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Phytotoxic Effect, DPPH Radical Scavenging Activity and Chlorogenic Acid Level of Methanol Extracts from Aerial Parts of Several Korean Salad Plants

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Abstract - A series of aqueous or methanol extracts from four different Korean salad plants were assayed to determine their allelopathy and antioxidant activity. The extracts applied on filter paper in a Petri-dish bioassay significantly inhibited root growth of against alfalfa (*Medicago sativa*) seedlings. Leaf extracts from 40 g dry tissue L^{-1} of *Aster yomena* was most phytotoxic to alfalfa root growth, and followed by that of *Cirsium japonicum*, *Taraxacum officinale*, and *Ixeris dentate*. Methanol extracts of plants dose-dependently increased DPPH free radical scavenging activity *in vitro*. Antioxidant activity of methanol extracts from the same plant species was investigated, and the result showed high DPPH free radical scavenging activity in *Cirsium japonicum*, *Aster yomena*, and *Ixeris dentate*, however, in *Taraxacum officinale* was least activity. By means of HPLC analysis, chlorogenic acid, *p*-coumaric acid, and total phenolics with 7.68, 17.47 and 18.64 mg 100 g^{-1}, respectively, showed the highest amounts in methanol extracts from *Cirsium japonicum* leaves. These results suggest that Compositae salad plants contain water-soluble substances with allelopathic potential as well as antioxidant activity.

Key words - Allelopathy, Antioxidant activity, DPPH radical scavenging activity, Plant parts, Extracts, Natural antioxidant

Introduction

Recently, there has been a worldwide trend towards the use of the phytochemicals from wild plants. Some plants have biologically-active substances that cause serious yield losses in spring sown-small grains row crops, and pastures (Hodgson, 1968), however, others such as Korean salad plants are being used as promising phytochemicals that are antioxidant to foods. Phenolic compounds are considered as secondary metabolites that are synthesized by plants during normal development and in response to stress conditions such as infection, wounding, and UV radiation. These compounds occur ubiquitously in plants and are very diversified group of phytochemicals derived from phenylalanine and tyrosine (Harborne and Turner, 1984; Shahidi and Maczk, 2004).

Allelopathy is the chemical interaction between including stimulatory as well as inhibitory influences (Molisch, 1937). Allelopathy plays an important role in both natural and agro-ecosystems, especially eco-friendly weed management. Most of scientific studies have been concentrated to exploit the positive significant roles, this phenomenon, if suitably managed, can play in enhancing crop productivity and use naturally occurring substances as natural replacements for synthetic pesticides or antioxidant from a number of plant resources. Growth and germination of wheat (*Triticum aestivum* L.) and flax (*Linum usitatissimum* L.) were inhibited by aqueous extracts of Canada thistle (*Circium arvense*) roots and shoots (Helgeson and Konzak, 1950). Stachon and Zimdal (1980) in their greenhouse experiments found that Canada thistle litter reduced the growth of redroot pigweed (*Amaranthus retroflexus* L.) and green foxtail (*Setaria viridis* L.) more than that of cucumber (*Cucumis sativus* L.) or barley (*Hordeum vulgare* L.). Chon *et al.* (2003) reported that the aqueous leaf extracts of 16 Compositae plant species, including common thistle, significantly inhibited hypocotyls and root lengths of alfalfa. In their earlier study, trans-cinnamic acid was found as the greatest amount at ethyl acetate fraction from methanol extracts of common

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thistle plant.

It is generally accepted that water extracts of top growth (especially leaves) produce more allelopathy for seedlings than those from roots and crowns of alfalfa (*Medicago sativa* L.) (Miller, 1996), and that shoot extract from the reproductive stage was more inhibitory than from the vegetative stage under laboratory conditions (Chung and Miller, 1995; Hedge and Miller, 1992). Chung and Miller (1995) ranked autotoxic effects of water extracts of plant parts of alfalfa as leaf (greatest), seed, root, flower, and stem (least). Chou and Leu (1992) reported that extracts from flowers of *Delonix regia* (BOJ) RAF exhibited highest inhibition against three test plants, alfalfa, lettuce (*Lactuca sativa*), and Chinese cabbage (*Brassica chinensis*). However, not many studies are conducted on antiosicdant activitity as affected by different plant parts.

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; 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).

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)

Probable major biosynthetic pathways leading to production of natural antioxidants have been known to be shikimic acid or acetate pathway (Rice, 1984). The objective of this research was to determine allelopathic effects of extracts or residues from leaves, stems, roots, and flowers of common thistle. This research will promote a better understanding of the mechanisms of allelopathy in agroecosystem, and contribute to development of eco-friendly alternative weed control mean. In this paper, we now report isolation of the causative components from the methanolic extract of common thistle 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 through Rancimat, TBARS, and DPPH methods. This research will make attractive the research for antioxidant and scavenger natural compound.

Materials and Methods

Preparation of aqueous and methanolic extracts

Four salad plants, *Aster yomena*, *Cirsium japonicum*, *Ixeris dentate*, and *Taraxacum officinale*, grown in a mountain of the Suncheon area, Korea were harvested at a vegetative stage in June 2005. The four leaf samples were directly freeze-dried at -40 (C for 5 days, ground with a Wiley mill to pass a 1-mm screen, and stored in a refrigerator at 2 (C until used. Forty grams of dried samples were separately extracted by soaking in 1L distilled water at 24 (C for 24 hours in a shaker to give a concentration of 40 g dry tissue L^{-1} (hereafter referred to as '$g\ L^{-1}$'). The extract was filtered through two layers of cheesecloth to remove the fiber debris, and centrifuged at 5000 rpm (x 4530 *g*) for 2 hours. The supernatant was vacuum filtered again through Whatman No. 42 paper. The aqueous extracts were used for alllelopathy bioassay.

To prepare methanol extracts the ground samples 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 °C using a rotary evaporator (N-1000V-W, Eyela, Japan). After evaporation, the yield of dried extracts (methanol extract) was about 10% of the original plant sample. The methanol extracts from each plant part were used for measuring DPPH radical scavenging activity.

Phytotoxic effects of aqueous plant extracts

Each stock extract was diluted appropriately with sterile distilled water to give the final concentrations of 10, 20, 30, and 40 $g\ L^{-1}$. EC, pH, and osmotic potential (Boyer and Knipling, 1965) for each ex-

tracts at 40 g L^{-1} were measured on stock extracts 2 days after extraction. Seeds were rinsed four times with deionized water, imbibed in deionized water at 22 °C for 12 h, and carefully blotted using a folded paper towel. Twenty swelled seeds were evenly placed on filter paper wetted with the extract in each Petri dish. The Petri dishes were covered, sealed by wrapping in parafilm, and placed flat in a growth chamber held at 24 °C during the14-h light period and 22 °C during the 10-h dark period. Plates were illuminated at 400 (mol photons m^{-2} s^{-1} photosynthetically active radiation (PAR) provided by a mixture of incandescent and fluorescent lamps. Root and hypocotyls lengths were measured on all seedlings in each Petri dish 5 days after placing seeds on the filter paper. The experiment was conducted with four replications.

DPPH radical scavenging activity of 4 salad plants

DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay was carried out according to the procedure described by Blosi (1958). Methanol extracts from each plant part, and four fractions from leaf extracts at various concentrations (0.10, 100, 250, 500 and 1000 μg ml^{-1}) were added to a 1.5 x 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 (%) = $\{(OD_{control} - OD_{sample}) / OD_{control}\}$ x 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, *p*-coumaric acid and total phenolics

The standard phenol compounds used for HPLC analysis were chlorogenic and p-coumaric acids, which are well documented to be antioxidant to food (Deshpande et al., 1984; Naczk and Shahidi, 2004). The standard chemicals were purchased as a high purity standard and the used solvents were HPLC spectral grade (Aldrich Co., CA, USA). All solvents and distilled water were degassed before use. All solvent ratios were based on volume. Chlorogenic and *p*-coumaric acids were 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 and *p*-coumaric acids from each fraction were identified by retention times or standard addition, and their amounts were calculated by comparing peak area with that of standard (Banwart et al., 1985).

The concentration of total phenolics (TP) was measured using the Folin-Ciocalteu assay (Singleton and Rossi, 1965). Briefly, 5 mL of Nanopure water, 05-1.0 mL of sample, and 1.0 mL of Folin-Ciocalteu reagent were added to a 25 mL volumetric flask. The contents were mixed and allowed to stand for 5-3 min at room temperature. Next, 10 mL of a 7% sodium carbonate solution was added, followed by the addition of Nanopure water filled to volume. Solutions were mixed and allowed to stand at room temperature for 2 h. Sample aliquots were filtered through a Whatman 0.45 m poly (tetrafluoroethylene) filter prior to the determination of TP concentration using a Beckman DU 7400 spectrophotometer monitoring 750 nm. TP content was standardized against ferulic acid and expressed as ppm of ferulic acid equivalents (FAE). The linearity range for this assay was determined as 0.5-5.0 mg/L FAE (R^2 = 0.9990), giving an absorbance range of 0.050-0.555 AU.

Results and Discussion

Phytotoxic effects of aqueous plant extracts

Electrical conductivity (EC), pH, and osmotic for leaf extracts at 40 g L^{-1} were ranged from 0.14 to 0.27 S/m, from 5.23 to 7.84, and from -0.003 to -0.110 MPa, respectively (Table 1). It was thought that EC, pH, and osmotic potential of thistle extracts did not affect seedling growth of test plants, indicating allelopathic effects of the plant extracts could go beyond the osmotic effects. Our experience in other study demonstrated that no significant growth reduction was observed at all concentrations of PEG 8000, corresponding to same osmotic potential of alfalfa leaf extracts. Osmotic stress less than –0.2

Table 1. Change in electric conductivity (EC), pH, and osmotic potential of aqueous leaf extracts at 40 g L^{-1} for 4 different salad plant species

Plant species	EC (dS/m)	pH	Osmotic potential (-MPa)
Aster yomena	0.149	5.23	0.004
Cirsium japonicum	0.268	5.80	0.110
Ixeris dentate	0.174	7.84	0.020
Taraxacum officinale	0.137	5.30	0.003

MPa of PEG 8000 is known to have little effect on root growth at concentrations of extract normally used. Reduction of root length can be explained mainly by allelopathic effect from extracts, not by osmotic effect (Chon et *al.*, 2004). Although it is often assumed that the response of seeds or seedlings to plant extracts is due entirely to allelopathy, extract may also exert negative osmotic effects on the test species (Bell, 1974), and some investigators have qualitatively assessed the relative importance of the osmotic influence and allellopathic potential of plant extracts on seed germination (Wardle *et al.*, 1992). They concluded in their study using aqueous leaf extracts of four pasture grass species that bioassays are more realistic when they are compared to control values that are adjusted to the same osmotic potential as the plant extract being tested.

Aqueous leaf extracts showed more inhibitory effect on root growth of test plant than shoot growth. The degree of inhibition was increased with increasing the extract concentration. At higher extract concentrations above 30 g L^{-1}, leaf extracts from *Aster yomena* and *Cirsium japonicum* reduced root length completely, while the extracts from *Ixeris dentate* and *Taraxacum officinale* reduced root length of alfalfa each around 20% (Fig. 1). Such differences might be related to specific allelopathic compounds being produced in larger quantities in a plant species, imparting a higher level of allelochemicals. Release of phytotoxic compounds could also be affected by plant species.

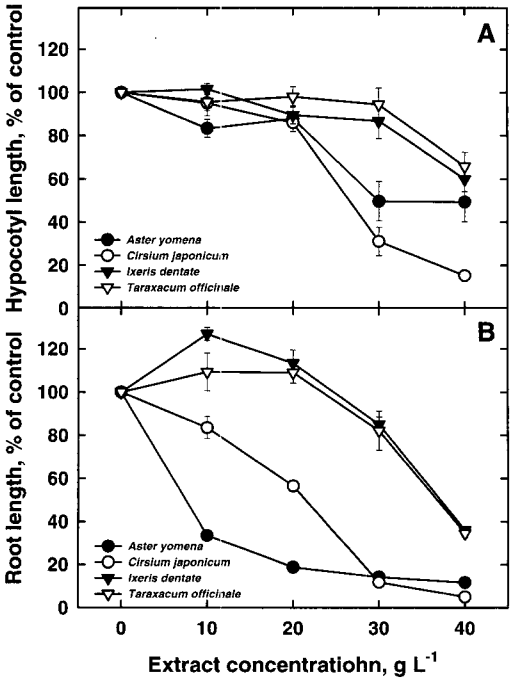

Fig. 1. Effects of aqueous extracts from Compositae Korean salad plant species on shoot and root lengths (% of control) of alfalfa 6 days after seeding. Each experiment was performed at least three times and data are expressed as average percent changes versus the control ± S.D. Within an extract concentration, means followed by the same letter are not significantly different at $p<0.05$. Each bar represents standard error of the mean.

DPPH radical scavenging activity of 4 salad plants

Methanol extracts of *Ixeris dentate* leaves had the highest DPPH radical scavenging activity, with an IC_{50} value of 148μg ml^{-1}, and followed by *Cirsium japonicum* and *Aster yomena*, with IC_{50} value of each 201 μg ml^{-1}, respectively (Fig. 2). However, IC_{50} value of *Taraxacum officinale* extracts was the least (1,879μg ml^{-1}). All samples of plant species showed DPPH radical scavenging activity in a dose-dependent manner. The results show that various compounds that cause antioxidant activity could be produced with different amount from plant species. Radical scavenging activity of phenolic compounds isolated from natural plants has been widely studied (Yoshida *et al.*, 1989), and the antioxidative potency and phenolic acids are generally known to be interrelated.

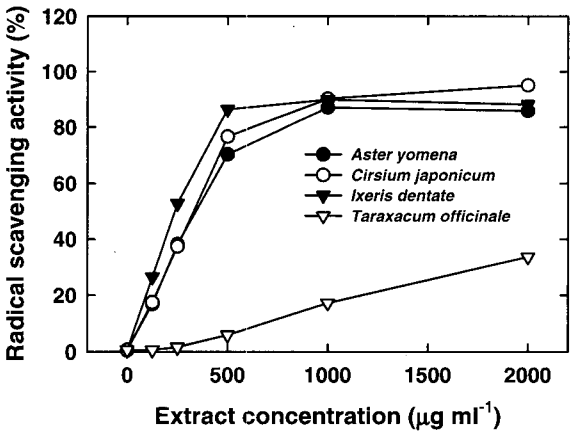

Fig. 2. DPPH radical-scavenging activity of methanol extracts from leaves of Compositae Korean traditional salad plants as affected by different extract concentrations. Each experiment was performed at least three times and data are expressed as average percent changes versus the control ± S.D. Within 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, *p*-coumaric acid and total phenolics

Chlorogenic acid and *p*-coumaric acid present in the fractions of methanol leaf extracts of 4 plant species were analyzed by HPLC using standard. Chlorogenic acid was more abundant in *Cirsium japonicum* and *Aster yomena* than in *Ixeris dentate* and *Taraxacum officinale*. And *p*-coumaric acid was detected as the highest amount in *Cirsium japonicum* (17.47 mg 100 g^{-1}) and followed by in *Ixeris dentate* (2.89 mg 100 g^{-1}) (Table 2). However, level of *p*-coumaric acid in

Table 2. Quantitative determination of some phenolic acids and total phenolic compounds (mg 100 g^{-1}) present methanol extracts from leaves of 4 Korean salad plants

Compound	HPLC analysis		
	Chlorogenic acid	*p*-coumaric acid	Total phenol compound
Aster yomena	1.61	0.00	11.95
Cirsium japonicum	7.68	17.47	18.64
Ixeris dentate	0.35	2.89	16.41
Taraxacum officinale	0.12	0.00	5.20

Aster yomena and *Taraxacum officinale* showed no detection. The results show that findings of quantification through HPLC were associated with the antioxidant activity. The antioxidative potency and phenolic acids are generally interrelated (Yoshida *et al.*, 1989). These phenolic compounds react with the free radicals formed during autooxidation, and generate a new radical which is stabilized by the resonance effect of the aromatic nucleus (Cuvelier *et al.*, 1992). On the other hand, total phenolics (TP) content of *Aster yomena*, *Cirsium japonicum*, *Ixeris dentate*, and *Taraxacum officinale* were 11.95, 18.64, 16.41 and 5.20 ppm, respectively, showing the highest amount in *Cirsium japonicum*. This result is highly consistent with the contents of *p*-coumaric acid and chlorogenic acid (Table 2). Chou and Leu (1992) concluded that the findings of bioassay on phytotoxic effect, and the quantitative and qualitative traits of responsible allelopathic compounds found in *Delonix regia* are well correlated.

In conclusion, the four Korean salad plants showed a potent allelopathic effect and antioxidant activity through Petri-dish bioassay and measurement of DPPH free radical scavenging activity. Extracts of 4 salad plants dose-dependently increased allelopathic potential and DPPH free radical scavenging activity, in vitro. The compounds that cause the activities could be produced with different amounts depending on plant species. Such differences might be related to compounds being produced in larger quantities in certain plant species. The results suggest that the extracts of 4 salad plants had allelopathic potential and antioxidant activity with important values for an alternative natural antioxidant or plant growth regulator based on natural plant extracts.

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