Antioxidant Potential and Chlorogenic Acid Level of Lettuce (*Lactuca sativa* L.) Cultivars

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ABSTRACT

Lettuce (*Lactuca sativa* L.) is known to contain water-soluble substances that improve antioxidant status due to the richness in antioxidants. Greenhouse experiment was carried out under different shading conditions during spring lettuce growing season. Shade significantly reduced shoot weight, number of leaves and chlorophyll content, while it increased shoot length of lettuce plants. The antioxidant potential of the individual fraction was in order of n-butanol > ethyl acetate > water > n-hexane fraction, although was less than that of commonly used antioxidants, BHT and ascorbic acid. Fractions from lettuce plants dose-dependently increased DPPH free radical scavenging activity, *in vitro* test. By means of HPLC analysis, BuOH fraction of cultivar "Hwahyang" (57.93 mg 100 g^{-1}) had the highest amount of antioxidant chlorogenic acid. Shading treatment increased average amount of chlorogenic acid of all cultivars in BuoH, EtOAc, hexane and water fractions by 33, 120, 144, and 58%, respectively. These results suggest that lettuce plants had potent antioxidant activity, and their activities were differently exhibited depending on cultivar and fraction.

Key words : antioxidant activity, chlorogenic acid, extracts, fractionation, Lettuce, shading

INTRODUCTION

Antioxidants, inhibitors of lipid peroxidation, are important not only for food protection but also for the defense of living cells against oxidative damage. The toxic and otherwise unfavorable effects of synthesized food antioxidants have been widely noted. Phenolic compounds, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ), have been widely used as synthetic antioxidants in food lipid. Although those antioxidants are considered as safe natural antioxidants, they do not always provide effective protection against in vitro oxidation (Frankle, 1980). Nevertheless, the phenolic antioxidants are still used extensively as food antioxidants because of their excellent results and low cost. When slightly larger doses (50mg/kg/day) of these phenolic antioxidants are administered to rodents and monkeys, however, certain pathological, enzyme and lipid alterations as well as carcinogenic effects have been observed (Branen, 1975). Therefore, research on other natural antioxidants has gained momentum as they are considered, rightly or wrongly, to pose no health risk to consumers (Wanasundara and Shahidi, 1994;

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Wanasundara *et al.*, 1997). The development of alternative natural antioxidants has, therefore assumed as increased importance. Many investigators have found different types of antioxidants in various sources of plants (Larson, 1988)

Lettuce is an annual herbaceous plant of Compositae, one of the largest and most diverse families of flowering plants. Compositae plant in Korea is known to be an increasingly important medicinal resources and new functional agent, mainly due to antioxidant activity (Lee *et al.*, 1997). Few studies on antioxidant effects of lettuce plants have been reported. Lettuce consumption increases the total cholesterol end-products excretion and improves antioxidant status due to the richness in antioxidants (vitamin C, E and carotenoids) (Nicolle *et al.*, 2004). Lettuce contains appreciable amounts of water-soluble antioxidants such as vitamin C and various phenolic compounds (phenolic acids, anthocyanins), as well as lipid-soluble antioxidants such as lutein or tocopherols (Souri *et al.*, 2004; Rice-Evans *et al.*, 1996; Szeto *et al.*, 2002). Naturally-occurring antioxidative components in foods or plants include flavonoids, phenolic acids, lignan precursors, terpenes, mixed tocopherols, phospholipids, polyfunctional organic acids and also plant extracts such as those of rosemary and sage (Schuler, 1990; Wanasundara *et al.*, 1997). Chlorogenic acid, a naturally-occurring polyphenol compound, is reported as a clastogenic agent in hamster cells (Stich *et al.*, 1981) and to participate in enzymatic browing treactions in potatoes, sunflower seed, leaf protein concentrates, milk proteins, and other foods (Deshpande *et al.*, 1984)

Some crop and plant species have been reported to change their biological potential depending on ionizing radiation (Alsaadawi *et al.*, 1985; Balakumar *et al.*, 1993). Generally, stressed donor plants increased biological activity due to increased production of the chemicals (Niemeyer, 1988). Thus, it was concluded that stress-induced elevation of biological activity was a strategy for defense of plants to environmental stress (Bell, 1981; Rice, 1984).

Probable major biosynthetic pathways leading to production of natural antioxidants have been known to be shikimic acid or acetate pathway (Rice, 1984). In this paper, we now report isolation of the causative components from the methanolic extract of lettuce and describe their antioxidant effects on DPPH radical. The objective of this research was to determine their antioxidant activities of the dried samples or extracts. This research will promote a better understanding of natural chemical production in the natural- and agro-ecosystems through investigating antioxidant activity.

MATERIALS AND METHODS

Plant material and growth conditions

Lettuce cultivars, "Ddukseom", "Jeokchima", and "Hwahyang" were grown in plastic pots (20 cm dia. x

Table 1. Light intensity of shading treatment in greenhouse during 15-day experiment

Shading treatment	No. of shading net	Light intensity (μmol $m^{-2}s^{-1}$)*
0 % shade (control)**	0 layer	706.5
70 % shade	2 layers	211.9

* Light intensity in shading net was measured at 2-hour interval from 8:00 AM to 4:00 PM in a day and averaged for all measurements.

** Light intensity of control in greenhouse was measured without shading net in April, 2003. At the same time, light intensity outside of greenhouse was 785 μmol m-2s-1.

15 cm high) filled with horticultural soil (Hanter 21, Seoul, Korea) in greenhouse for 40 days at Dongshin University in 2002. Shading nets were used for shading treatment. No shading (0% shade) and 2 folds (70% shade) of shading nets, corresponding to 490 and 147 μ mol photons m^{-2} s^{-1} photosynthetically active radiation (PAR), respectively, were mulched over the lettuce plants (Table 1).

Growth parameters and chlorophyll concentration

The plants were harvested at a vegetative stage of development 15 days after shading treatment. Plant height, shoot fresh weight, and number of leaves of each cultivar were measured in all plants after shading treatment. Chlorophyll content was measured spectrophotometrically by the method of Lichtenthaler (Lichtenthaler, 1987).

Fractionation of methanol extracts

Ground leaf samples from 3 lettuce cultivars were extracted with 95% methanol at room temperature. The extract was then filtered through a Whatman No. 1 filter paper. The collected filtrate was evaporated to dryness under vacuum at 40℃ using a rotary evaporator (N-1000V-W, Eyela, Japan). The yield of dried extracts from the original leaves was about 10%. For fractionation, crude methanol extracts were diluted with distilled water and n-hexane to collect hexane fraction using a separating funnel. After hexane collection, the distilled water fractions were added with ethylacetate (EtOAc) to obtain EtOAc fraction in the same way. The same procedure was used in preparing butanol (BuOH) and water fractions. The fractions were taken to dryness on a rotary evaporator at 40-50℃, and transferred into vacuum freeze dryer to obtain dry matters.

Antioxidant effects of the fractions from methanol extracts

DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging assay was carried out according to the procedure described by Blosi (1958). Fractions of each cultivar at various concentrations (0.10, 100, 250, 500 and 1000 $\mu g/m\ell$) were added to a 1.5×10^{-4} M solution of DPPH in methanol and the reaction mixture was shaken vigorously. The amount of DPPH remaining was determined at 520 nm, and the radical scavenging activity was obtained from the following equation: Radical scavenging activity (%) = $\{(OD_{control} - OD_{sample}) / OD_{control}\} \times 100$. The antioxidant activity of plants extracts was expressed as IC50, which was defined as the concentration (in μg ml^{-1}) of extract required to inhibit the formation of DPPH radicals by 50 %. When the F-test was significant ($P<0.05$) means were separated on the basis of least significant difference (LSD) (SAS Institute, 2000).

Quantification of chlorogenic acid

The standard phenol compound used for HPLC analysis was chlorogenic acid (Aldrich Co., CA, USA). The chemical was purchased as a high purity standard and the used solvents were HPLC spectral grade. All solvents and distilled water were degassed before use. All solvent ratios were based on volume. Chlorogenic acid was identified by a HPLC system (SPP 10AVP, Shimadzu, Japan) with a flow rate of 1 mL min-1, the column was CAPCELL PAK C18 SG120 (4.6 x 250 mm) and an autoinjector with a 10 ?l sample loop was employed. The mobile phase consisted of water, methanol and acetic acid in the ratio of 12:15:1 volume, respectively. The UV detector wavelength was set at 275 nm. Standard compound was chromatographed. Retention time for the standard compound and the major peaks in the extract were recorded. Chlorogenic acid from each fraction was identified by retention times or standard addition, and its amount was calculated by comparing peak area with that of standard (Banwart *et al.*, 1985).

RESULTS AND DISCUSSION

Growth parameters and chlorophyll concentration

The effect of shading on growth was studied for 3 lettuce cultivars in greenhouse giving 0 and 70% shade in the short term study. Plant height of lettuce plants under shading condition significantly increased by 21 ? 33% as compared to the control, while shoot fresh weight and number of leaves extremely decreased by 36 -44% and 4-21%, respectively. Especially, "Hwayang" was the most sensitive cultivar to shading treatment (Table 2). Shade treatments reduced chlorophyll content significantly in all cultivars ($p<0.05$). However, no significant difference in chlorophyll content among cultivars was observed (Fig. 1). Some crop and plant species have been reported to change their biological potential depending on ionizing radiation (Alsaadawi *et al.*, 1985; Balakumar *et al.*, 1993). Generally, stressed donor plants increased biological activity due to increased production of the chemicals (Niemeyer, 1988).

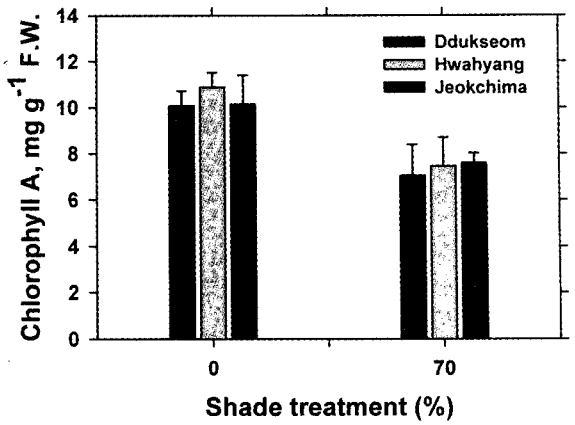

Fig. 1. Shade effects on chlorophyll a content of three lettuce cultivars. Each bar represents standard error of the mean.

Antioxidant effects of the fractions from methanol extracts

All samples of fractions showed DPPH radical scavenging activity in a dose-dependent manner. After fractionation, BuOH fraction showed the highest DPPH radical scavenging activity, with an IC50 value of 668 ㎍㎖, and followed by EtOAc fraction (IC50 = 706 ㎍

Table 2. Plant height, shoot fresh weight, and number of leaves in three lettuce cultivars under shading condition.

Shade treatment	'Ddukseom'	'Hwahyang'	'Jeokchima'	Mean
Plant height, cm				
0 %	16.3 (100)	16.8 (100)	18.3 (100)	17.1 (100)
70 %	19.8 (121)	20.6 (123)	24.0 (133)	21.5 (125)
Shoot F.W., g/plant				
0 %	5.4 (100)	6.7 (100)	7.2 (100)	6.4 (100)
70 %	3.4 (64)	4.0 (60)	4.0 (56)	3.8 (59)
Number of leaves				
0 %	3.2 (100)	3.7 (100)	4.5 (100)	3.8 (100)
70 %	3.0 (96)	2.9 (79)	3.6 (81)	3.2 (83)

Values in parentheses represent % of no shading (control).

mℓ). These values were much lower than those of BHT or ascorbic acid, with IC50 values of 25 and 92μgmℓ$^{-1}$, respectively. However, water and hexane fractions had lower DPPH radical scavenging activity (Fig. 2). The results show that causative antioxidant components were present as the highest amount in the BuOH fraction and followed by EtOAc, water, and hexane fractions, resulting in inhibitory effects on DPPH radicals. These results also indicate that various compounds that cause antioxidant activity could be produced with different amount from the fractions.

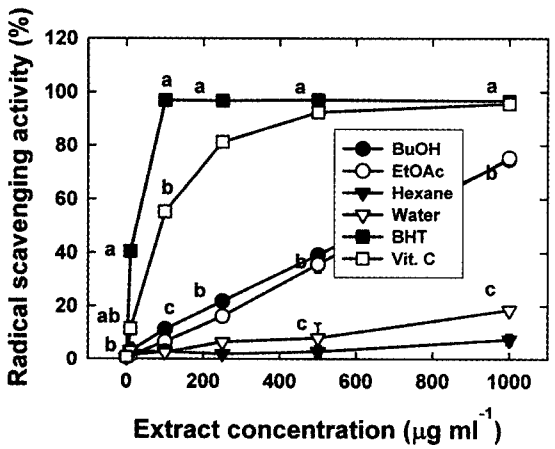

Fig. 2. Radical scavenging effect of the fractions from cultivar "Hwahyang" leaf methanol extracts on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. Within an extract concentration, means followed by the same letter are not significantly different at $p<0.05$. Each bar represents standard error of the mean.

Quantification of chlorogenic acid

Chlorogenic acid present in the fractions of methanol extracts from 3 lettuce cultivars were analyzed by HPLC using standard. In control, chlorogenic acid was detected in the BuOH fraction as the highest amount and followed by water and EtOAc fractions (Table 3). However, level of chlorogenic acid showed the lowest amount in the hexane fraction. Also chlorogenic acid in cultivar "Hwayang" was detected as the greatest amount (57.93 mg 100 g^{-1}) and in cultivar "Ddukseom" the lowest (15.15 mg 100 g^{-1}). Shading treatment increased average amount of chlorogenic acid of all cultivars in BuOH, EtOAc, hexane and water fractions by 33, 120, 144, and 58%, respectively. The results show that findings of quantification by fraction through HPLC were considerably associated with the antioxidant activity. Radical scavenging effect of phenolic compounds isolated from natural sources has been widely studied (Yioshida *et al.*, 1989). The antioxidative potency and phenolic acids are generally inter-related. These phenolic compounds react with the free radicals formed during autoxidation, and generate a new radical which is stabilized by the resonance effect of the aromatic nucleus (Cuvelier *et al.*, 1992).

In conclusion, the present assessment demonstrated that the lettuce plants had potent antioxidant activity and high amount of antioxidant chlorogenic acid,

Table 3. Shading effect on quantitative determination of chlorogenic acid present in leaves of three lettuce cultivars through HPLC analysis.

Cultivar	BuOH		EtOAc		Hexane		Water	
	0%	70%	0%	70%	0%	70%	0%	70%
				mg 100 g -1				
Ddukseom	15.15	30.67	0.50	1.87	0.09	0.54	2.64	8.85
Hwahyang	57.93	63.73	3.19	4.39	0.04	0.21	10.96	17.55
Jeokchima	29.78	42.51	0.25	2.40	0.36	0.43	8.87	9.05
Mean	34.29	45.64	1.31	2.89	0.16	0.39	7.49	11.84

showing inhibitory effects on DPPH radicals. Different natural compounds that cause antioxidant activity could be produced with different types and amounts depending on fractionation method or cultivar that produces the antioxidant chemicals. Such differences also might be related to specific allelopathic compounds being produced in larger quantities in certain fractions, imparting a higher level of allelopathy. The present results suggest that the antioxidant activity of lettuce may be a valuable alternative mean based on natural plant component.

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