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PC12세포와 동물모델에서의 기억력 장애를 유도하는 산화적스트레스에 대한 취나물과 모과 복합추출물의 개선 효과

박찬규[1] · 최수정[2] · 신동훈[3†]

Ameliorative Effect of *Aster scaber* Thunberg and *Chaenoleles sinensis* Koehne Complex Extracts Against Oxidative Stress-induced Memory Dysfunction in PC12 Cells and ICR Mice

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ABSTRACT

Background: Oxidative stress plays an important role in neuro-degenerative disorders such as Alzheimer's disease. Oxidative stress is mediated by reactive oxygen species (ROS), which are implicated in the pathogenesis of numerous diseases, and account for the toxicity of a wide range of compounds.
Methods and Results: In order to study the neuro-protective effect of the complex extracts of *Aster scaber* Thunberg (AS) and *Chaenoleles sinensis* Koehne (CSK) against hydrogen peroxide in PC12 cells, cell viability was evaluated by the MTT assay using tetrazole, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and the intracellular ROS levels were determined the by 2',7'-dichlorofluorescein diacetate (DCF-DA) assay. In order to examine the anti-amnesic effects of the complex extracts of AS and CSK, behavioral tests were performed on male ICR mice. The ameliorating effect of the complex extracts against $A\beta_{1-42}$-induced learning and memory impairment was analyzed by y-maze and passive avoidance tests. The AS and CSK extracts showed neuro-protective activity both *in vitro* and *in vivo*, and the neuro-protective effect of their 60 : 40 (AS : CSK) mixture was better than that of the other mixtures. Moreover, the complex extracts synergistically inhibited acetylcholinesterase activity and rapid peroxidation.
Conclusions: A mixture of the AS and CSK extracts could be used to develop functional foods and serve as raw materials for the development of therapeutics against Alzheimer's disease.

***Key Words*:** *Aster scaber* Thunberg (AS), *Chaenoleles sinensis* Koehne (CSK), Complex Extracts, Oxidative Stress, Reactive Oxygen Species, Synergy Effect

INTRODUCTION

One of the characteristics of Alzheimer's disease (AD) is increased levels of intracellular reactive oxygen species (ROS), resulting from the accumulation of amyloid beta peptide (Aβ). Aβ is produced through the amyloidogenic pathway, *via* sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretases (Choi *et al.*, 2013). Accumulation of this peptide could be the main pathogenic mechanism of AD and may trigger neurotoxicity (Jin *et al.*, 2009; Xiaowei *et al.*, 2015).

ROS are considered to be the main cause of a wide range of common diseases, including cardiovascular diseases, inflammatory conditions, cancer, mutations, and neuro-degenerative diseases (George *et al.*, 2002). Increased ROS levels lead to oxidative stress and modify the intracellular components, causing DNA

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breakage, lipid peroxidation, and protein carbonylation (George *et al.*, 2002; Cheignon *et al.*, 2018). This induces cytotoxicity and fatal cellular damage.

Antioxidants are vital substances that protect the body from ROS-mediated damage. Plants have complex antioxidant systems to inhibit the oxidative chain reaction. They also produce phytochemicals, which are bioactive compounds obtained from secondary metabolites (Sridhar and Charles, 2019). Some of these phytochemicals exhibit high antioxidant activity and exert other possible effects to decrease the risk of neuro-degenerative diseases caused by Aβ-induced oxidative stress (Jeong *et al.*, 2010; Sridhar and Charles, 2019).

Aster scaber Thunberg (AS) is a perennial plant belonging to the chrysanthemum family and grows in mountainous areas and grasslands of Asia, including Korea. It is rich in various nutrients, including calcium, iron, flavonoids, and saponin. It is reported that the anti-cancer and anti-inflammatory activity of these constituent in AS extracts. Also, another study confirmed acetylcholinesterase inhibition activity of AS extracts according to different extraction processes (Kim and Youn, 2014).

Chaenoleles sinensis Koehne (CSK) belongs to the Rosaceae family found in the East Asia region, including Korea. It is rich in dietary fibers, organic acids and bioactive triterpenes, such as oleanolic acid and ursolic acid. It also contains large amounts of bioactive phenolic acids and vitamins. In particular, the flavonoids of CSK are the main phytochemical constituents that have proven effective in preventing oxidative damage caused by free radicals (Zhang *et al.*, 2009). According to Sancheti and Seo's study, ethanol extracts of CSK showed antiacetylcholinesterase effects in diabetic rats (Sancheti *et al.*, 2013).

To develop preventive strategies for neuro-degenerative diseases like AD, we investigated the antioxidative and neuroprotective effects of AS and CSK complex extracts against H_2O_2-induced cytotoxicity in PC12 cells. We also investigated the cognitive-enhancing effect of AS and CSK complex extracts on Aβ-induced learning and memory impairment in ICR (Institute of Cancer Research) mice.

MATERIALS AND METHODS

1. Chemicals

Dimethyl sulfoxide (DMSO), hydrogen peroxide (H_2O_2), L-ascorbic acid (vitamin C), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichlorofluorescein diacetate (DCF-DA), 2-thiobarbituric acid (TBA), amyloid beta peptide ($A\beta_{1\text{-}42}$, $A\beta_{42\text{-}1}$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Pure Chemical (Richmond, VA, USA). All other chemicals used were of analytical grade.

2. Sample preparation

Aster scaber Thunberg and *Chaenoleles sinensis* Koehne were obtained from a local market (Sancheong and Cheongdo, Korea). After drying, all the samples were ground to powder and extracted with 10 volumes of water at 90℃ for 4 h respectively. The extracts were filtered through a Whatman filter paper No.1 (Whatman Co., Maidstone, England) and dried in a rotary evaporator (EYELA, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) under reduced pressure at 37℃. As a result, the dried sample AS (6.4 ㎏) and CSK (3 ㎏) were extracted using hot water extraction, the percentage yield of these extracts were 14.5% and 29.6% w/w, respectively. And then, AS and CSK extracts were prepared at eleven different concentrations (AS : CSK = 100 : 0, 90 : 10, 80 : 20, 70 : 30, 60 : 40, 50 : 50, 40 : 60, 30 : 70, 20 : 80, 10 : 90, 0 : 100, w/w).

3. DPPH radical scavenging activity assay

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was carried out according to Blois method with a slight modification (Blois, 1958). Briefly, a 0.2 mM solution of DPPH radical solution in ethanol was prepared, and then, 1.5 mL of this solution was mixed with 0.5 ㎖ of the extract solution; finally, after 30 min, the absorbance was measured at 517 ㎚ (SpectraMax M2, Molecular Devices Llc., Sunnyvale, CA, USA). Vitamin C was used as the positive control. The scavenging activity is shown as percentage DPPH scavenging, calculated as

$$\%\ \text{DPPH scavenging} = \left[\frac{(\textit{Control absorbance} - \textit{Sample absorbace})}{\text{Control absorbance}}\right] \times 100$$

4. Cell culture

The PC12 cell line (CRL-1721) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The PC12 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) horse serum, 5% (v/v) fetal bovine serum, and 1% (v/v) antibiotic-antimycotic (Gibco BRL

Co., Gaithersburg, MD, USA). Cells were cultured in 100 ㎜ tissue culture dishes (Falcon Inc., NY, USA). Cultures were maintained at 37℃ with 5% CO_2 and water saturation.

5. Assessment of cell viability

Cell viability was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] tetrazolium reduction assay, wherein the yellow tetrazole is reduced to a purple insoluble compound, known as formazan, in the mitochondria of metabolically active cells. Cells were seeded in 96-well plates at a density of 10^5 - 10^6 cells/㎖ (Choi *et al.*, 2009).

In the cytotoxicity studies, PC12 cells were treated with H_2O_2 at the indicated concentration for 2 h at 37℃. Finally, cells were incubated for 3 h at 37℃ with MTT (2.5 ㎎/㎖ final concentration). Then, the formazan crystals were dissolved in DMSO. The absorbance was measured on a microplate reader (SpectraMax M2, Molecular Devices Llc., Sunnyvale, CA, USA) using a reference wavelength of 630 ㎚ and a test wavelength of 570 ㎚.

6. Measurement of oxidative stress

Levels of cellular oxidative stress were measured using DCF-DA. PC12 cells were pretreated for 12 h with *Aster scaber* Thunberg and *Chaenoleles sinensis* Koehne complex extracts. Cells were then treated with or without H_2O_2 for 2 h. At the end of the treatment, cells were incubated in the presence of 50 μM DCF-DA for 50 min (Choi *et al.*, 2009). After incubation, DCF was quantified using the SpectraMax M2 (Molecular Devices Llc., Sunnyvale, CA, USA). The results were expressed as percent relative to the oxidative stress level of the control cells, which was set to 100%.

7. Animals

The ICR mice (males, 5-week-old) were purchased from Daehan Biolink (Chungnam, Korea). Eight mice were housed per cage in a room maintained at 24 ± 1℃ and 55% humidity with an alternating 12 h light-dark cycle. They had free access to feed and water. All experiments were conducted according to the guidelines of the Committee on Care and Use of Laboratory Animals of Korea University (certificate: kuiacuc-2018-75).

Subsequently, $A\beta_{1-42}$ was administered via intracerebroventricular (ICV) injection. The control group was injected with $A\beta_{42-1}$. Briefly, Aβ was dissolved in 0.85% sodium chloride solution (v/v). Each mouse was injected at the bregma with a 25 ㎕ Hamilton microsyringe fitted with a 26-gauge needle that was inserted to a depth of 2.5 ㎜. The injection volume was 5 ㎕ (dose 410 ㎮ol/mouse) (Yan *et al.*, 2001). The sample groups were injected with $A\beta_{1-42}$ after their diets were supplemented with AS and CSK extract mixture (AS 400; AS : CSK = 100 : 0, 400 ㎎/㎏, CSK 400; AS : CSK = 0 : 100, 400 ㎎/㎏, AS-CSK 400; AS : CSK = 60 : 40, 400 ㎎/㎏, AS-CSK 800; AS : CSK = 60 : 40, 800 ㎎/㎏).

8. Y-maze test

The Y-maze test was carried out 3 days after the Aβ injection. The maze was made of black opaque Y-shaped plastic, and each arm of the maze was 33 ㎝ long, 15 ㎝ high, 10 ㎝ wide, and positioned at an equal angle. Each mouse was placed at the end of one arm and allowed to move freely through the maze during an 8 min period. The sequence of the arm entries was recorded manually.

A spontaneous alternation behavior was defined as entry into all three arms on consecutive choices in overlapping triplet sets. The percentage of spontaneous alternation behavior was calculated as the ratio of actual to possible alternations (Zhang *et al.*, 2009; Choi *et al.*, 2017).

$$\text{Alternation behavior (\%)} = \left[\frac{(\textit{Possible alternations})}{(\textit{Total number of arm entries - 2})}\right] \times 100$$

9. Passive avoidance test

After the Y-maze test, the passive avoidance test was conducted to examine short-term memory. The apparatus consisted of a light room and a dark room. The dark room had a grid of steel rods connected to a shock generator. The rooms were separated by a wall with a connecting passage. For the acquisition trial, each mouse was put into the light room and allowed to explore freely (for 1 min, with no light or shock). Each mouse was allowed to explore further for 2 min with light and no shock.

A mouse that had completely entered into the dark room received a foot shock of 0.5 ㎃ for 1s through the steel grid. One day later, the same mouse was again put into the light room, and the latency time taken to enter the dark room was measured without any foot shock. The maximum cut-off latency was set to 300 s. Doses of samples were designed 400 ㎎/㎏ and 800 ㎎/㎏ according to previous study (Kwon *et al.*, 2016; Choi *et al.*, 2017).

10. Determination of lipid peroxidation in mouse brain tissue

The brains were homogenized in cold PBS in an ice bath. The homogenates were directly centrifuged twice at a 30 s interval at 10,000 rpm for 10 s. Aliquots of the supernatant were used to determine the malondialdehyde (MDA) levels and protein content in the brain.

The MDA level was assayed for lipid peroxidation products by using a previously described method (Blois, 1958). Briefly, 80 ㎕ of each homogenate was mixed with 480 ㎕ phosphoric acid (1%, v/v), followed by the addition of 160 ㎕ thiobarbituric acid solution (0.67%, w/v). The mixture was incubated at 95℃ in a water bath for 45 min. After cooling, the colored complex was extracted with n-butanol. The butanol phase was separated by centrifugation, and the absorbance was measured using tetramethoxypropane as a standard at 532 ㎚.

11. Measurement of acetylcholinesterase (AChE) activity in mouse brain tissue

AChE activity was measured by Ellman's modified method. Briefly, PC12 cells were homogenized with Tris-HCl buffer (20 mM Tris-HCl, pH 7.5 containing 150 mM NaCl, 10 mM $MgCl_2$, and 0.5% Triton X-100), and then, the samples were centrifuged at 10,000 × g for 15 min. The supernatant was used as an enzyme source, and acetylcholine iodide (ACh iodine) was used as the reaction substrate. 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) was used to quantify the thiocholine produced from the hydrolysis of AChE.

A 10 ㎕ liquot of each sample was mixed with 10 ㎕ of enzyme solution, which was subsequently added to 70 ㎕ of the reaction mixture (50 mM sodium phosphate buffer, pH 8.0, containing 1 mM DTNB and 0.5 mM ACh iodine). This mix was then incubated at 37℃ for 15 min (Ellman *et al.*, 1961). The final enzyme reaction was monitored at a wavelength of 405 ㎚ using a microplate reader (SpectraMax M2, Molecular Devices Llc., Sunnyvale, CA, USA). The percentage of inhibition was calculated by comparing the rates of absorbance.

12. Quantification of ACh content in mouse brain tissue

The ACh content in the brain was measured using the method of Hestrin, as described previously, based on the reaction of ACh and hydroxylamine. Mice were sacrificed, and their brains maintained at -80℃, prior to use. The brains were homogenized in cold phosphate buffered saline (PBS) in an ice bath. The homogenates were directly centrifuged twice at a 30 s interval at 10,000 rpm for 10 s.

Subsequently, 1 ㎖ of the homogenate was mixed with 2 ㎖ alkaline hydroxylamine reagent. After at least 1min, the pH was adjusted to 1.2 ± 0.2 with 1 ㎖ HCl solution and 1 ml iron solution. The density of the purple brown color was determined at 540 ㎚ (Hestrin, 1949).

13. Statistical analysis

All results were presented as means ± standard deviation (SD). Statistical analyses were performed using the Statistical Analysis System (SAS 9.4) software package (Cary, NC, USA).

Significant differences between groups were examined by one-way ANOVA followed by the Scheffe's Multiple Range Test. A p-value < 0.05 was considered statistically significant.

RESULTS

1. DPPH radical scavenging activity

The DPPH radical scavenging activities of the AS and CSK extracts were estimated through comparing the percentage inhibition of the formation of DPPH radicals by the concentrations of AS, CSK, and vitamin C. The reduction capability of DPPH was determined by the decrease in its absorbance at 517 ㎚, which is induced by antioxidants. A 1 ㎎/㎖ of AS, CSK, and vitamin C exhibited 77.7, 71.3, and

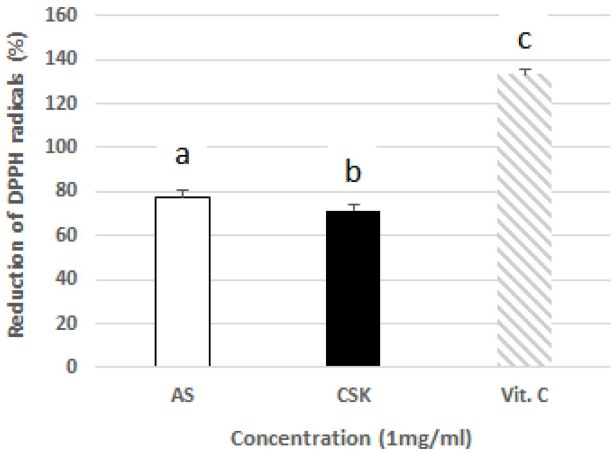

Fig. 1. DPPH radical scavenging activities of AS and CSK extracts. Vit. C group; L-ascorbic acid (1 ㎎/㎖), the sample groups (□; AS 1 ㎎/㎖, ■; CSK, 1 ㎎/㎖). Each value represents the means ± SD (n = 3). Between groups comparisons were conducted using One-way ANOVA with Scheffe's Multiple Range Test (Different superscripts indicate significant difference among groups at $p < 0.05$).

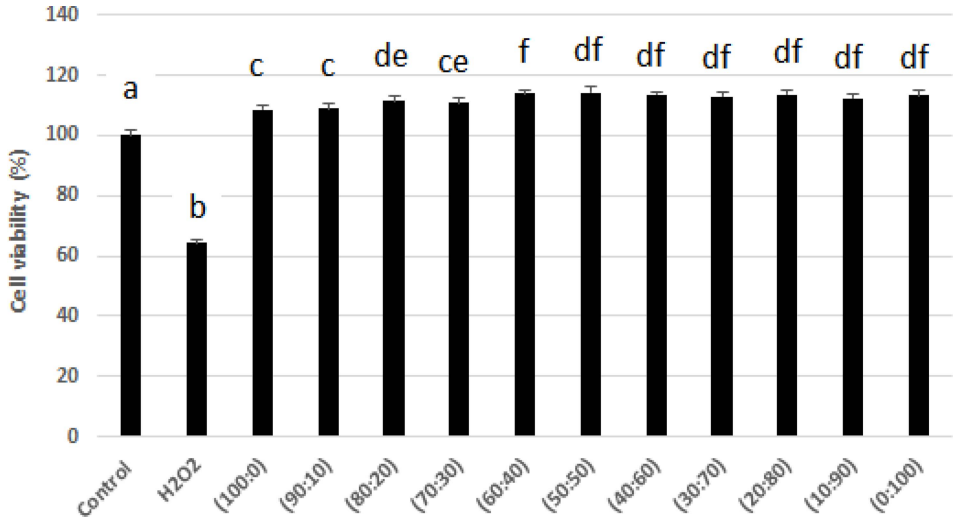

Fig. 2. Protective effect of AS and CSK complex extracts against H_2O_2-induced cytotoxicity. Control group; untreated, H_2O_2 group; 200 ?M H_2O_2, Sample group; treatment with various mixtures of AS and CSK complex extracts (100 : 0, 90 : 10, 80 : 20, 70 : 30, 60 : 40, 50 : 50, 40 : 60, 30 : 70, 20 : 80, 10 : 90, 0 : 100, w/w). The concentration of all the samples was 1 ㎎/㎖. Each value represents the means ± SD (n = 4). *Mean within a column followed by the same letters are not significantly different based on One-way ANOVA with Scheffe's Multiple Range Test (Different superscripts indicate significant difference among groups at $p < 0.05$).

133.3% inhibition, respectively (Fig. 1). The RC_{50} values of AS, CSK, and vitamin C for DPPH radical scavenging activity were 0.207 ㎎/㎖, 0.168 ㎎/㎖, 0.016 ㎎/㎖, respectively.

2. Cell viability

To confirm the synergistic effect of AS and CSK complex extracts on the cell viability, MTT reduction assay was performed. The yellow tetrazole is reduced to a purple insoluble compound, known as formazan, in the mitochondria of metabolically active cells. As shown in Fig. 2, the H_2O_2 group represented low cell viability (35% decrease) compared to the control group (100%). In the sample groups, the mixture was tested at various ratios, and the extract mixture groups (AS : CSK = 60 : 40 – 40 : 60) showed high cell viability against H_2O_2-induced oxidative stress.

3. Oxidative stress

To measure the neuro-protective effects of the AS and CSK extract mixtures on H_2O_2-induced oxidative stress, we evaluated ROS production levels using DCF-DA. The DCF-DA assay was performed to confirm the reduction of H_2O_2-induced intracellular ROS production in PC12 cells.

The H_2O_2 group displayed a significant increase in intracellular ROS production (1,860%) compared with the control group (100%). Specifically, the 60 : 40 group exhibited remarkable intracellular ROS reduction (384%) (Fig. 3).

4. Y maze test results

To confirm the ameliorating effect of AS and CSK extract mixture on $A\beta_{1-42}$-induced learning- and memory-impaired mice, the Y-maze test was conducted. The Y-maze results displayed impairment of the spatial cognitive function of the Aβ group (58.16%) compared to the alternation behavior of the control group (73.20%). The AS and CSK extract diet group showed slightly increased alternation behavior (AS; 63.41%, CSK; 61.87%) (Fig. 4A).

In contrast, the AS and CSK extract mixture diet group significantly increased spontaneous alternation behavior after Aβ injection (AS-CSK 400 = 68.20%, AS-CSK 800 = 76.695%). The number of arm entries did not change in all the experimental groups (Fig. 4B).

5. Passive avoidance test results

The passive avoidance test reflects short-term learning and memory function. The $A\beta_{1-42}$ injected mice exhibited a significant reduction (21% decrease) in the step through latency compared to the control group (Fig. 5).

Similar to the Y-maze test, the AS and CSK extract mixture diet group effectively reversed the $A\beta_{1-42}$ induced cognitive impairment. In particular, the 60 : 40 mixture groups exhibited

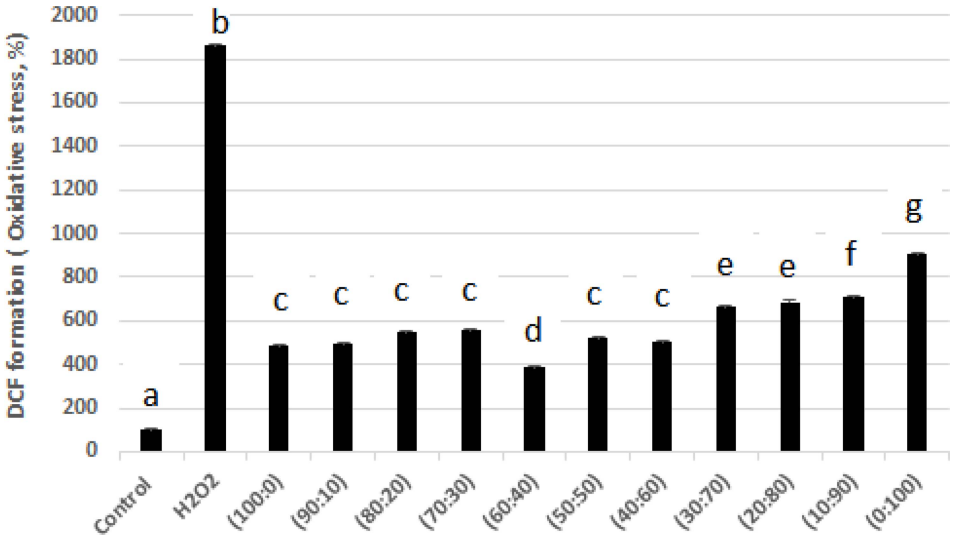

Fig. 3. Protective effect of AS and CSK complex extracts against oxidative stress in PC12. Control group; untreated, H_2O_2 group; 200 mM H_2O_2, Sample group; treatment with various mixtures of AS and CSK complex extracts (100 : 0, 90 : 10, 80 : 20, 70 : 30, 60 : 40, 50 : 50, 40 : 60, 30 : 70, 20 : 80, 10 : 90, 0 : 100, w/w). ㎎/㎖. Each value represents the means ± SD (n = 4). *Mean within a column followed by the same letters are not significantly different based on One-way ANOVA with Scheffe's Multiple Range Test (Different superscripts indicate significant difference among groups at $p < 0.05$).

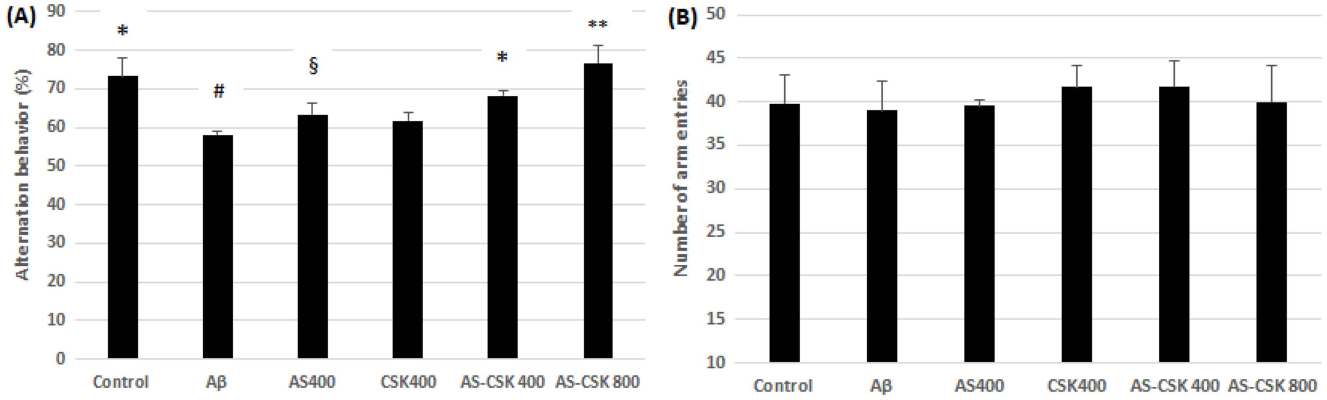

Fig. 4. Memory ameliorating effects of AS and CSK complex extracts against $Aβ_{1-42}$ induced cognitive impairment in the Y-maze test. The control group was injected with $Aβ_{42-1}$. The Aβ group was injected with 410 pmol of $Aβ_{1-42}$ per mouse. The sample groups (AS400, CSK400, AS-CSK 400, AS-CSK 800) were injected with $Aβ_{1-42}$ followed by feeding with AS and CSK complex extracts (AS400; AS : CSK = 100 : 0, 400 ㎎/㎏, CSK400; AS : CSK = 0 : 100, 400 ㎎/㎏, AS-CSK400; AS : CSK = 60 : 40, 400 ㎎/㎏, AS-CSK 800; AS : CSK = 60 : 40, 800 ㎎/㎏ body weight per day). The spontaneous alternation behaviors were measured during 8 min. Each value represents the means ± SD. (n = 4 - 8). Mean within a column followed by the same letters are not significantly different based on One-way ANOVA with Scheffe's Multiple Range Test (*,#,§,**Different superscripts indicate statistical differences among the groups at $p < 0.05$).

a dose-dependent (24.3%, 33.8%) attenuation of Aβ-induced memory impairment.

6. Lipid peroxidation

Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) present in the sample. The levels of MDA were significantly increased in the Aβ-injected group (0.718 ㎛ol/㎎·protein) when compared with those of the control group (0.436 ㎛ol/㎎·protein). In contrast, the AS and CSK extract mixture diet groups (AS-CSK 400 : 0.31 ㎛ol/㎎·protein, AS-CSK 800 : 0.247 ㎛ol/㎎·protein) significantly decreased the MDA contents in a dose-dependent manner (Fig. 6).

7. AChE activity

The Aβ group in this experiment showed an increase

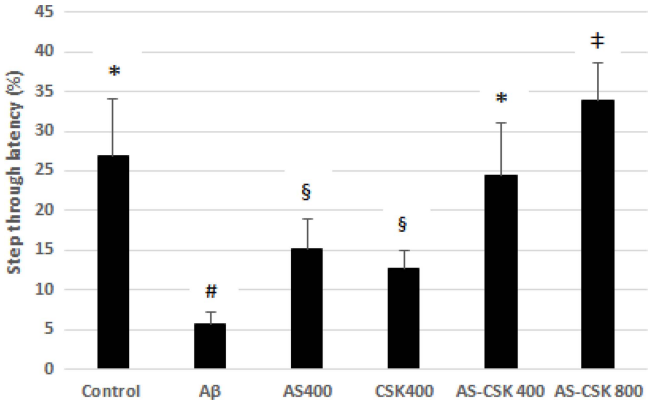

Fig. 5. Memory ameliorating effects of AS and CSK complex extracts against $A\beta_{1-42}$ induced cognitive impairment through passive avoidance test. Control group was injected with $A\beta_{42-1}$. Aβ group was injected with 410 pmol of $A\beta_{1-42}$ per mouse. Sample groups (AS400, CSK400, AS-CSK 400, AS-CSK 800) were injected with $A\beta_{1-42}$ followed by feeding with AS and CSK complex extracts (AS400; AS : CSK = 100 : 0, 400 ㎎/㎏, CSK400; AS : CSK = 0 : 100, 400 ㎎/㎏, AS-CSK 400; AS : CSK = 60 : 40, 400 ㎎/㎏, AS-CSK 800; AS : CSK = 60 : 40, 800 ㎎/㎏ body weight per day). The step-through latency was measured during 5 min. Each value represents the means ± SD. (n = 4 - 8). Mean within a column followed by the same letters are not significantly different based on one-way ANOVA with Scheffe's Multiple Range Test ($^{*,\#,\S,\ddagger}$Different superscripts indicate statistical differences among the groups at $p < 0.05$).

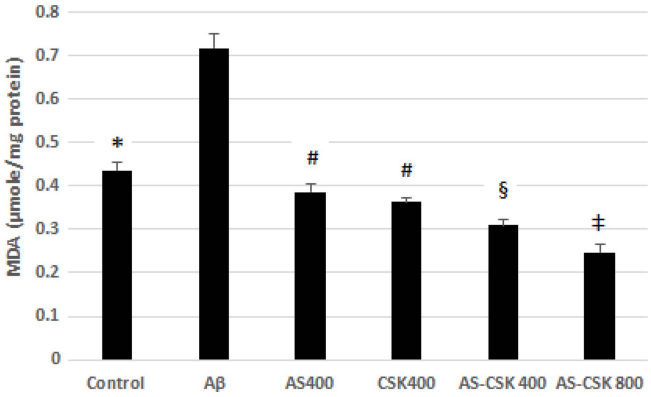

Fig. 6. Effect of AS and CSK complex extracts on lipid peroxidation in mice brains. Control group was injected with $A\beta_{42-1}$. Aβ group was injected with 410 pmol of $A\beta_{1-42}$ per mouse. Sample groups (AS400, CSK400, AS-CSK 400, AS-CSK 800) were injected with $A\beta_{1-42}$ followed by feeding with AS and CSK complex extracts (AS400; AS : CSK = 100 : 0, 400 ㎎/㎏, CSK 400; AS : CSK = 0 : 100, 400 ㎎/㎏, AS-CSK400; AS : CSK = 60 : 40, 400 ㎎/㎏, AS-CSK 800; AS : CSK = 60 : 40, 800 ㎎/㎏ body weight per day). Each value represents the means ± SD. (n = 4 - 8). Mean within a column followed by the same letters are not significantly different based on One-way ANOVA with Scheffe's Multiple Range Test ($^{*,\#,\S,\ddagger}$Different superscripts indicate statistical differences among the groups at $p < 0.05$).

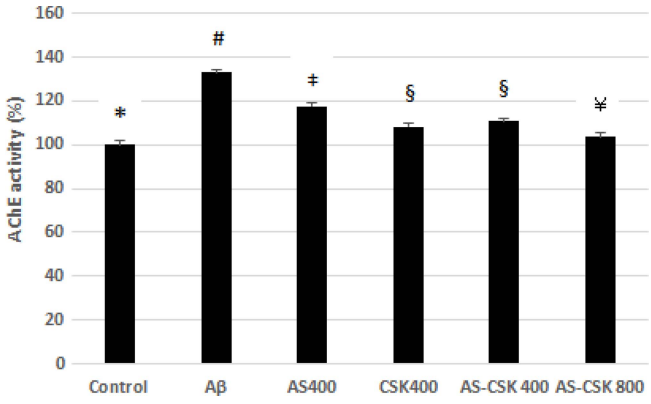

Fig. 7. Effect of AS and CSK complex extracts on AChE activity in mice brains. Control group was injected with $A\beta_{42-1}$. Aβ group was injected with 410 pmol of $A\beta_{1-42}$ per mouse. Sample groups (AS400, CSK400, AS-CSK 400, AS-CSK 800) were injected with $A\beta_{1-42}$ followed by feeding with AS and CSK complex extracts (AS400; AS : CSK = 100 : 0, 400 ㎎/㎏, CSK400; AS : CSK = 0 : 100, 400 ㎎/㎏, AS-CSK 400; AS : CSK = 60 : 40, 400 ㎎/㎏, AS-CSK 800; AS : CSK = 60 : 40, 800 ㎎/㎏ body weight per day). Mean within a column followed by the same letters are not significantly different based on one-way ANOVA with Scheffe's Multiple Range Test ($^{*,\#,\S,\ddagger,¥}$ Different superscripts indicate statistical differences among the groups at $p < 0.05$).

(32.8%) in AChE activity compared with the control group. Treatment with extract mixture mitigated the Aβ-induced impairment of the cholinergic system by decreasing AChE activity (Fig. 7).

8. ACh content

The ACh content in the mouse brain tissue was determined by spectrophotometric analysis. As shown in Fig. 8, the ACh level was lower (39.29 ㎎/㎎·protein) in Aβ-treated mice than in the control group (102.48 ㎎/㎎·protein). In contrast, administration of the AS and CSK extract mixture reversed the decrease in ACh content compared to that of mice treated with Aβ only (AS-CSK 400: 96.69 ㎎/㎎·protein, AS-CSK 800: 94.95 ㎎/㎎·protein).

9. AST, ALT activity

After behavioral test, serum was investigated by using a serum transaminase reagent kit. The results of serum transaminases did not represent significant differences among different groups (Fig. 9). It was normal levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) ranges for all groups.

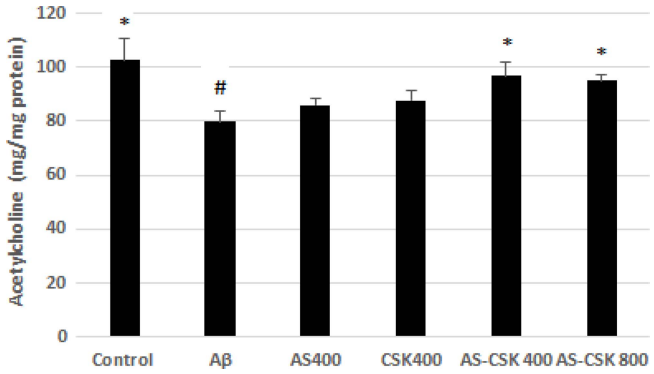

Fig. 8. Effect of AS and CSK complex extracts on ACh contents in mice brains. The control group was injected with $A\beta_{42\text{-}1}$. The Aβ group was injected with 410 pmol of $A\beta_{1\text{-}42}$ per mouse. The sample groups (AS400, CSK400, AS-CSK 400, AS-CSK 800) were injected with $A\beta_{1\text{-}42}$ followed by feeding with AS and CSK complex extracts (AS400; AS : CSK = 100 : 0, 400 ㎎/㎏, CSK400; AS : CSK = 0 : 100, 400 ㎎/㎏, AS-CSK 400; AS : CSK = 60 : 40, 400 ㎎/㎏, AS-CSK 800; AS : CSK = 60 : 40, 800 ㎎/㎏ body weight per day). Mean within a column followed by the same letters are not significantly different based on One-way ANOVA with Scheffe's Multiple Range Test ($^{*}p < 0.05$ as compared with the Aβ group, $^{\#}p < 0.05$ as compared with the control group).

DISCUSSION

AD is one of the most common forms of dementia affecting approximately 10% of the population over the age of 65 years (Hestrin, 1949). However, the mechanism of Aβ-induced neurotoxicity is still poorly understood (Alzheimer's Association, 2019). Aβ is known to induce free radicals and oxidative stress and lead to the apoptosis of neuronal cells by disrupting the function of mitochondria and lysosomes. Moreover, Aβ increases ROS production and oxidative stress due to an excess of oxidants, reducing the antioxidant levels (Anand and Singh, 2013; Halliwell and Gutteridge, 1984). Aβ-induced cytotoxicity has been shown to be caused by the intracellular accumulation of H_2O_2, leading to cell death.

Reactive oxygen species (ROS) cause oxidative damage in biological macromolecules such as DNA, protein and fatty acids. Thus, in order to examined the memory impairment and neuronal dysfunction against oxidative stress, ROS that cause oxidative damage is used as H_2O_2 in PC12cells and Aβ in ICR mice, respectively.

In previous research, the AS extract showed a protective effect against oxidative damage-induced neurotoxicity in both *in vitro* and *in vivo* models of AD (Choi *et al.*, 2014). Also, AE (low temperature high pressure extraction) and LTPE (ultrasonication extraction) of 70% ethanol extracts from *Aster scaber* represented significant inhibitory activity on acetylcholinesterase (Kim and Youn, 2014). The CSK extract is an effective activator of choline acetyltransferase (ChAT), and it might protect against TMT-induced memory and cognition deficit (Kwon *et al.*, 2015). And ethyl acetate fraction of Chaenomeles sinensis (Thouin) Koehne fruit showed AChE inhibitor activity in streptozotocin-induced diabetic rats (Sancheti *et al.*, 2013).

In this study, different ratios of the AS and CSK complex extracts were used. Specifically, the 60 : 40 mixture of the AS

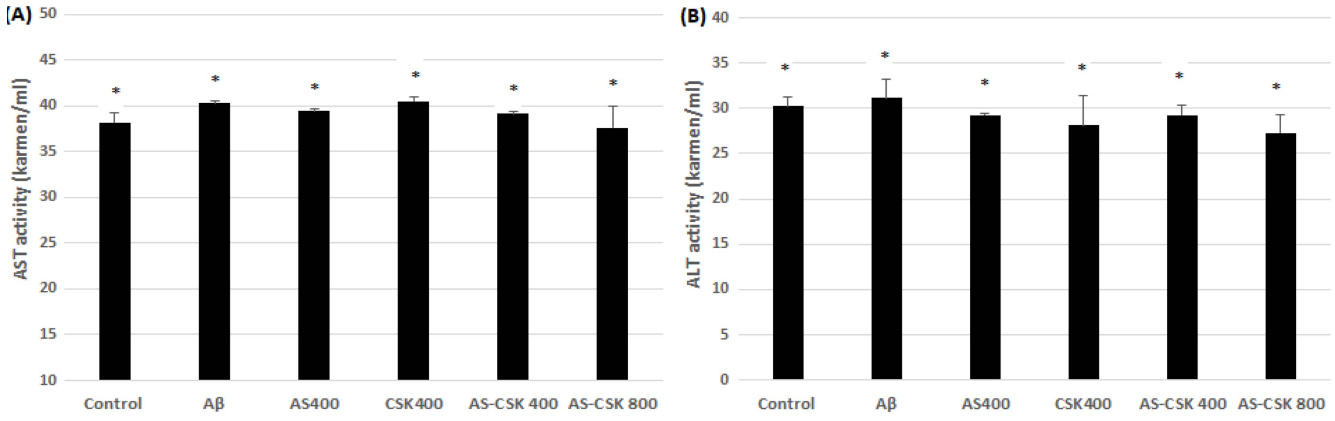

Fig. 9. Aspartate aminotransferase activity in the serum of ICR mice (A). Alanine aminotransferase activity in the serum of ICR mice (B). The control group was injected with $A\beta_{42\text{-}1}$. The Aβ group was injected with 410 pmol of $A\beta_{1\text{-}42}$ per mouse. The sample groups (AS400, CSK400, AS-CSK 400, AS-CSK 800) were injected with $A\beta_{1\text{-}42}$ followed by feeding with AS and CSK complex extracts (AS400; AS : CSK = 100 : 0, 400 ㎎/㎏, CSK 400; AS : CSK = 0 : 100, 400 ㎎/㎏, AS-CSK 400; AS : CSK = 60 : 40, 400 ㎎/㎏, AS-CSK 800; AS : CSK = 60 : 40, 800 ㎎/㎏ body weight per day). Each value represents the means ± SD. (n = 4 - 8). Mean within a column followed by the same letters are not significantly different based on One-way ANOVA with Scheffe's Multiple Range Test (Different superscripts indicate statistical differences among the groups at $p < 0.05$).

and CSK showed a significant neuroprotective effect. The scavenging activity of the AS and CSK was measured using the DPPH radical scavenging assay. The DPPH radical is considered to be a model lipophilic radical. A chain of lipophilic radicals is generated by the lipid auto-oxidation. The DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Shukla *et al.*, 2009).

In MTT assay, the AS and CSK complex extract groups did not exhibit significant differences. However, extract mixture groups displayed an increase in cell viability. The data clearly demonstrated the protective effect of the extract mixture against H_2O_2.

The test of Aβ-induced cognitive deficit in the mice model was carried out using Y-maze test, which reflects working memory. The $A\beta_{1-42}$ ICV-injected mice group showed impaired spatial working memory compared to the control group. The path-tracing results showed that the control group mice engaged in similar path tracing in each arm, whereas the line of the Aβ group showed that they tended to lean toward one arm. These results also suggest that spatial cognitive impairments were caused by Aβ injection because the innate inclination of normal mice is to explore new environments (Bae *et al.*, 2014). The behavioral test results suggest that the complex extracts had excellent ameliorating effects on spatial cognitive function as learning and memory ability. Our results also confirm that Aβ injection in mice induced severe behavioral dysfunction, but the administration of the extract mixture effectively ameliorated learning and memory impairment by Aβ-induced neurotoxicity (Ali *et al.*, 1992).

The passive avoidance test was performed to determine the short-term memory recovery in mice with Aβ-induced neurotoxicity, and the results are shown in Fig. 5. The passive avoidance test estimates the amygdala-dependent short-term learning and memory cognitive function. The $A\beta_{1-42}$ ICV-injected mice group represented a significant reduction through latency compared to the control group. In contrast, the AS and CSK complex extract groups attenuated the $A\beta_{1-42}$-induced impairment of mice in the passive avoidance test. This result showed that learning and memory function were effectively improved when compared with the Aβ-treated group. Doses of AS and CSK on behavioral test were designed 400 ㎎/㎏ and 800 ㎎/㎏ according to previous study (Choi *et al.*, 2014; Kwon *et al.*, 2015).

The brains of AD patients are associated with an increase in the deposition of Aβ-induced oxidative stress. Malondialdehyde is used as an indicator of lipid peroxidation, induced by oxidative stress. In this test, MDA content in the Aβ-injected mice brain tissues was examined, and the results are shown in Fig. 6. The levels of MDA were increased in the $A\beta_{1-42}$-injected mice group compared to the control group. The AS and CSK complex extract groups significantly decreased MDA contents in a dose-dependent manner. Oxidative stress induced by Aβ may eventually destroy the antioxidant system in the brain. Therefore, the collapse of the antioxidant system increased the MDA levels and induced cognitive dysfunction.

Cognitive functions have been considered to be highly dependent on central cholinergic neurotransmission (Hu *et al.*, 2003). It means that the cholinergic system plays a key role in the regulation of learning and memory processes (Geoffrey and Angela, 2002). According to the cholinergic hypothesis, reductions in choline acetyltransferase (ChAT) activity and ACh synthesis are closely related to cognitive impairments like those in AD (Shivarajashankara *et al.*, 2002).

In the AChE activity test, the Aβ-injected mice showed increased activity compared to the control group. However, the AChE activity was reduced to the level of the control groups when the mice were fed a diet containing the AS and CSK extract mixture. The ACh contents in the mouse brain tissue are shown in Fig. 8. The Aβ-injected mice group showed a decrease in ACh contents compared to the control group. Administration of the AS and CSK complex extracts slightly reversed the decrease in ACh content compared to that in mice treated with Aβ only.

Amyloid peptides reduce ACh synthesis through the leakage of choline across cell membranes and immediately affect the surface and intracellular AChE expression around amyloid plaques in AD brain tissues (Bidchol *et al.*, 2011; Bridoux *et al.*, 2013). Moreover, ACh and AChE activities in the central nervous system play an important role in behaviorally relevant sensory signaling. This neuronal signaling is controlled by multiple neuronal productions and secretions. ACh is hydrolyzed to acetate and choline by AChE in the cholinergic synaptic cleft in the brain (Sims *et al.*, 1983).

In this study, we demonstrated that the (60 : 40, v/v) AS and CSK complex extract exerts an ameliorative effect against oxidative stress in PC12 cells and in mice. Furthermore, since the deposition of Aβ as toxic clumps in AD patients is promoted by AChE, this study demonstrates the effects of the complex extracts against Aβ from a perspective of counteracting

oxidative stress and its underlying mechanisms, which, however, need further clarification.

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