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Antioxidant Activities of Mulberry (*Morus alba* L.) Leaf Extracted with Different Concentrations of EtOH

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Abstract Antioxidant activities of mulberry leaf extracted with different concentrations of EtOH were investigated. Total phenolic content and electron donating abilities of extract from 70% EtOH were the highest. Extracts obtained from EtOH-water mixture were shown to be significantly higher superoxide dismutase (SOD)-like activities than other treatment ($p<0.05$). Angiotensin-converting enzyme (ACE) inhibition was the greatest at 50% EtOH concentration ($p<0.05$). The extracts from 30-70% EtOH exhibited higher ferric reducing ability of plasma (FRAP) value than rest of the concentration ($p<0.05$). In case of nitrite scavenging activity, much higher scavenging activities were observed when the extraction was performed with EtOH or EtOH-water mixture ($p<0.05$). The results indicate that concentration of EtOH as extraction solvents can affect the antioxidant activity of mulberry leaf, which may provide useful information on the optimal solvent conditions for the extraction.

Keywords: mulberry leaf, electron donating ability, superoxide dismutase (SOD)-like activity, angiotensin-converting enzyme (ACE) inhibition rate, nitrite scavenging activity, reducing power

Introduction

Mulberry (*Morus alba* L.) plant is a fast-growing deciduous plant that grows under different climatic conditions (i.e., tropical, subtropical, and temperate) (1). Mulberry leaf is manly used for rearing of silkworms in sericulture and is also used to feed cattle without causing adverse effects. In Japan, consumption of mulberry leaf as a tea or powdered juice has been increasing (2). The leaf is nutritious, palatable, and non-toxic, and is stated to improve milk yield when fed to dairy animals (3). Reports indicate that mulberry leaves contain proteins, carbohydrates, calcium, iron, ascorbic acid, β-caroteine, vitamin B_1, folic acid, and vitamin D (4). The presence of rutin, quercetin, isoquercetin, and other flavonoids in mulberry leaves also has been reported (5). The total antioxidant activity of plant foods is the result of individual activities of each of the antioxidant compounds present such as vitamin C, tocopherols, carotenoids, and phenolic compounds, the latter being the major phytochemicals responsible for antioxidant activity of plant materials (5). Moreover, these compounds render their effects via different mechanisms such as radical scavenging, metal chelation, inhibition of lipid peroxidation, quenching of singlet oxygen, and so on to act as antioxidants (5).

Typically, various solvents of water, alcohols, acetone, and ether, etc are used to extract bioactive substances from natural products due to their broad solubility propensity on solvents. Thereby, alcohol-water mixtures are used to extract out various ingredients having broad range of solubility propensity for the investigation of the specific functionality of the molecular compounds from extracted ingredients (6). Therefore, this work was aimed to evaluate the effect of concentrations of EtOH as extraction solvents on the total phenolic contents and the antioxidant capacities, such as electron donating ability, superoxide dismutase like activity, nitrite scavenging activity, and reducing power.

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Materials and Methods

Materials Mulberry (*M. Alba* L.) leaf was harvested at Yongcheon, Korea, in May 2008.

Preparation of extracts Two g of dried powder of mulberry leaf was extracted with 150 mL EtOH (Junsei, Tokyo, Japan) of different concentration (0, 30, 50, 70, and 100%) about 3 hr at 70°C. The extracts were centrifuged at 1,372×g for 20 min to obtain the supernatant and the residue was re-extracted under the same conditions. The supernatants were collected together for the filtration using membrane filter (Millipore Corp., Billerica, MA, USA) and stored at 4°C for further analysis.

Total phenolic content Total phenolic content was determined by the method of Folin and Denis (7). Each extract (0.2 mL) was mixed with distilled water (Sigma-Aldrich, St. Louis, MO, USA) (1.8 mL) and Folin reagent (Sigma-Aldrich) (0.2 mL) for 3 min, and 0.4 mL of Na_2CO_3 (Junsei) was added. The final volume was adjusted to 4 mL with distilled water. After holding the mixed solution for 1 hr, absorbance was measured at room temperature at 725 nm using a UV-VIS spectrophotometer (V-570; Jasco, Tokyo, Japan). Results were expressed as tannic acid (Sigma-Aldrich) equivalents (mg TAE/g sample).

Electron donating ability (EDA) EDA toward 1,1-diphenyl-2-picyrylhydrazyl (DPPH) (Sigma-Aldrich) radical was measured according to the method of Blois (8). One mL of extract was added to 1 mL of DPPH solution (4×10^{-4}

M in EtOH) as the free radical source and kept for 30 min at room temperature. The decrease in the solution absorbance, due to proton donating activity of extract, was measured at 535 nm. The percentage EDA was calculated using the following equation:

$$EDA\ (\%)=(1-A_1/A_0)\times 100$$

A_0 is the absorbance of the control and A_1 is the absorbance of extract or standard sample.

Superoxide dismutase (SOD)-like activity SOD-like activity was assayed by the method of Marklund and Marklund (9). The reaction mixture was prepared by mixing 0.2 mL of the sample solution, 3 mL of the Tris-HCl buffer (50 mL Tris[hydroxymethyl]amino-methane+ 10 mM EDTA, pH 8.5) (Sigma-Aldrich), 0.2 mL of 7.2 mM pyrogallol (Sigma-Aldrich) and left stand at room temperature for 10 min. The oxidized pyrogallol was measured at 420 nm, using a UV-VIS spectrophotometer (V-570; Jasco), after stopping the reaction by adding 0.2 mL of 1.0 N HCl (Junsei). The SOD-like activity was calculated using the following equation:

$$SOD\text{-like activity}\ (\%)=(1-A/B)\times 100$$

A is the absorbance of testing solution and B is the absorbance of control solution.

Nitrite scavenging activity Nitrite scavenging activity was measured according to the method of Gray and Dugan (10). One mL of 1 mM $NaNO_2$ (Sigma-Aldrich) solution was added to 1 mL of each sample, and pH values of the resulting mixtures were adjusted to 1.2, 3.0, 4.2, and 6.0 using 7 mL of buffer solutions. 0.1 N HCl for pH 1.2 and 0.2 N citric acid (Junsei) for pH 3.0, 4.2, and 6.0. Each sample was allowed to react at 37°C for 1 hr, after which 1 mL of each sample was extracted from the solutions, after which 1 mL of each sample was extracted from the solutions, mixed thoroughly with 5 mL of 2% acetic acid (Junsei) with 0.4 mL of Griess reagents, and maintained at room temperature for 15 min. Griess reagent was prepared by mixing equal amounts of 1% sulfanilic acid (Sigma-Aldrich) and 1% napthylamine (Sigma-Aldrich), which were made with 3% acetic acid. A blank was prepared by adding 0.4 mL of distilled water instead of the Griess reagent. The nitrite scavenging activity was evaluated using UV-VIS spectrophotometer (V-570; Jasco) at a wavelength of 520 nm based on the following equation;

$$\text{Nitrite scavenging activity}\ (\%)=[1-(A-C)/B]\times 100$$

A is the absorbance of the mixture sample during a reaction with 1 mM $NaNO_2$ after a 1 hr reaction, B is the absorbance of a mixture distilled water and 1 mM $NaNO_2$ after a 1 hr reaction, and C is the absorbance of the sample.

Determination of the reducing power The reducing power of the mulberry leaf extracts was determined by using ferric reducing ability of plasma (FRAP) assay described by Benzie and Strain (11) with a slight modification. Briefly, the FRAP reagent contained 2.5 mL of 10 mM TPTZ (Sigma-Aldrich) solution in 40 mM HCl plus 2.5 mL of 20 mM $FeCl_3$ (Sigma-Aldrich) and 25 mL of 0.3 M acetate buffer, pH 3.6, was freshly prepared. The extracts were dissolved in EtOH at a concentration of 1 mg/mL. An aliquot of 20 μL test of solution was mixed with 180 μL of FRAP reagent. The absorption of the reaction mixture was measured at 593 nm by a microtitre plate reader. Ethanolic solutions of known Fe(II) concentration, in the range of 50-1,000 μM ($FeSO_4\cdot 7H_2O$) (Junsei), were used to prepare the calibration curve. The reducing power was expressed as the equivalent concentration. This parameter was defined as the concentration of antioxidant having a ferric reducing ability equivalent to that of 1 mM $FeSO_4\cdot 7H_2O$.

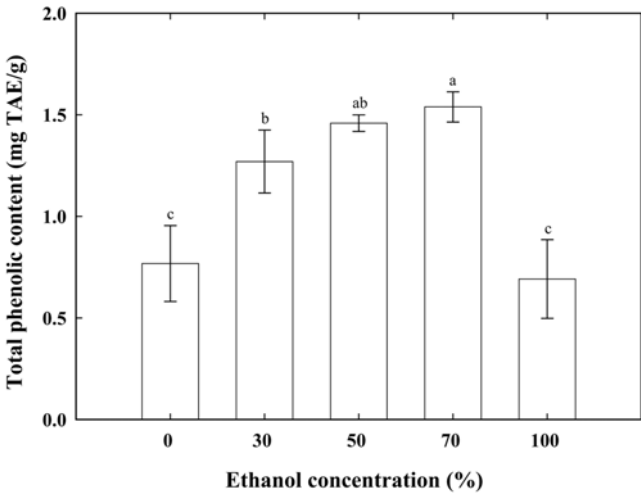

Fig. 1. Total phenolic content of mulberry leaf extracted with different concentrations of EtOH. Values are the means of triplicate±SD; Means with different letters are significantly different at $p<0.05$ by Duncan's multiple range test.

Table 1. Correlation coefficients between assays

	DPPH	SOD	ACE	FRAP	TPC
DPPH	1				
SOD	0.553	1			
ACE	0.168	0.667	1		
FRAP	0.975**	0.387	0.067	1	
TPC[1)]	0.992**	0.595	0.254	0.950*	1

[1)]Total phenolic content; *$p<0.05$, **$p<0.001$.

Statistical analysis Data are expressed as mean±standsrd deviation (SD) of 3 parallel measurement. All data were analyzed by one-way analysis of variances (ANOVA) using Statistical Package for Social Science (SPSS version 10.0, Chicago, IL, USA). The Pearson correlation analysis and principal component analysis were also performed by SPSS 10.0 for determination of the correlations among means and visualizing the differences and similarities among varieties in term of antioxidant activities, respectively.

Results and Discussion

Total phenolic content Total phenolic content of mulberry leaf extracted with different concentrations of EtOH is presented in Fig. 1. Total phenolic contents of the mulberry leaf extracted with EtOH concentration over 30 to 70% (1.27, 1.46, and 1.54 mg TAE/g for 30, 50, and 70% EtOH,

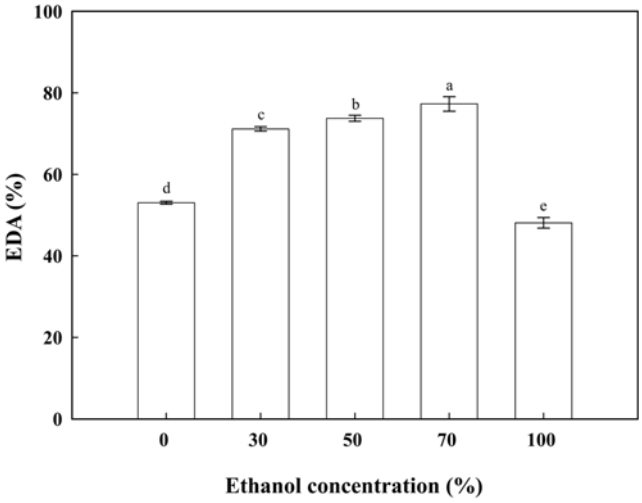

Fig. 2. Electron donating abilities (EDA) of mulberry leaf extracted with different concentrations of EtOH. Values are the means of triplicate±SD; Means with different letters are significantly different at $p<0.05$ by Duncan's multiple range test.

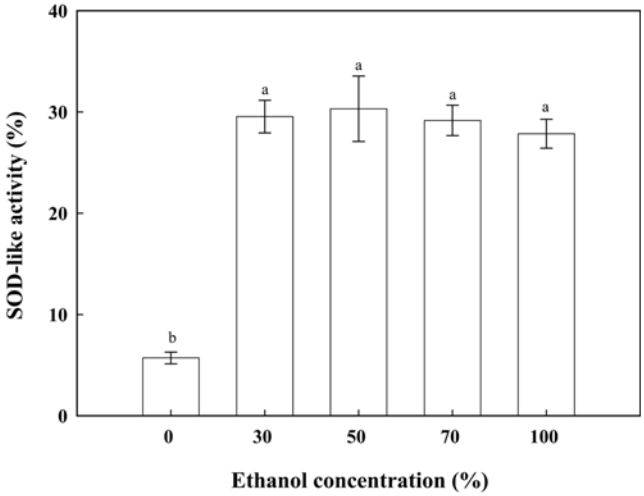

Fig. 3. SOD-like activity of mulberry leaf extracted with different concentrations of EtOH. Values are the means of triplicate±SD; Means with different letters are significantly different at $p<0.05$ by Duncan's multiple range test.

respectively) were significantly higher than other concentrations (0.77 and 0.69 mg TAE/g for 0 and 100%, respectively) ($p<0.05$). Total phenolic content of mulberry leaf extracts is highly correlated with EDA ($R^2=0.992$, $p<0.05$) as well as with FRAP value ($R^2=0.950$, $p<0.05$) (Table 1).

Phenolic phytochemicals are secondary metabolites of plant origin which constitute one of the most abundant groups of natural metabolites and are synthesized by plants in order to protect themselves from biological and environmental stress (12). Recent studies showed that phenolic phytochemicals had high antioxidant activity and certain therapeutic properties, including anti-diabetic and anti-hypertension activity (13) and could be obtained through dietary herbs, spices, fruits, and vegetables. Youwei *et al.* (14) reported excellent correlation between antioxidant activity and total phenolic content in certain fresh flowers in southern China.

These results suggest that the mulberry leaf extracts obtained from EtOH-water mixture might have high antioxidant properties.

EDA The EDA of mulberry leaf extracted with different concentrations of EtOH is presented in Fig. 2. The extracts of mulberry leaf were capable of scavenging the DPPH radicals via hydrogen donating activity by 53.04, 71.15, 73.76, 77.28%, and 48.11% at EtOH concentration of 0, 30, 50, 70, and 100%, respectively, indicating extracts obtained from 70% EtOH showed the highest effect ($p<0.05$). Previous studies reported that radical scavenging activity is an index for the antioxidant effectiveness of phenolic compounds (15,16), and the higher total phenolic content from 70% EtOH concentration than other treatment might explain the high antioxidant properties of mulberry leaf extracts. The EDA correlated strongly with total phenolic content ($R^2=0.992$, $p<0.05$) or FRAP value ($R^2=0.975$, $p<0.01$) (Table 1). The results show that mulberry leaf extracted with EtOH or EtOH-water mixture possesses higher EDAs than water extracts.

SOD-like activity SOD-like activity of mulberry leaf extracted with different concentrations of EtOH is shown in Fig. 3.

SOD-like activities of mulberry leaf extracts obtained from different EtOH concentrations of 0, 30, 50, 70, and 100% were 5.7, 29.6, 30.3, 29.2, and 22.9%, respectively, indicating higher activity with EtOH extracts and EtOH-water mixture extracts ($p<0.05$). It might be due to the higher content of antioxidative materials capable of repressing the reactivity of superoxide (16,17). Previous researches also reported that the EtOH extract of the cherry elaeagnus leaves had significantly higher SOD-like activity compared to the water extract at all concentrations (16). The results show that EtOH extracts and EtOH-water mixture extracts of mulberry leaf possess higher SDO-like activity than water extracts ($p<0.05$).

Angiotensin-converting enzyme (ACE) inhibition rate ACE inhibition rate of mulberry leaf extracts obtained from the extraction solvent containing EtOH concentration of 0, 30, 50, 70, and 100% were 68.3, 75.8, 91.8, 74.8, and 84.2%, respectively, indicating the highest activity with 50 and 100% EtOH extracts, and the lowest activity of water extracts ($p<0.05$) (Fig. 4).

One of the most important intermediary factors for controlling hypertension is the action of the ACE (18,19). Since ACE converts angiotensin I to angiotensin II, inhibition of ACE is considered a useful therapeutic approach in the treatment of high blood pressure in both diabetic and non-diabetic patients (20).

Recent studies indicate that phenolic compound rich food and plants have the ability to inhibit ACE activity, both *in vitro* and *in vivo* (13,19,20).

Nitrite scavenging activity The nitrite scavenging activities of mulberry leaf extracts obtained from the extraction solvent containing different EtOH concentration is shown in Fig. 5. The nitrite scavenging activities were affected by

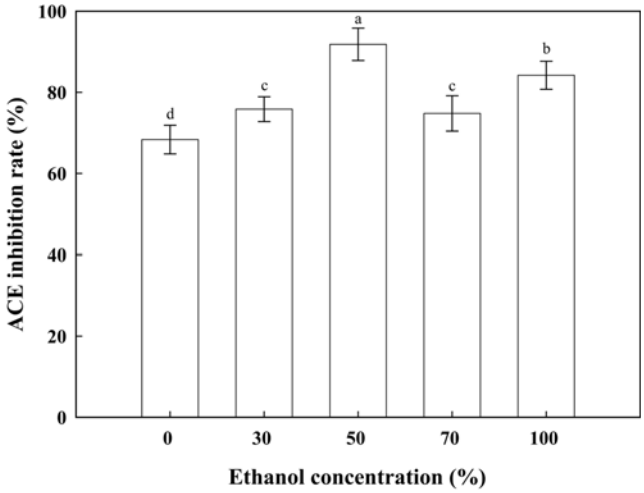

Fig. 4. ACE inhibition rate of mulberry leaf extracted with different concentrations of EtOH. Values are the means of triplicate±SD; Means with different letters are significantly different at $p<0.05$ by Duncan's multiple range test.

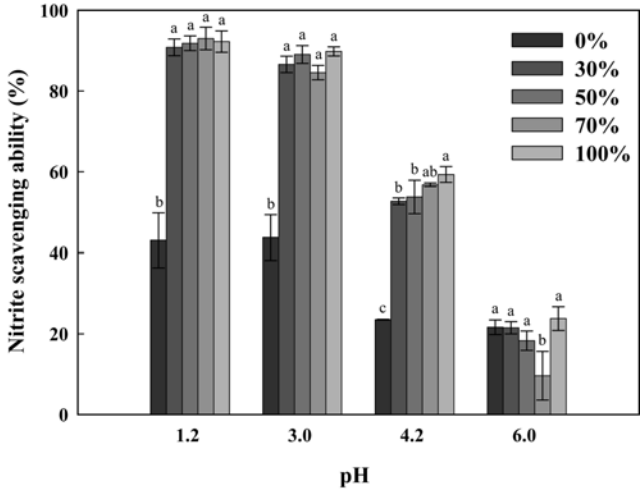

Fig. 5. Nitrite scavenging ability of mulberry leaf extracted with different concentrations of EtOH. Values are the means of triplicate±SD; Means with different letters are significantly different at $p<0.05$ by Duncan's multiple range test.

pH. More specially, the nitrite scavenging activity was decreased with the increase of pH, highest activity at a pH of 1.2 and lowest activity at pH of 6.0. The fact that the nitrite scavenging activity was high at pH 1.2 suggests that nitrosamine production can be inhibited *in vivo* (21). This finding is similar to the results from the study of pine needle extracts and mugwort extracts where demonstrated higher nitrite scavenging activities from pH below 3 (22). When it comes to extraction solvent, very low scavenging activities were observed with water extracts, while much higher scavenging abilities were found with EtOH extracts or EtOH-water mixture extracts ($p<0.05$).

Reducing power Reducing power of mulberry leaf extracts obtained from the extraction solvent containing different EtOH concentration is shown in Fig. 6. Extracts

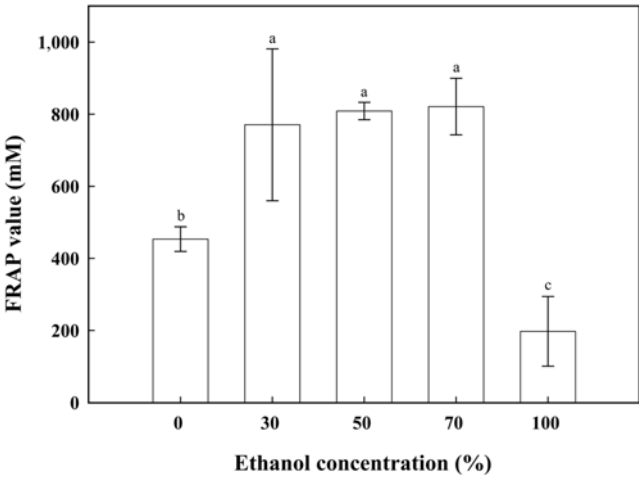

Fig. 6. FRAP value of mulberry leaf extracted with different concentrations of EtOH. Values are the means of triplicate±SD; Means with different letters are significantly different at $p<0.05$ by Duncan's multiple range test.

from EtOH-water mixture (770.7, 808.8, and 821.1 mM for 30, 50, and 70% of EtOH concentration, respectively) showed higher FRAP value than water extracts (453.4 mM) and EtOH extracts (197.4 mM), indicating strong reducing activity of extracts from EtOH-water mixture ($p<0.05$). The FRAP value is correlated strongly with total phenolic content ($R^2=0.950$, $p<0.05$) or EDA ($R^2=0.975$, $p<0.01$) (Table 1). The results show that mulberry leaf extracts obtained from EtOH-water mixture possess higher reducing power than water extracts and EtOH extracts ($p<0.05$).

In summary, it was shown that EtOH concentration as extraction solvents significantly affected the total phenolic content and antioxidant activities. These results suggest that EtOH extracts can be used as an easily accessible source of natural antioxidants and as a possible food supplement. However, other components responsible for the antioxidant activity of mulberry leaf are still unknown. Therefore, further studies would be required for isolation and identification of the antioxidant components in mulberry leaf.

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