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Antioxidant Activity of Fractions from 70% Methanolic Extract of *Sonchus oleraceus* L.

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Abstract The potential antioxidant activities of different fractions from a 70% methanolic (MeOH) extract of *Sonchus oleraceus* were assayed *in vitro*. All of the fractions exception of *n*-hexane showed a strong antioxidant activity, especially the ethyl acetate (EtOAc) fraction, which showed the highest 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity (IC_{50}=19.25 μg/mL). The results of hydroxyl radical scavenging activity and a reducing power assay showed concentration dependence, the EtOAc fraction demonstrating a better result than the other fractions at the same concentration in the studies. Additionally, the fractions' total phenolic (TP) contents was measured, phenolic compounds such as tannic acid, *p*-coumatric acid, quercetin, epicathchin, and kaempferol being detected by high performance liquid chromatography (HPLC). Meanwhile, a regression analysis revealed a moderate-to-high correlation coefficient between the antiradical activity and the TP contents, suggesting that fractions obtained from the 70% MeOH extract of *S. oleraceus* are of potential use as sources of antioxidant material.

Keywords: *Sonchus oleraceus*, fraction, antioxidant activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH), high performance liquid chromatography (HPLC)

Introduction

Free radicals are produced in normal and/or pathological cell metabolism. Oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, uncontrolled production of oxygen-derived free radicals is involved in the onset of many diseases such as cancer, rheumatoid arthritis, cirrhosis, and arteriosclerosis as well as in degenerative processes associated with ageing. Exogenous chemical and endogenous metabolic processes, in the food system and human body, respectively, might produce highly reactive free radicals, particularly oxygen-derived radicals, capable of oxidizing biomolecules, resulting in cell death and tissue damage (1,2). The harmful action can, however, be blocked by antioxidant substances that scavenge the free radicals and, thereby, detoxify the organism. Antioxidant-rich foods have the prevention of cardiovascular diseases, cancers (3-5), and neurodegenerative diseases including Parkinson's and Alzheimer's diseases (6).

Numerous types of natural antioxidants expressing various activities have been identified, but much attention has been drawn to the issue of adding polyphenols to foods and biological systems, due to their known abilities to scavenge free radicals (7,8). Phenolic compounds are widely distributed in plant secondary products, and found in many plants used as food and feed, and they are involved in many biological activities, including the chelating of metals, the scavenging of active oxygen species, and antioxidative activity (9).

Sonchus oleraceus L. (Compositae), a herb native to North Africa, Asia, and Europe, is widely found in moist areas of fields, orchards, roadside, gardens, or cleared land (10). Its plant has been used in folk medicines to treat diseases such as diarrhea, pneumonia, hepatitis, enteritis, appendicitis, chronic bronchopneumonia, throat swelling, haematemesis, and uraemia (11). Previously, some researches were reported the antioxidant activity of *S. oleraceus* (12) and also, we demonstrated that *S. oleraceus* exerts significant antioxidant activity, especially in its 70% methanolic (MeOH) crude extract (13). However, to date, antioxidant activity of its organic soluble fractions, such as hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH), and the water (H_2O) layer of *S. oleraceus* have not been reported. In the view of fact that many of the phenolic compounds remain in the 70% MeOH extract, our interest is focused on its fractions as potential sources of bioactive phenolics, which could be used for purposes in the food and pharmaceutical industry. The primary objectives of this study were to establish the antioxidative activity of fractions against stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) and reactive hydroxyl radicals and to investigate the composition of phenolic compounds determined by high performance liquid chromatography (HPLC). Additionally, the correlation between the total phenolic (TP) contents in the tested fractions and their antioxidant activities was investigated.

Materials and Methods

Chemicals DPPH, butyrated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), Folin-Ciocalteu reagent, ethylenediamine tetraacetic acid (EDTA), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine), tannic acid, caffeic acid, epicatechin, quercetin, *p*-coumaric acid, and kaempferol were purchased from Sigma-Aldrich (St. Louis, MO, USA). These chemicals were analytical

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and HPLC grades.

Materials The whole herb of *S. oleraceus* was collected at Chuncheon in South Korea in August, 2006. The materials were air dried under shade at 27°C and cut into small pieces (270 mesh), followed by being grinded into powder.

Extraction and fractionation Two kg of dried powder of *S. oleraceu* was extracted with solvent of 70% MeOH for 3 hr (5 L×3). The crude extract was collected by filtering and evaporating using a rotary evaporator at 45°C. The crude extract was subsequently fractionated with *n*-hexane, CH_2Cl_2, EtOAc, *n*-BuOH, and H_2O with a separatory funnel and then each fraction was freeze-dried to give 9.34 g of *n*-hexane, 23.13 g of CH_2Cl_2, 12.39 g of EtOAc, 37.97 g of *n*-BuOH, and 256.36 g of H_2O partitioned fractions after removal of the solvents in vacuum, respectively.

DPPH free radical scavenging activity The free radical scavenging activity of various fractions was evaluated by the method described by Guo and Wang (14) with some modifications. Briefly, fractions were mixed with a 0.3 mM DPPH ethanol solution, to give final concentrations of 5, 10, 25, 50, and 100 μg of fraction/mL DPPH solution. After 30 min at room temperature, the absorbance values were measured at 517 nm. This scavenging activity was expressed as the inhibition concentration at 50% (IC_{50}). The IC_{50} value, defined as the concentration of sample required for 50% scavenging of DPPH radical under experimental condition employed, was calculated using the % scavenging activities of serially diluted extract concentration. L-Ascorbic acid and BHA were used as a standard control.

Hydroxyl radical (·OH) scavenging activity The scavenging activity of fractions from the *S. oleraceus* 70% MeOH extract on the ·OH was based on the deoxyribose method (15) with some modifications. The ·OH were generated by direct addition of iron (II) salts to the reaction mixture. The reaction mixture contained 200 μL of 10 mM $FeSO_4 \cdot 7H_2O$, 200 μL of 10 mM EDTA, 200 μL of 10 mM 2-deoxyribose, 200 μL of 10 mM H_2O_2, mixed with 1.2 mL of 100 mM phosphate buffer (pH 7.4) containing 200 μL of investigated fractions. The reaction mixture was incubated for 4 hr at 37°C in a water bath. After incubation, 1 mL of 1% thiobarbituric acid and 1 mL of ice-cold 2.8% trichloroacetic acid were added to the resultant reaction mixture followed by being heated in a boiling water bath (95-100°C) for 10 min. After cooling down to room temperature and being centrifuged at 395×g for 5 min, the absorbance at 532 nm was measured. BHT and vitamin E were used as the positive control.

The scavenging activity on hydroxyl radicals (HRSA) was expressed as:

$$\text{HRSA}(\%)=[1-(As-Ac)/A_b]\times 100\%$$

Where As was the absorbance with the presence of deoxyribose and samples; A_b was the absorbance with the presence of deoxyribose but without test samples; and Ac was the absorbance with the presence of test samples but without deoxyribose.

Reducing power assay The reducing power was determined according to the method of Oyaizu (16). Various concentrations of different fractions (2.5 mL) were mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After 2.5 mL of 10%(w/v) trichloroacetic acid were added, the mixture was centrifuged at 650×g for 10 min. The upper layer (5 mL) was mixed with 5 mL deionised water and 1 mL of 0.1% of ferric chloride, and the absorbance was measured at 700 nm: higher absorbance indicates higher reducing power. The assays were carried out in triplicate and the results were expressed as mean±standard deviation (SD). BHA and vitamin C were used as standards.

Determination of total phenolic contents The phenolic compounds were determined according to a protocol similar to that of Athukorala *et al.* (17). Sample (1 mL) was mixed with 1 mL of ethanol 95%, 5 mL of distilled water, and 0.5 mL of 50% of Folin-Ciocalteu reagent. The mixture was reacted its 1 mL of 5% Na_2CO_3 for 5 min. Finally, each sample was mixed thoroughly and placed in dark for 1 hr and absorbance was measured at 725 nm with a ultraviolet (UV)-Vis spectrophotometer. A gallic acid standard curve was obtained for the calculation of phenolic contents. The concentration of TP compounds in the tested fractions determined as mg gallic acid equivalent (GAE)/g fraction.

HPLC analysis of *S. oleraceus* phenolic compounds HPLC was used to separate and determine individual phenolic compounds in different tested fractions by the method of Zheng *et al.* (18). Various solvent fractions were dissolved in 1 mL of acidified water (3% formic acid), and then passed through a 0.2-μm membrane filter and 20 μL was analyzed by HPLC. The samples were determined using a Waters Corp. (Milford, MA, USA) HPLC system coupled with a photodiode array detector (Waters 990 Series) and equipped with 2 pumps (600E system controller; Waters). Samples were injected at ambient temperature (20°C) into a reversed-phase NOVA-PAK C18 column ($250\times4.6\ mm^2$). The mobile phase consisted of 2.5% aqueous formic acid (A) and HPLC grade acetonitrile (B). The mobile phase was acidified water containing 2.5% formic acid (A) and acetonitrile (B) in a linear gradient from 5 to 20% B in the first 15 min, followed by a linear gradient from 20 to 30% B for 5 min, an isocratic mixture for 5 min, followed by a linear gradient from 30 to 90% B for 5 min, and an isocratic mixture for 5 min before returning to the initial conditions, followed by another isocratic mixture for 10 min. The flow rate was 0.8 mL/min, and the wavelengths of detection were set at 320 and 350 nm. Scanning between 240 and 550 nm was performed, and data were collected by the Waters 990 3D chromatography data system. Retention times (RT) and spectra were compared to those of authentication standards.

Ferrous ions chelating activity assay The chelating of ferrous ions of samples was estimated by the method of Oktay *et al.* (19) with some modifications. Different concentrations of various fractions of *S. oleraceus* species

(0.1-2 mg/mL) were added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by adding 5 mM ferrozine (0.2 mL) and the mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was measured spectrophotometrically at 562 nm. EDTA was used as a positive control.

All tests and analyses were run in triplicate. The % inhibition of ferrozine-Fe^{2+} complex formation is given by the formula:

$$\text{Inhibition } (\%) = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of samples and standards. The control did not contain $FeCl_2$ or ferrozine.

Statistical analysis All tests were performed in independent triplicate and data were expressed as mean±standard derivation (SD). Statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS 7.5 (Window Version 7.5 Software Inc., New York, NY, USA) and individual comparisons were obtained by Duncan's multiple-range test which was used to determine the difference of means, and $p<0.05$ was considered to be statistically significant.

Results and Discussion

DPPH free radical scavenging activity Free radicals are involved in a number of diseases in which their oxidative damage to DNA, lipids, and proteins, can result in the failure of cellular functions (20). DPPH has been widely used to measure free radical scavenging activity. The IC_{50} value is a parameter widely used to measure that activity (21). Table 1 shows the IC_{50} values of DPPH free radical scavenging activity tested with various fractions. All except the *n*-hexane fraction demonstrated strong antiradical activity (IC_{50} range: 19.25-92.2 μg/mL), the EtOAc fraction showing the highest scavenging activity (IC_{50}: 19.25 μg/mL). In this study, BHA and L-ascorbic acid were measured as the positive controls, showing the IC_{50} values of 4.34 and 6.81 μg/mL, respectively.

·OH scavenging activity Among the reactive oxygen species, the ·OH is the most reactive, inducing severe damage to adjacent biomolecules (22). The ·OH scavenging activity of different fractions was increased with the sample concentrations (Fig. 1). All except the H_2O fraction showed a high and dose-dependent ·OH scavenging activity, though the activity was weaker than those of the positive controls at the same concentration (vitamin E and BHT, at the concentration of 100 μg/mL, scavenged 83.3 and 84.5% of the available free radicals, respectively). The EtOAc fraction demonstrated the highest scavenging activity (65.6%) for the same concentration. At concentrations from 100 to 1,000 μg/mL, all except the H_2O fraction scavenged more than 60% of the available free radicals.

Table 1. DPPH free radical scavenging activity of different fractions from 70% MeOH extract of *S. oleraceus*

Fraction	IC_{50}[1] (μg/mL)
EtOAC	19.25
n-BuOH	49.95
H_2O	53.93
CH_2Cl_2	92.25
n-Hexane	1,152.12
Positive control antioxidant	
L-Ascorbic acid	6.81
BHA	4.34

[1]The effective concentration at which DPPH radical was scavenged by 50%.

Reducing power assay Antioxidant activity is reported to be concomitant with the reducing power, or the capability of reducing oxidized intermediates of the lipid peroxidation processes (23), and the reducing activity is generally associated with the presence of reductones (24), which have been shown to exert an antioxidant effect by donating a hydrogen atom and thereby breaking the free radical chain. As shown in Fig. 2, all of the fractions exhibited a moderate and concentration dependent reducing activity, though it was weaker than those of vitamin C and BHA at

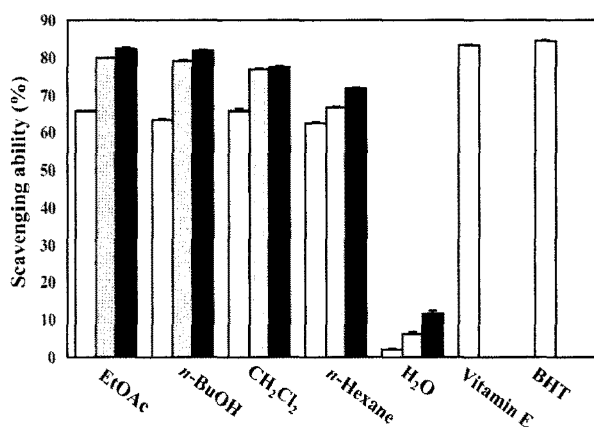


Fig. 1. Effects of different fractions from 70% MeOH extract of *S. oleraceus* on hydroxyl radical (·OH) scavenging assay. The concentrations of different fractions were 100 (□), 500 (▨), and 1,000 (■) μg/mL, respectively. BHT and vitamin E at the concentration of 100 μg/mL were used as the positive control.

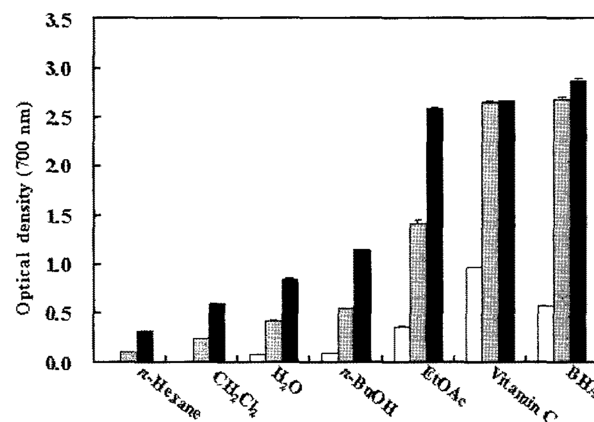


Fig. 2. Effects of various fractions from 70% MeOH extract of *S. oleraceus* on reducing power assay. The concentrations of different fractions were 100 (□), 500 (▨), and 1,000 (■) μg/mL, respectively.

Table 2. Total phenolic (TP) contents of different fractions from 70% MeOH extract of *S. oleraceus*

Fraction	TP contents (mg/g)
EtOAC	129.64±3.48[1)]
n-BuOH	108.84±4.99
H_2O	62.27±1.74
CH_2Cl_2	48.18±2.04
n-Hexane	32.98±2.93

[1)]Each value is expressed as mean±SD (n=3).

the same concentration. Notably, the EtOAc fraction demonstrated a higher reducing activity than did the other fractions at the same concentration, which was lower than that of the positive control. The 5 fractions in descending order of strength of reducing activity, were EtOAc>*n*-BuOH>H_2O>CH_2Cl_2>*n*-hexane fraction (Fig. 2). The reducing activity of the tested fractions was in the same descending order as the free radical scavenging activity, and this fact might be associated with the relationship between the antioxidant activity and the reducing power of fractions.

Total phenolic contents Phenolic compounds are found in both edible and inedible plants, which have multiple biological effects, including antioxidant activity. The antioxidant activity of phenolic compounds is due to their redox properties, which play an important role in absorbing and neutralizing free radicals, quenching single and triple oxygen, and decomposing peroxides (25-27). The Folin-Cioacalteu method used to investigate TP contents was employed on the different fractions. The phenolic contents of EtOAc, *n*-BuOH, H_2O, CH_2Cl_2, and *n*-hexane fractions were 129.64, 108.84, 62.27, 48.18, and 32.98 mg/g, respectively (Table 2). A strong positive correlation has been reported between total polyphenolic contents and DPPH radical scavenging activity (28,29), which had been strongly demonstrated in this study. DPPH radical scavenging activity and the phenolic contents of the 5 fractions were, in the same descending order: EtOAc>*n*-BuOH>H_2O>CH_2Cl_2>*n*-hexane fraction.

Ferrous ions chelating activity Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted, with the result that the red color of the complexes fades. Measurement of the rate of color reduction therefore allows estimation of the chelating activity of the coexisting chelator (30). The ferrous ions chelating activity was investigated in each fraction. In this assay, both the fraction and EDTA interfered with the formation of ferrous and ferrozine complexes, suggesting that it expresses chelating activity and capture ferrous ion before ferrozine. The absorbance of the Fe^{2+}-ferrozine complex was decreased dose-dependently, whereas the activity was increased the concentration from 0.1 to 2 mg/mL the concentration. Figure 3 presents the ferrous ions chelating of soluble fractions of MeOH extract from *S. oleraceus*. The ferrous ions chelating activity soluble fractions of the *S. oleraceus* and standard compound follwed the order EDTA>*n*-hexane>H_2O>*n*-BuOH>CH_2Cl_2>EtOAc. EDTA was an excellent chelating agent for ferrous ions; its chelating activity was 95.8% at 0.1 mg/mL. The metal chelating capacity was significant, since the extract reduced the concentration of the catalyzing transition metal in lipid peroxidation (31). It has been reported that chelating agents, which form σ-bonds with a metal, are as effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion (32). The data shown in Fig. 3 reveals that the tested fractions have the capacity for iron binding, suggesting that their actions as antioxidants might be related to their iron-binding capacity.

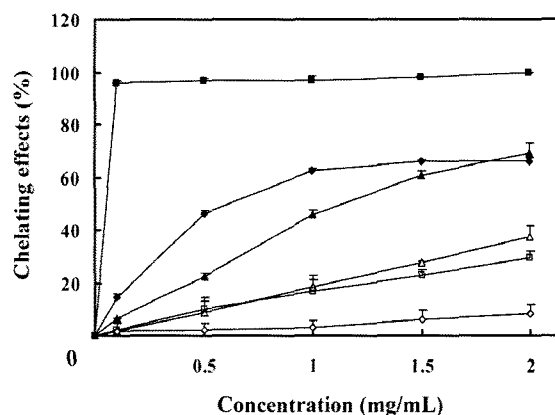
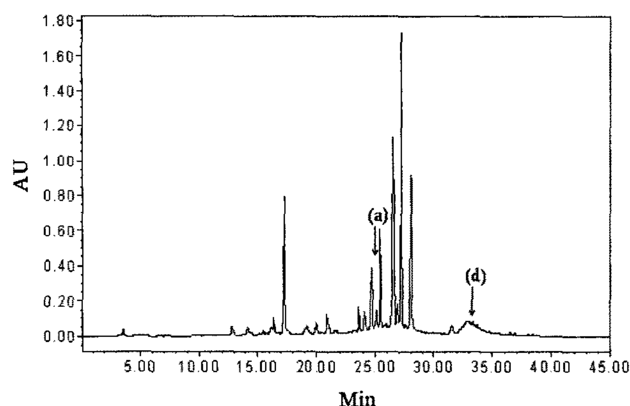


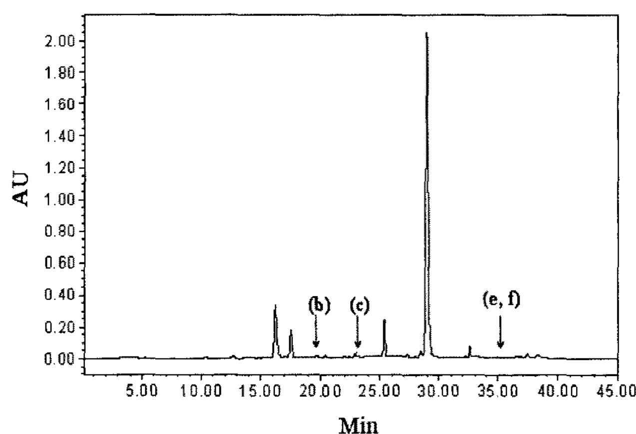
Fig. 3. Chelating ability of tested fractions from 70% MeOH extract of *S. oleraceus* on ferrous ions. (▲) H_2O fraction; (◆) *n*-hexane fraction; (△) *n*-BuOH fraction; (□) CH_2Cl_2 fraction; (◇) EtOAc fraction; (■) EDTA. All the values in the same group are not different.

HPLC analysis of *S. oleraceus* phenolic compounds HPLC analysis was employed to identify and quantify the major polyphenols present in the tested fractions. Figure 4 shows the typical HPLC chromatograms of some of the fractions. Phenolic acids such as tannic acid, caffeic acid, kaempferol, quercetin, epicathchin, and *p*-coumaric acid were identified in some fractions by RT and spectra with those of the standards. The contents of individual phenolic compounds was expressed as mg/g dry weight of fractions. Caffeic acid, *p*-coumaric acid, kaempferol, and epicathchin were detected in small amounts in the H_2O fraction, where their concentrations were 0.5, 2.1, 0.1, and 0.1 mg/g, respectively. Quercetin was detected in the EtOAc, *n*-BuOH, and *n*-hexane fractions where its concentrations were 20.0, 2.9, and 20.1 mg/g, respectively. Additionally, *p*-coumaric acid was detected in the CH_2Cl_2 fraction at the concentration of 1.2 mg/g, and tannic acid was detected in the *n*-BuOH fraction at 59.7 mg/g. The sums of the individual phenolics identified by HPLC were lower than the TP contents determined by the Folin-Ciocalteu method (Tabel 2, Fig. 4). This difference might be explained by the fact that the Folin-Ciocalteau method, because some other substances such as organic acids, residual sugars, amino acids, proteins, and other hydrophilic compounds interfere with this assay, is not an absolute measurement of the amount of phenolics. Various phenolic compounds have different responses in the Folin-Ciocalteau assay (33,34).

(1) *n*-BuOH fraction



(2) H_2O fraction



(3) EtOAc fraction

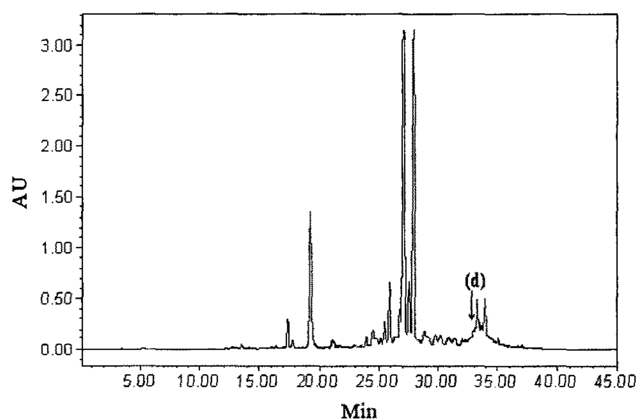


Fig. 4. HPLC chromatograms of phenolic compounds of different fractions from *S. oleraceus*. Detection at $A_{320\ nm}$: (a) tannic acid; (b) caffeic acid; (c) *p*-coumaric acid; (d) quercetin; (e) kaempferol; (f) epicathchin.

Assessment of total phenolic contents and antiradical activity The studies had shown a linear relationship between TP contents and antioxidant capacity in some plants and fruits (35). It is interested to consider the correlation between phenolic composition and the antioxidant activities of fractions, as phenolic compounds contribute directly to antioxidant activity (36). $1/IC_{50}^{DPPH}$ is representative of the antioxidant activity, because the more

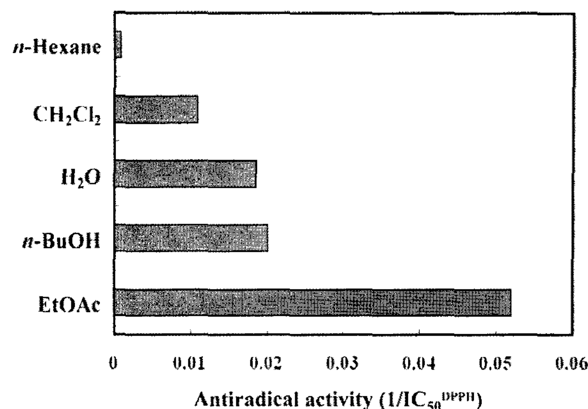


Fig. 5. Antiradical activities defined as $1/IC_{50}^{DPPH}$ of various fractions.

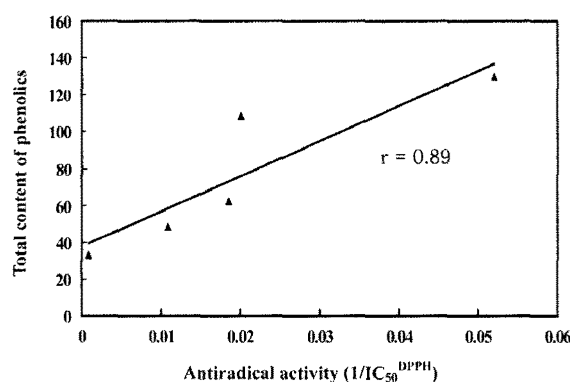


Fig. 6. Correlation between total contents of phenolic and antiradical activity-$1/IC_{50}^{DPPH}$.

it increases, the more efficient is the fraction (Fig. 5). In order to determine the relative importance of individual phenolic compounds in radical scavenging activity, a correlation analysis was conducted to determine the relationship between the $1/IC_{50}^{DPPH}$ value and the TP contents. As shown in Fig. 6, we can see that there was a distinct correlation between the 2 parameters (TP and $1/IC_{50}$) in the selected fractions. In the case of DPPH free radical scavenging activity, the correlation coefficient for the total phenolics, calculated from a linear regression analysis, was high. The $1/IC_{50}^{DPPH}$ value was highly correlated with the TP contents (r=0.89).

According to the results of the present study, it is clear that the selected fractions from *S. oleraceus* 70% MeOH extract exert significant antioxidant activity against various antioxidant systems *in vitro* so that, they possess considerable amounts of phenolic compounds. Phenols and polyphenolic compounds are sidely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities (37,38). The various antioxidant mechanisms of the fractions can be attributed to a strong hydrogen donating activity, a metal chelating ability, as well as their effectiveness as good scavengers of free radicals. Moreover, the correlation coefficient exhibited a positive relationship between antiradical activity and TP contents. *S. oleraceus* should be regarded as a potential value-added ingredient for functional foods.

Acknowledgments

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