be beneficial for human health.

Conclusively, ESE can protect against oxidative stress, particularly lipid peroxidation, and it activates the body's antioxidant defence system through increases in catalase, SOD, and the GSH/GSSG ratio. Oxidative stress-induced deterioration of the antioxidant defense system in organelles has been suggested to play an important role in carcinogenesis. Therefore, food supplements containing ESE may be useful in the maintenance of good health given that DMBA-induced oxidative stress can be attenuated by ESE.

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