In vitro Antioxidant Effect of *Aster scaber* Thunb. Extract

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Methanol extract and solvent fractions of *Aster scaber* Thunb. were tested to elucidate antioxidative characteristics *in vitro*. Methanol extract showed strong inhibition on linoleic acid autoxidation and conjugated diene production. Hexane and diethyl ether fractions showed stronger chelating effect on 10^{-4} M $FeCl_3$ than the other fractions. Butanol fraction had the strongest capacity on 10^{-3} M $CuSO_4$ similar to α-tocopherol. Synergistic effect of butanol fraction with histidine, methionine, and lysine on linoleic acid autoxidation had been observed. Contents of total phenol, vitamin E, vitamin C, and carotenoid were evaluated. From the results, *Aster scaber* Thunb. could be expected to play a role as an antioxidant *in vivo*.

Key words: *antioxidant, extract,* Aster scaber *Thunb., metal ion chelating activity, synergistic effect.*

Living organisms, which sustain their lives through aerobic respiration, including human beings, have been subjected to oxidative stress due to the chemical property of oxygen.[1] Moreover, increased environmental pollution during the recent years have also increased the oxidative stress. Oxidative stress was revealed to cause such diseases as cancer, atherosclerosis, neuronal disease, rheumatic arthritis, and aging.[2] Use of antioxidants such as α-tocopherol with scavenging property against oxidative stress could be the counter-plan to prevent and cure these diseases. Several researches including our study have thus been conducted to search for new powerful antioxidants from natural resources.[3,4]

Aster scaber Thunb. (*Chamchwi*), a perennial herb of the *Compositea* family, is popular vegetable in Korea. In addition, the root and aerial part of *Chamchwi* have been used in korean traditional medicine to detoxify, ameliorate pains, stimulate blood circulation, and reduce throat inflammation. Non-volatile and volatile components of *Chamchwi* were reported by several researchers; their physiological effects include reduction of hyperlipidemia and cardiovascular disease, inhibition of cellular mutagenicity and carcinogenecity, microorganisms, and oxidation.[5-14] Results of research by various institutes world wide and the accumulated knowledge based on traditional use in Korea, *Chamchwi* is expected to possess an antioxidant property. Thus the antioxidant characteristics of methanol, and water extracts, hexane, diethylether, ethylacetate, butanol, and aqueous fractions of *Aster scaber* Thunb. were tested *in vitro* through various experiments.

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Abbreviations: OD, optical density; BHT, buthlated hydroxy toluene.

Materials and Methods

Materials. *Chamchwi*, aerial part of *Aster scaber* Thunb., was purchased at Pujeon Market, Pusan, Korea and identified based on comparison with dried specimen in Department of Biology, Pusan National University, Korea. It was washed under running water, freeze dried at -80°C, and stored at -20°C before use.

Reagents. First-grade solvent was used for extraction and fractionation and was distilled twice before use. α-Tocopherol and linoleic acid were purchased from Sigma Chemical Co. Other reagents used for experiments were guaranteed grade reagent.

Quantification of total phenol, vitamin E, vitamin C, and carotenoid. Methanol extract and solvent fractions were used for the quantification of total phenol. They were each prepared into a final concentration of 100 $\mu g \cdot ml^{-1}$ in reaction mixture by adding solvents. Each sample (0.1 m*l*) was mixed with 2 m*l* of 2% Na_2CO_3. After 2 min, 0.1 m*l* of 50% Folin-Ciocalteau reagent was added to this reaction mixture. The reaction mixture was sustained for 30 min at room temperature and analyzed at 750 nm through a spectroscopic method using tannic acid as a standard for calculation. Using the method of Combs and Combs[15] for the quantification of vitamin E, 5 g of dried *Chamchwi* powder was saponified with ethanol containing 50% KOH and 10% pyrogallol at 70°C for 15 min. Tocopherol from the saponified sample was extracted with hexane, which included 0.2% BHT. The hexane extract was dried with nitrogen gas and dissolved in ethanol to a final volume of and massed up to 50 m*l*. Using the method of Desai,[16] 1 m*l* of the hexane extract was diluted to 4 m*l* with hexane. Two milliliters of this diluted hexane extract was placed in a test tube and dried and the remnant was dissolved with ethanol. The preparation was mixed thoroughly with 0.2

m*l* of 0.2% bathophenanthroline reagent, then mixed with ferric chloride reagent. The reaction mixture was analyzed at 536 nm via spectroscopy. Vitamin E concentration of each sample was calculated as follows.

$$\text{Vitamin E } (\mu g \cdot 100\ ml^{-1}) = \frac{\text{OD of test - OD of blank}}{\text{OD of standard - OD of blank}} \times \text{concentration of standard}$$

One gram of dried *Chamchwi* powder was analyzed through the hydrazine colorimetry method for the quantification of ascorbic acid. Using the method of A.O.A.C[17] for the quantification of carotenoid, 20 g of dried *Chamchwi* powder was extracted three times with 100 m*l* of acetone. The acetone extract was saponified with 60% KOH for 24 h and extracted with petroleum ether. At 450 nm, the maximum absorption wavelength of carotenoid, optical density of this extract was evaluated. Content of total carotenoid was calculated by multiplying with $E^{1cm}_{1\%}$ 2500, the absorption coefficient of λ_{max} visible spectrum of carotenoid.

Preparation of extract and solvent fractions. One hundred grams of dried *Chamchwi* powder was used for preparing the methanol extract. Methanol extract was successively separated into hexane, diethylether, ethylacetate, butanol, and aqueous fractions.

Inhibition effect on linoleic acid autoxidation. Reaction mixture was made by adding 120 μl of extract or fractions, 9 m*l* of phosphate buffer (0.04 M, pH 7.0), and 2.88 m*l* of 2.51% linoleic acid in 99.9% ethanol. The mixture was incubated at 40°C in darkness for 4 days and analyzed through the ferrithiocyanate method reported by Haraguchi *et al.*[18] A part of the reaction mixture (0.01 m*l*) was mixed with 9.7 m*l* of 75% ethanol and 0.1 m*l* of 30% ammonium thiocyanate, then with 0.1 m*l* of 2×10^{-2} M ferrous chloride in 3.5% HCl. After 3 min, optical density of this mixture was measured at 500 nm through spectroscopy. The inhibition capacity on linoleic acid autoxidation is shown as by percent inhibition (%) was determined by calculating the difference between optical density of control and sample divided by that of control and multiplied by one hundred.

$$\text{Inhibition (\%)} = \frac{\text{OD of control - OD of sample}}{\text{OD of control}} \times 100$$

Inhibition effect on conjugated diene production. An aliquot of the reaction mixture prepared via the method of Haraguchi et al, was tested with 3 m*l* of methanol for measuring the conjugated diene production optically at 232 nm.[19] The optical density was calculated into 10^{-6} M conjugated diene using molar extinction coefficient ($2.6 \times 10^{-4}\ M^{-1} \cdot cm^{-1}$) of the conjugated diene.

Synergistic effect with amino acids and commercial antioxidants. For measuring the synergistic effect, each solvent fraction of *Aster scaber* was tested with commercial antioxidants, such as α-tocopherol and ascorbic acid, and amino acids, including asparagine, histidine, lysine, methionine, and tryptophan, in a linoleic acid-phosphate buffer system using the method of Haraguchi *et al.* The synergistic effect was calculated through the same method used for measuring the inhibition capacity on linoleic acid autoxidation.

Chelating effect on $CuSO_4$ and $FeCl_2$ in linoleic acid-phosphate buffer system. Against $CuSO_4$ and $FeCl_2$ at concentrations of 10^{-4} and 10^{-3} M in linoleic acid-phosphate buffer system, respectively scavenging capacity of solvent fractions was tested using the method of Haraguchi *et al.*

Statistical analysis. The result of the experiment mentioned above was exhibited as mean ± standards deviation. Significance of control and sample was verified through Duncan's multiple range test and one way ANOVA (analysis of variance) test at a level of $p<0.05$.

Results and Discussion

Yields of solvent fractions prepared from *Aster scaber* Thunb. Table 1 exhibits the yields of hexane, diethylether, ethylacetate, butanol, and aqueous fractions separated from the methanol extract (22.6 g), which was prepared using 100 g of powdered *Chamchwi*. Content of the aqueous fraction was the highest among the fractions, followed by hexane, butanol, ethylacetate, and diethylether fractions in decreasing order. Content of total phenol, and antioxidant vitamins. Table 2 shows the content of total phenol of the methanol extract and several solvent fractions prepared from *Chamchwi*. Total phenol content of the ethylacetate fraction was 75.2% based on the content of tannic acid, the highest among the solvent fractions. Because phenol compounds have a potential to scavenge free radical,[20,21] and to reduce *in vivo* lipid peroxidation,[22] ethylacetate fraction which exhibited high in total phenol content from this result, is also expected to have a

Table 1. Yields of hexane fraction, diethylether, ethylacetate, and butanol fractions of dried *Aster scaber* Thunb.

	Yield (g)	(%)*
Hexane fraction	3.93	17.39
Diethylether fraction	0.58	2.57
Ethylacetate fraction	1.89	8.36
Butanol fraction	3.70	6.37
Aqueous fraction	12.50	55.31

*Percentage of each fraction to methanol extract content (22.6 g) obtained from 100 g of powdered *Aster scaber* Thunb.

Table 2. Total phenol content of methanol extract, diethylether, ethylacetate, and butanol fractions prepared from the dried *Aster scaber* Thunb.

Sample	Tannic acid (%)
Methanol extract	18.31 ± 0.11
Diethylether fraction	9.25 ± 0.13
Ethylacetate fraction	75.20 ± 0.48
Butanol fraction	38.71 ± 0.19

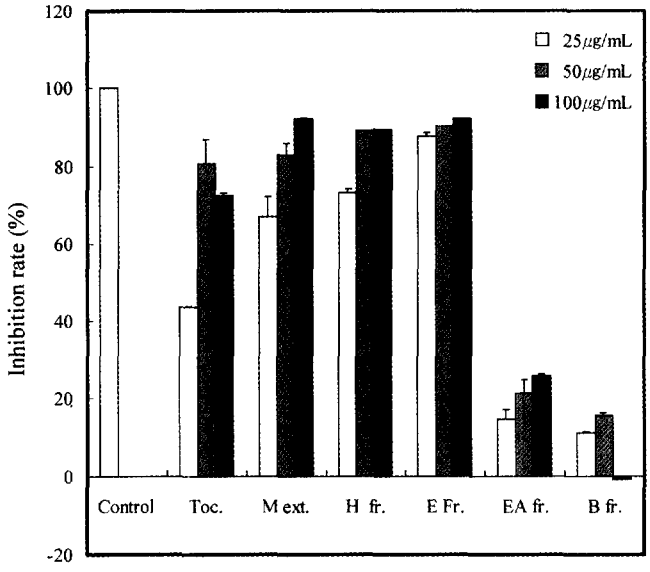

Fig. 1. Antioxidative effect of methanol extract (M ext.), hexane (H fr.), diethylether (E fr.), ethylacetate (EA fr.), butanol fractions (B fr.) from *Aster scaber* Thunb. and α-tocopherol (Toc.) on linoleic acid autoxidation. Data was analyzed through the ferrithiocyanate method on the 4th day of incubation at 40°C on linoleic acid autoxidation.

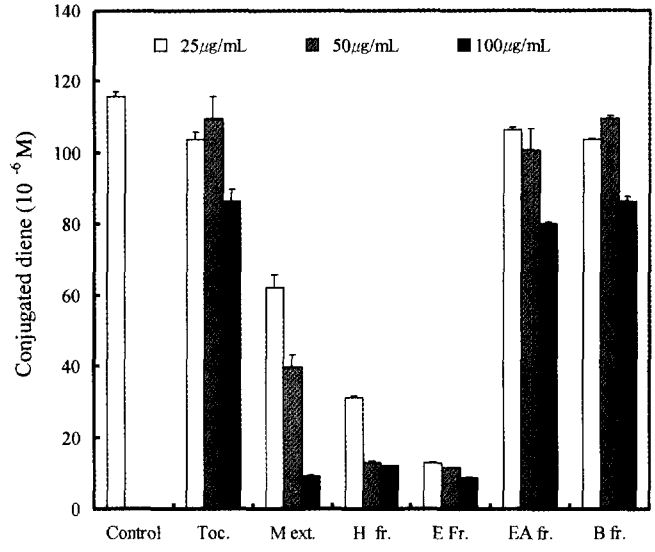

Fig. 2. Inhibition effect of methanol extract (M ext.), hexane (H fr.), diethylether (E fr.), ethylacetate (EA fr.), butanol fractions (B fr.) from *Aster scaber* Thunb. and α-tocopherol on conjugated diene production. Data was analyzed on the 4th day of incubation at 40°C.

potential antioxidative power. Vitamins E, C, and carotenoid, known as antioxidants were quantified in *Chamchwi*. Content of diketogulonic acid (DKA) form of vitamin C was 748.33 mg%. Because ascorbic acid, α-tocopherol, and β-carotene have antioxidant activities, these antioxidants in *Aster scaber* are expected to play crucial role, which include protecting human cells against damages due to ultraviolet light.[23] It was reported that the antioxidant action of these vitamins was affected by their concentration, O_2 tension, and solubility.[24]

Antioxidant effect of methanol extract and solvent fractions on linoleic acid autoxidation. Methanol extract of *Chamchwi* at concentrations of 100, 50, and 25 μg · ml^{-1}, inhibited linoleic acid autoxidation by 91.48, 85.51, and 63.01%, respectively, higher than those (73, 81, and 44%) of α-tocopherol (Fig. 1). Antioxidant effects of several solvent fractions on linoleic acid autoxidation are shown in Fig. 1. At a concentration of 100 μg · ml^{-1}, diethylether and hexane fractions showed inhibition rates of 92 and 89%, respectively, higher than those of α-tocopherol. The result suggest that methanol extract, diethylether, and hexane fractions of *Chamchwi* could play roles as scavengers of hydroperoxy-octadecadienoic acid and oxooctadienoic acid isomers which were the main product of linoleic acid autoxidation.[25] Because lipid peroxidation is involved in the atherosclerosis, hemolytic disease processes, chronic fatigue syndrome, and hepatocarcinogenesis,[26-29] *Chamchwi* is expected to be a useful material for inhibiting these processes as an antioxidant.

Antioxidant effect of methanol extract and solvent fractions on conjugated diene production. Inhibition profile of methanol extract and solvent fractions on conjugated diene production resemble that of the linoleic acid autoxidation.

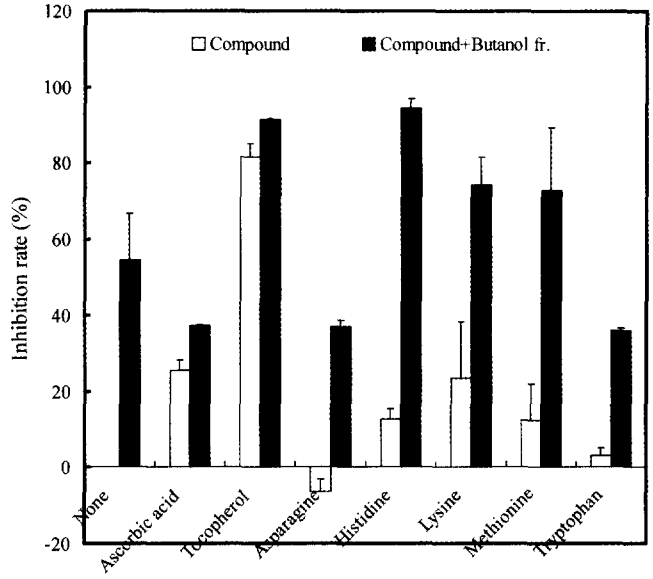

Fig. 3. Synergistic effect of butanol fraction separated from methanol extract with ascorbic acid, α-tocopherol, and amino acids on linoleic acid autoxidation. Data was analyzed using the ferrithiocyanate method on the 3rd day of incubation at 40°C, and final concentration of each sample in reaction mixture was 100 μg · ml^{-1}.

Methanol extract at concentrations of 100, 50, and 25 μg · ml^{-1} showed inhibition capacities of 92, 66, and 46% on the 3rd day of incubation, respectively. Solvent fractions also inhibited the conjugated diene production in the same manner on linoleic acid autoxidation. Methanol extract, diethylether, and hexane fractions showed stronger inhibition capacities on the conjugated diene production than α-tocopherol. In addition, conjugated diene level was increased in patients with β-thalasemia and chronic renal failure.[30,31]

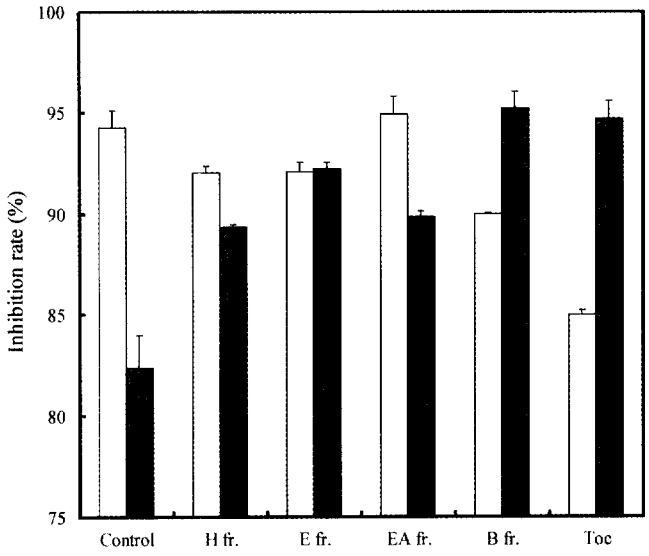

Fig. 4. Chelating effects of hexane (H fr.), diethylether (E fr.), ethylacetate (EA fr.), butanol fractions (B fr.) separated from methanol extract and α-tocopherol (Toc.) on 10^{-4} M (□) and 10^{-3} M (■) $CuSO_4$. Data was analyzed using the ferrithiocyanate method on the 3rd day of incubation at 40°C, and final concentration of each sample in reaction mixture was 100 μg · ml^{-1}.

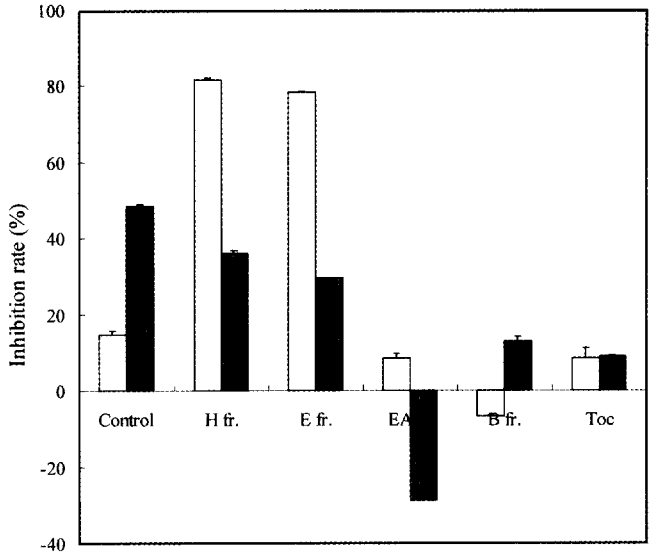

Fig. 5. Chelating effects of hexane (H fr.), diethylether (E fr.), ethylacetate (EA fr.), butanol fractions (B fr.) separated from methanol extract and α-tocopherol (Toc.) on 10^{-4} M (□) and 10^{-3} M (■) $FeCl_3$. Data was analyzed using the ferrithiocyanate method on the 3rd day of incubation at 40°C, and final concentration of each sample in reaction mixture was 100 μg · ml^{-1}.

Synergistic effect of butanol fraction with commercial antioxidant and five amino acids. Among the solvent fractions, butanol fraction showed synergistic effects to histidine, lysine, and methionine on the linoleic acid autoxidation (Fig. 3). Synergistic effect of amino acids was revealed to be affected by pH.[32] On the other hand, methanol extract and the remaining fractions did not show this effect. Amino acids such as tryptophan, cystein, alanine, and glycine were also reported to have synergistic properties.[33] In this experiment, we determined that *Chamchwi* has synergistic property with lysine, methionine, and histidine, which are known to have antioxidative activities.[34]

Chelating effect on $CuSO_4$ and $FeCl_2$ in linoleic acid-phosphate buffer system. Transition metal ions such as Co, Cu, Fe, Mn, and Ni, are known to be principal prooxidants.[35] We evaluated the chelating capacity of *Chamchwi* on these ions. Metal ion-chelating effects of hexane, diethylether, ethylacetate, and butanol fractions are shown in Figs. 4 and 5. The solvent fractions had stronger chelating effect on 10^{-3} M $CuSO_4$ than 10^{-4} M $CuSO_4$. Among the fractions, butanol fraction had the strongest capacity on 10^{-3} M $CuSO_4$ similar to α-tocopherol (Fig. 4). Hexane and diethylether fractions showed higher chelating effects on 10^{-4} M $FeCl_3$ than α-tocopherol. On 10^{-3} M $FeCl_3$, all fractions and α-tocopherol had little chelating effect as compared with the control used with only 10^{-3} M $FeCl_3$ (Fig. 5). The inhibition of control in 10^{-4} M $CuSO_4$ was higher than that of 10^{-3} M $CuSO_4$. This can be explained through the result reported by Kanner *et al.* who reported that Cu^{2+} act as a prooxidant at below 10^{-4} M concentration and as an antioxidant at above 10^{-3} M concentration.[36] As the result of this experiment revealed, 10^{-3} M Cu^{2+} reacted with components of *Aster scaber* could play a role as an antioxidant. Thus, *Chamchwi* could play a role as an antioxidant with Cu^{2+} through metal-chelate, produced between Cu^{2+} and ascorbic acid. It can terminate the reaction of lipid oxidation through the action as a hydroperoxide decomposer and the reaction with free radicals produced via lipid oxidation as reported by Kanner. Oxidative stress induced by iron could cause Parkinson's disease.[37] Copper, the component of ferritin-inducing lipid peroxidation, is a powerful accelerating agent of atherosclerosis. The reduction from Cu (II) to Cu (I) evokes lipid peroxidation.[38] A small amount of selenium, copper, zinc, and manganese included in enzyme, protect animal tissues from oxidative stress.[39] As the transition metal has properties related with the induction of several diseases and oxidative stress mentioned above, *Chamchwi*, revealed to have chelating capacity in this experiment, could be used as an antioxidant.

Table 3. Contents of vitamin C, E, and carotenoid determined from the dried *Aster scaber* Thunb.

Components		Content (mg%)
Vitamin C	DKA[1]	748.33 ± 5.84
	DHA[2]	5.33 ± 2.51
	ASA[3]	47.65 ± 5.80
Vitamin E		17.18 ± 0.70
Carotenoid		13.02 ± 1.51

[1]DKA: Diketogulonic acid.
[2]DHA: Dehydroascorbic acid.
[3]ASA: Ascorbic acid.

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