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Radical Scavenging Activities of Fruits of *Crataegus pinnatifida* BUNGE Major. from Korea

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Abstract – Screenings of potential antioxidant activities of *Crataegus pinnatifida* BUNGE Major. fruits extracted 80% methanol were performed using four antioxidant assays. Significant differences were observed both in total phenolic contents (TPC) and total flavonoid contents (TFC), DPPH radical scavenging activity, nitric oxide scavenging activity, ABTS radical scavenging assay, and reducing power assay. The total polyphenol content and total flavonoid content in the extract were measured to be 224.4 ± 0.52 mg GAE/100 g and 12 ± 0.25 mg QE/100 g, respectively. When the tested concentration was 500 μg/mL, DPPH and ABTS radical-scavenging activities of methanolic extracts were 84.15% and 88.8%, respectively. The reducing power and nitric oxide scavenging activity were increased at the manner of dose-dependently. These results suggest that methanolic extracts of *Crataegus pinnatifida* Bge. fruits possess excellent radical scavenging activities and may serve as a potential source of natural antioxidant.
Keywords – *Crataegus pinnatifida* BUNGE Major. Fruits, Radical scavenging activities, DPPH, Nitric oxide, ABTS, Antioxidant activity

Introduction

Free radicals play detrimental roles in peroxidation of lipid, denaturation of protein, tumor, transformation, mutation, aging, and cancer (Simic *et al*., 1988). To maintain a healthy life and to prevent deterioration in the quality of food by peroxidation of lipid, effective prevention of various diseases caused by free radical is necessary. Researches are going on for the development of antioxidants that inhibits the generation and activity of free radicals (Choe *et al*., 1982). 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 radical 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 (Halliwell *et al*., 1985, Mahfuz *et al.*, 2007). Antioxidant-rich foods helps in the prevention of cardiovascular diseases, cancers (Gerber *et al*., 2002, Serafini *et al*., 2002), and neurodegenerative diseases including Parkinson's and Alzheimer's diseases (Di *et al*., 2003). Natural antioxidants like vitamin C, vitamin E, carotenes, phenolic acid, phytate and phytoestrogens are mostly derived from grains, fruits and vegetables, and have been identified to have the potential in reducing disease risk (Jacob *et al*., 1996, Knight *et al*., 1988). *Crataegus pinnatifida* Bge. ver major N.E.Br. locally call Hawthorn, is widely distributed throughout the northern temperate regions of the world with approximately 280 species, primarily in East Asia, Europe and North America (Zhang *et al*., 2002). Hawthorn fruits have long been used in traditional Chinese medicine and European herbal medicine, and are widely consumed as food, in the form of juice, drink, jam and canned fruit (Chang *et al*., 2006). The extract of hawthorn has been shown to have many health benefits including being cardiovascular protective, hypotensive, hypocholesterolaemic and lowers serum cholesterol (Yao *et al*., 2008, Zhang *et al*., 2001, Zhang *et al*., 2002). Pharmacological and toxicological studies have demonstrated that consumption of hawthorn fruits is associated with long-term medicinal benefits to cardiovascular function with little side effect (Ammon *et al*., 1981a, Ammon *et al*., 1981b, Ammon *et al*., 1981c). Hawthorn fruits and leaves have a curative effect on blood vessels of the heart which have been extensively reported (Frishman *et al*., 2009, Frishman *et al*., 2004, Long *et al*., 2006, Pittle *et al*., 2003). Recently,

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there has been a great increase of interest in natural antioxidant of plant origin since they are viewed as promising therapeutic agents for free radical pathologies and also found to be useful as nutraceuticals due to their impact on the status of human health and disease prevention (Jayaprakasha *et al*., 2000, Kitts *et al*., 2000, Nogochi *et al*., 2000). The major objective of this study was to investigate the radical scavenging activities of methanolic extract from the fruits of *C. pinnatifida* BUNGE Major. by employing various in-vitro assay systems. Total polyphenol, flavonoid contents, and various antioxidative properties including DPPH radical scavenging activity, Nitric oxide scavenging activity, ABTS radical scavenging assay and the reducing power were measured. These pro-screening experiments reported herein will be a basis to selectively identify the most appropriate species for further characterization and to evaluate suitability of active components from *C. pinnatifida* BUNGE Major. fruit extracts as a natural antioxidant for application in food industry.

Experimental

Plant material – Korean *C. pinnatifida* BUNGE Major. fruits (10 kg) were collected from Jincheon-gun, Chungbuk, Korea. After harvesting, the fruits were dried and the seeds were removed. A voucher specimen has been deposited in Duksung Women's University, Seoul, South Korea. The air-dried powder of *C. pinnatifida* BUNGE Major. fruits (9.3 kg) were extracted with 80% MeOH (20 L) at room temperature for one week and filtered. The residue was re-percolated again. This Process was repeated three times. The combined methanol extracts were concentrated under reduced pressure at temperature not exceeding 45°C to yield a dry extract (1043.24 g).

Chemicals – 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid (ABTS), potassium persulphate, potassium ferricyanide, sodium nitroprusside, sodium carbonate, hydrogen peroxide, sulfanilic acid, sulfanilamide, phosphoric acid, glacial acetic acid, trichloroacetic acid, naphthylethylenediamine dichloride, gallic acid, trolox, and quercetin were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Other chemicals including methanol and phosphate buffer were purchased from Merck, USA. All the chemicals used in this study were of analytical grade.

Determination of total polyphenol content – Total polyphenol content was estimated using the Folin-Ciocalteu colorimetric method (Cai *et al*., 2004) with a slight modification. Briefly, the appropriate dilutions of the filtered extract were oxidized with Folin–Ciocalteu reagent and the reaction solution was neutralized with saturated sodium carbonate (20 g/L). The absorbance of the resulting blue color was measured at 760 nm with a UV-VIS spectrophotometer after incubation for 1 h at room temperature. Quantification was conducted on the basis of the standard curve of gallic acid (200 - 1.563 mg/mL). Total polyphenol content in *C. pinnatifida* Bge. fruits was expressed as mg gallic acid equivlents (GAE) per 100 g fresh weight.

Determination of total flavonoids content – Total flavonoid was determined using the method of M.S Taga (Taga *et al*., 1984) on the formation of a complex flavonoidaluminium. A volume of 0.5 mL of 2% $AlCl_3$-methanol solution was mixed with 0.5 mL of the extract (1 mg/mL). The resultant mixture was incubated for 15 min for yellow color development which indicated the presence of flavonoid. The absorbance was measured at 420 nm using UV-VIS spectrophotometer. Total flavonoid content in *C. pinnatifida* Bge. fruits was expressed as mg quercetin equivalent (QE) per 100 g fresh weight.

DPPH free radical scavenging activity – The method of Shen (shen *et al*., 2010) was used for the determination of scavenging activity of DPPH radical in the extract solution. A portion of 0.2 mM DPPH prepared in methanol was added to 0.0157 to 1 mg of the plant extracts, and ascorbic acid was used as standard. The reaction mixture was vortexed thoroughly and left in dark at room temperature for 30 min. The absorbance was measured by UV-VIS spectrophotometer at 520 nm. The scavenging ability of the plant on DPPH was calculated using the equation: DPPH scavenging activity (%) = [(Abs control – Abs sample)] / (Abs control)] × 100, where Abs control is the absorbance of DPPH + methanol; Abs sample is the absorbance of DPPH radical + sample extract or standard.

Nitric oxide scavenging activity – The method of Marcocci (Marcocci *et al*., 1994) used for the determination of scavenging activity of nitric oxide in the extract solution. Scavenging of NO was determined using sodium nitroprusside (SNP) as NO donor. SNP (10 mM) in phosphate buffered saline was mixed with different concentrations of methanolic extract (62.5 to 1000 μg/mL), ascorbic acid was used as standard (15.625 μg/mL to 500 μg/mL) and incubated at 25 °C for 150 min, then equal volume of Griess reagent (2% sulfanilamide in 4% phosphoric acid and 0.2% naphylethylenediamine dihydrochloride in 4% phosphoric acid) was added. The absorbance was immediately measured at 542 nm. The NO scavenging activity was calculated using the formula,

percentage NO scavenging activity = [(Abs of Control – Abs of Sample) / Abs of Control] × 100. Each experiment was carried out in triplicate and results were expressed as mean % NO scavenging activity ± SD.

ABTS radical scavenging assay – Standard TEAC (Trolox Equivalent Antioxidant Capacity) assay was performed according to the method of Re R (Re *et al*., 1999) with slight modification. Briefly, ABTS was prepared by mixing 7.4 mM aqueous ABTS with potassium persulfate (2.6 mM) in the dark at room temperature for 24 h. For the evaluation of antioxidant activity, the solution was diluted with ethanol to reach an absorbance of 0.70 ± 0.02 at 732 nm. Different concentrations of extracts were mixed with ABTS solution. The final absorbance was read at 732 nm after 6 min with 1 min intervals. The ABTS scavenging activity was calculated from the formula, percentage ABTS scavenging activity = [(Abs of Control – Abs of Sample) / Abs of Control] × 100. Each experiment was carried out in triplicate and results were expressed as mean % ABTS scavenging activity ± SD.

Reducing power assay – The reducing power was determined according to the method of Oyaizu (Oyaize *et al*., 1986). One milligram of the extract at 6 kinds of concentrations was mixed with 1 mL of 200 mM sodium phosphate buffer (pH 6.6) and 1 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After adding 1 mL of 10% (w/v) trichloroacetic acid, the mixture was centrifuged at 650 × g for 10 min. The upper layer (1 mL) was mixed with 1 mL of deionized water and 1 mL of 0.1% 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). Ascorbic acid was used as a standard.

Statistical analysis – All experiments were conducted in independent triplicate (n = 3) and data were expressed as mean ± SD. Statistical significance was evaluated by one-way analysis of variance using SPSS Win program (Version 19.0, Cary, NC), and individual comparisons were determined using Duncan's multiple range tests at the $p < 0.05$ level.

Results and Discussion

Total polyphenol and flavonoid contents – The yield of 80% methanolic extracts from *C. pinnatifida* BUNGE Major. fruits were 11.22%, this result is presented in Table 1. The total phenolic content (TPC) of *C. pinnatifida* BUNGE Major. fruits extract was determined through a linear gallic acid standard curve ($y = –0.0005x^2 + 0.0606x – 0.0824$; $R^2 = 0.9972$) and expressed as milligram of gallic acid equvialents (GAE) per 100 gram of dry *C. pinnatifida* BUNGE Major. fruits (mg GAE/100 g extract). TPC of *C. pinnatifida* BUNGE Major. fruits extract was observed ($p < 0.05$) to be 224.4 ± 0.52 mg GAE/100 g of extract (Table 1). In this study, the total flavonoids content (TFC) of *C. pinnatifida* BUNGE Major. fruits extract was evaluated by aluminium colourimetric assay, using quercetin as a standard compound ($y = –0.0003x^2 + 0.0495x – 0.0744$; $R^2 = 0.9971$) and then expressed as milligram of quercetin equivalents (QE) of dry *C. pinnatifida* BUNGE Major. fruits (mg QE/ 100 g extract). TFC of *C. pinnatifida* BUNGE Major. fruits extract was observed ($p < 0.05$) to be 12 ± 0.25 mg QE/100 g extract (Table 1). Polyphenol and flavonoid compounds constitute the primary class of natural antioxidants present in the plant kingdom, and they are endowed with free radical scavenging and antioxidative activities (Amin *et al*., 2007). Diverse biological activities related to their free radical-scavenging and antioxidant activities (e.g. anti-inflammatory, anticarcinogenic, and anti-atherosclerotic activities) were exhibited (Shetty *et al*., 1995).

Table 1. Total polyphenol and flavonoid contents of *C. pinnatifida* BUNGE Major. fruits extracts

Tatal plant materials (g)[1)]	9300
80% methanolic extracts (g)[2)]	1043.24
Yield (%, W/W)[3)]	11.22
Total polyphenols (mg GAE/100 g extract)[4)]	224.4 ± 0.52
Total flavonoids (mg QE/100 g extract)[5)]	12 ± 0.25

[1)] Air-dried powder weight
[2)] Freeze dried weight extracted
[3)] Based on Air-dried powder weight
[4)] Based on gallic acid as a standard
[5)] Based on quercetin as a standard

DPPH free radical scavenging activity – As a kind of stable free radical, DPPH can accept an electron of hydrogen radical to become a stable diamagnetic molecule, which is widely used to investigate radical scavenging activity. The antioxidants can react with DPPH, a deep-violet colored stable free radical, converting it into a yellow colored α,α-diphenyl-β-picrylhydrazine. The discoloration of the reaction mixture can be quantified by measuring the absorbance at 520 nm, which indicates the radical scavenging ability of the antioxidant (Braca *et al*., 2001). *C. pinnatifida* BUNGE Major. fruits extract showed antioxidant potential to scavenge DPPH radicals (Fig. 1.). Methanol extract of *C. pinnatifida* BUNGE Major. fruits

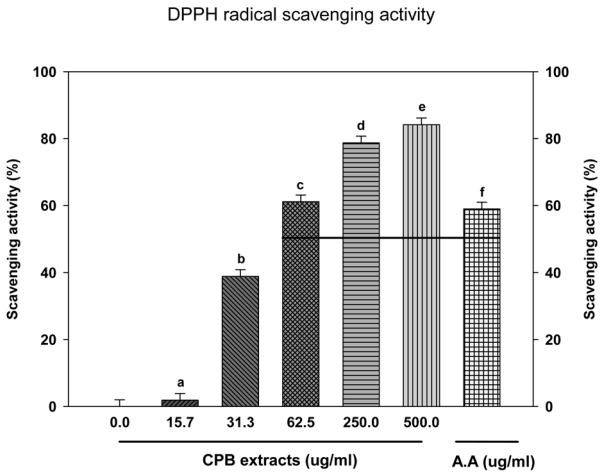

Fig. 1. DPPH radical scavenging activities of the fruits from *Crataegus pinnatifida* Bge. Data represent the means ± S.D. from triplication (CPB: *Crataegus pinnatifida* Bge., A.A: Ascorbic acid). Different subscript letters indicate a significant difference at the $p < 0.05$ by Duncan's multiple range tests. In the figure, the horizontal line appears IC_{50} concentration (CPB IC_{50} concentration: 48.5 μg/mL, ascorbic acid: IC_{50} concentration: 3.906 μg/mL).

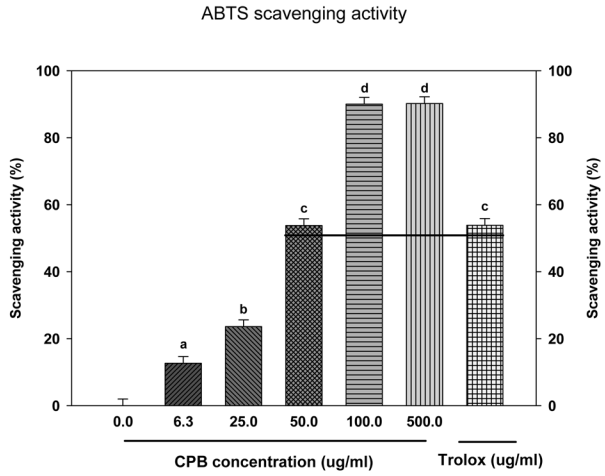

Fig. 2. ABTS radical scavenging activities of the fruits from *Crataegus pinnatifida* Bge. Data represent the means ± S.D. from triplication (CPB: *Crataegus pinnatifida* Bge). Different subscript letters indicate a significant difference at the $p < 0.05$ by Duncan's multiple range tests. In the figure, the horizontal line appears IC_{50} concentration (CPB IC_{50} concentration: 46.5 μg/mL, Trolox IC_{50} concentration: 97.66 μg/mL).

was measured at concentrations of 500, 250, 125, 62.5, 31.3, and 15.7 μg/mL. DPPH radical scavenging activity of the methanol extract of *C. pinnatifida* BUNGE Major. fruits increased depending on the sample concentration. In comparison with ascorbic acid (IC_{50} value 3.906 μg/mL) *C. pinnatifida* BUNGE Major. fruit was shown to have a reliable IC_{50} value 48.5 μg/mL. According to recent reports, glasswort seed extract (Kang *et al*., 2011) and dried jujube (Kim *et al*., 2011) showed higher antioxidant effect than those of Vitamin C and Vitamin E, even though their IC_{50} values of DPPH radical scavenging activity were showed to be around 800 μg/mL and 500 μg/mL, respectively. In comparison with these plants, *C. pinnatifida* BUNGE Major. fruit (IC_{50} value, 48.5 μg/mL) showed potential antioxidant activity.

ABTS radical scavenging activity – The ABTS method has the extra flexibility in that it can be used at different pH levels (unlike DPPH, which is sensitive to acidic pH) and thus is useful when studying the effect of pH on antioxidant activity of various compounds (Lemanska *et al*., 2001). It is useful for measuring antioxidant activity of samples extracted in acidic solvents. On the other hand, Perez-Jimenez and Saura-Calixto (Pacher *et al*., 1996) reported significant lower ABTS values in case of acidic condition. In the present study, as shown in Fig. 2, the methanol extract of *C. pinnatifida* BUNGE Major. fruits was measured concentrations at 500, 100, 50, 25, and 6.25 μg/mL. As shown in Fig. 2, ABTS radical scavenging activity of the methanol extract of *C. pinnatifida* BUNGE Major. fruits increased depending on the sample concentration, as they did in DPPH radical scavenging analysis. In comparison with trolox (IC_{50} value; 97.6 μg/mL) *C. pinnatifida* BUNGE Major. fruit was shown to have a reliable IC_{50} value, 46.5 μg/mL. According to recent reports, glasswort seed extract (Kang *et al*., 2011) and dried jujube showed higher antioxidant effect than those of Vitamin C and Vitamin E (Kim *et al*., 2011), even though their IC_{50} values of ABTS radical scavenging activity were showed around 1800 μg/mL and 100 - 1000 μg/mL, respectively. In comparison with these plants, *C. pinnatifida* BUNGE Major. fruit (IC_{50} value; 46.5 μg/mL) extracts demonstrated potent ABTS radical scavenging activity.

Nitric oxide scavenging activity – Despite the possible beneficial effects of $NO^{\cdot}$, its contribution to oxidative damage is increasingly becoming evident. This is due to the fact that $NO^{\cdot}$ can react with superoxide to form the peroxynitrite anion, which is a potentialy strong oxidant that can decompose to produce ·OH and NO_2 (Beckman *et al*., 1996, Pacher *et al*., 1996). $NO^{\cdot}$ released from SNP has a strong NO^{+} character which can alter the structure and function of many cellular components. *C. pinnatifida* BUNGE Major. fruits extract showed antioxidant potential to scavenge Nitric oxide radicals (Fig. 3.). Methanol extract of *C. pinnatifida* BUNGE Major. fruits was measured at concentrations of 1, 0.5, 0.25, and 0.125 mg/mL. In comparison with ascorbic acid, (IC_{50} value; 0.625 μg/mL) *C. pinnatifida* BUNGE Major. fruit did not show

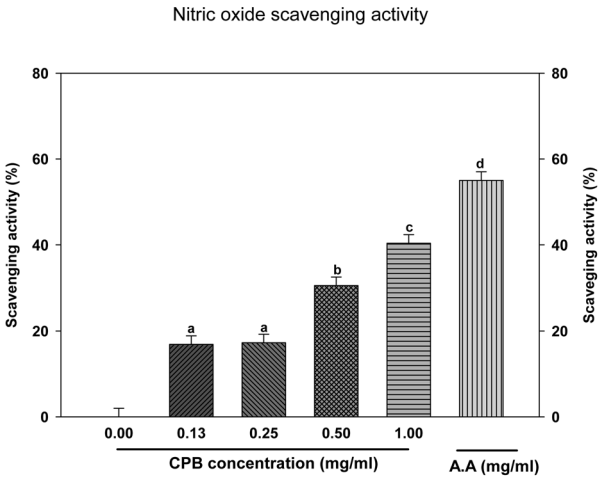

Fig. 3. Nitric oxide scavenging activities of the fruits from *Crataegus pinnatifida* Bge. Data represent the means ± S.D. from triplication (CPB: *Crataegus pinnatifida* Bge., A.A: Ascorbic acid). Different subscript letters indicate a significant difference at the $p < 0.05$ by Duncan's multiple range tests (ascorbic acid IC_{50} concentration: 62.5 μg/mL).

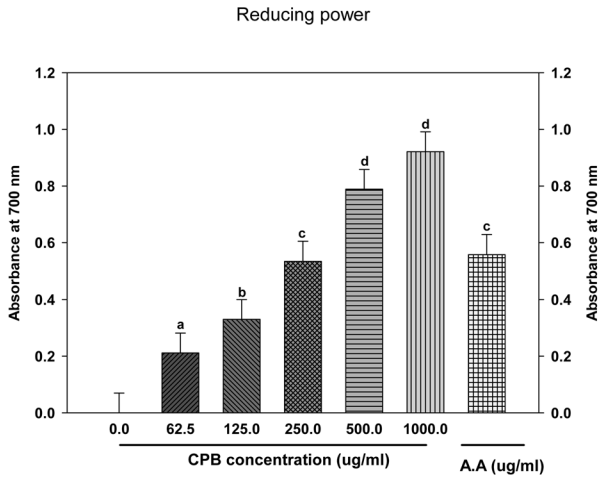

Fig. 4. Reducing power of the fruits from *Crataegus pinnatifida* Bge. Data represent the means ± S.D. from triplication (CPB: *Crataegus pinnatifida* Bge., A.A: ascorbic acid). Different subscript letters indicate a significant difference at the $p < 0.05$ by Duncan's multiple range tests (ascorbic acid concentration: 62.5 μg/mL).

strong scavenging activity, but as shown in Fig. 3, nitric oxide radical scavenging activity of the methanol extract of *C. pinnatifida* BUNGE Major. fruit increased depending on the sample concentration, as they did in DPPH and ABTS radical scavenging analysis.

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 (Ordonez *et al*., 2006), and the reducing activity is generally associated with the presence of reductions (Duh *et al*., 1998) which have been shown to exert an antioxidant effect by donating a hydrogen atom and thereby breaking the free radical chain. The reducing power of *C. pinnatifida* BUNGE Major. fruits extracts showed a dose-dependent response (Fig. 4). Compared to the positive control (ascorbic acid: 62.5 μg/mL), *C. pinnatifida* BUNGE Major. fruits extract (1000, 500, 250, 125, and 62.5 μg/mL) showed high reducing power. The reducing capability of a compound may serve as a significant indicator of its potential antioxidant activity (Meri *et al*., 1995), thus the significant antioxidant activity of *C. pinnatifida* BUNGE Major. appears to be at least partially related to its reducing power activity. Prasad *et al.* (Prasad *et al*., 2009) reported that reducing power depends on the presence of hydroxyl groups in the phenolic compounds, which act as electron donors, According to Lee and Goh (Lee *et al*., 2001), the reducing powder of red wine containing 1,667 - 2,537 mg/L of total polyphenolic compounds and that of white wine containing 247 - 339 mg/L of total polyphenolic compounds are in the range from 3.1 - 3.4 and 1.5 - 1.7, respectively. Overall reducing power trend was similar to those of DPPH and ABTS radical scavenging activities. The abundance of TPC might play an important role in the high reducing power of *C. pinnatifida* BUNGE Major. fruit extract.

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