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Phytotoxicity and DPPH Radical Scavenging Activity of Barley Seedling Extracts

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ABSTRACT : A series of methanol extracts from leaf and root parts in spring- and winter-barley plants were assayed to determine their allelopathy and antioxidant activity. The methanol extracts applied on filter paper in a Petri-dish significantly inhibited root growth of Chinese milkvetch (*Astragalus sinicus* L.) seedlings. Leaf extracts at 25 and 50 g L^{-1} inhibited root growth of Chinese milkvetch seedlings more than root extracts. No difference in phytotoxic effects of spring- and winter-barley seedlings extracts on root growth of Chinese milkvetch was observed. Methanol extracts dose-dependently increased DPPH free radical scavenging activity *in vitro*. DPPH free radical scavenging activity was higher in the methanol extracts from winter-barley seedlings than in those from spring-barley seedlings, and from leaf extracts than from root extracts. The antioxidant potential of the individual fraction from the methanol extracts of spring-barley seedlings was in order of *n*-butanol > ethyl acetate > water > chloroform > *n*-hexane fraction. By means of HPLC analysis, spring-barley (200.62 mg 100 g^{-1}) had more amount of total phenol acid than winter-barley (114.08 mg 100 g^{-1}). Especially, ferulic acid was detected in spring-barley extract (183.46 mg 100 g^{-1}) as the greatest amount. These results suggest that early seedlings of barley plants had potent allelopathy and antioxidant activity, and their activities were differently exhibited depending on plant parts and growing condition.

Keywords: barley extracts, phenolic compounds, allelopathy, antioxidant activity, bioassay

Allelopathy was defined by Molisch (1937) as a chemical interaction between plants or sometimes between microbes and higher plants that includes stimulatory as well as inhibitory influences. Later it was defined as any direct or indirect, harmful or beneficial effect of one plant as a donor plant on another as a recipient plant through the production of chemical compounds that escape into the environment (Rice, 1984). Allelopathy can play significant roles under both natural and manipulated ecosystems (Rice, 1984), such as weed control, crop protection, and crop re-establishment, mainly by adversely affecting seed germination and seedling growth. Suitable manipulation of allelopathy towards improvement of crop productivity and environmental protection through eco-friendly control of weeds, pests, crop diseases, conservation of nitrogen in crop land, and synthesis of novel agrochemicals based on natural products have gained prominent attention of scientists engaged in allelopathic research. Alternatives to synthetic chemical herbicides need to be developed, especially for organic or eco-friendly farming operations, landscape management systems, home gardens, and for situations where public policies mandate reduced pesticide use.

Most studies on allelopathy have focused on interference and allelopathic effects of several important weeds on crop yields. Plant seedlings of various crops possess allelopathic potential or weed-suppressing activity, including cucumber (*Cucumber sativs* L.) (Putnam and Duke, 1974), oat (*Avena* spp.) (Fay and Duke, 1977) and rice (*Oryza sativa* L.) (Dilday *et al.*, 1994; Olofsdotter and Navarez, 1996). A total of 538 accessions of cultivated and wild cucumber were screened in pot and field tests with several accessions causing inhibited growth of *Brassica hirt*a and *Panicum miliaceum* (Putnam and Duke, 1974). Out of more than 3000 accessions of oat, several exuded a large amount of an allelochemical, scopoletin (Fay and Duke, 1977). Also oat suppressed the growth of *Erysimum cheiranthoides* in both laboratory and field tests due at least in part to an allelopathic mechanism (Markova, 1972). The incorporation of an allelopathic character into a crop cultivar could provide the plant with a means of gaining a competitive advantage over certain important weeds (Putnam and Duke, 1974). Wu *et al.* (1999) suggested that genetically improving crops with allelopathic potential and the allelopathy can play an important role in future weed management.

Several researchers have reported that crops such as rice, barley, wheat, and oat release toxic substances into the environment either through root exudation or from decaying plant materials like residues (Borner, 1960; Chon and Kim, 2004). Borner (1960) indicated that cold water extracts of barley, rye, and wheat straws, as well as alcoholic extracts of

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roots, contained phenolic compounds toxic to plant growth. Some of these compounds were ferulic acid (4-hydroxyl-3-methoxy-cinnamic acid), *p*-coumaric acid (4-hydroxycinnamic acid), vanilic acid (4-hydroxy-3-methoxy-benzoic acid), and *p*-hydroxybenzoic acid. Recently, Chon and Kim (2004) reported that extracts from 60-day-old barley, oat, rice and wheat plants significantly reduced root growth of alfalfa, barnyard grass (*Echinochloa crus-galli*, Beauv. var. *oryzicola* Ohwi.), and eclipta (*Eclipta prostrata* (L.) L.), showing highest inhibition in barley extracts. The high-performance liquid chromatography (HPLC) analysis showed that caffeic acid, hydro-cinnamic acid, ferulic acid, *m*-coumaric acid, *p*-coumaric acid and coumarin were present in all the crop plant species, and hydro-cinnamic acid were detected as the highest amount.

Kwak and Kim (1984) studied allelopathic effects of barley residues including straws and roots, and the results showed the high inhibition in germination and shoot growth of rice and paddy weeds. Also, by means of paper chromatography, phenolic compounds identified from residues were *p*-coumaric acid, *p*-hydroxybenzoic acid, ferulic acid, vanillic acid, and salicylic acid, they showed allelopathic effects on test plants. Hoult and Lovett (1993) reported that significant differences in the ability of three lines of barley to produce hordenine and gramine were detected using HPLC techniques. Hordenine (N, N-dimethyltyramine) and gramine (N, N-dimethyllindolemethylamine), alkaloids have been identified and quantified by using HPLC (Kohl *et al.*, 1983; Renaudin, 1984; Liu and Lovett, 1990), both quickly and accurately. Liu and Lovett (1993) reported that effects of alkaloids, hordenine and gramine, released from the root of barley in hydroponic system, on white mustard included reduction of radicle length and apparent reduction in health and vigor of root tips. Overland (1966) showed that allelopathy was involved in the mechanism of weed control by barley used as a smother crop, with the greatest inhibition occurring with chickweed (*Stellaria media*) and less with *Capsella bruspastroris* and *Nicotiana tabaccum*, while no significant effect on wheat was observed.

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 were 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). Lee *et al.* (1994) reported that the antioxidant activity of solvent extracts isolated from barley leaves was investigated peroxide value, the results showed in decreasing order, methanol > ethyl ether > methylene chloride > ethyl acetate > acetone > hexane.

Recently, there has been a worldwide trend towards the use of the phytochemicals from wild plants. Phenolics are ubiquitous compounds found in all plants as their secondary metabolites. 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, was reported as a clastogenic agent in hamster cells (Stich *et al.*, 1981) and to participate in enzymatic browning reactions in potatoes, sunflower seed, leaf protein concentrates, milk proteins, and other foods (Deshpande *et al.*, 1984).

The objective of this research was (a) to determine allelopathic and antioxidant effects of the extracts from barley seedlings, and (b) to identify the causative compounds from the extracts of barley plants under two growing conditions, in greenhouse for spring barley and in field for winter-barley. This research will be useful for eco-friendly weed management using natural plant materials, and for utilization of early barley seedlings as a natural antioxidant.

MATERIALS AND METHODS

Plant sampling and preparation

Barley plants were grown at two places of Dongshin University. For greenhouse culture, barley seeds of 'Saechalssalbori' were planted in 20-cm diameter plastic pots filled with silt-loam soil on early February in 2005. The plants (around 15 cm high) with 4 - 5 leaves were harvested at a vegetative stage of development 20 days after planting and separated into shoot and root parts (hereafter "spring-barley"). For

outside culture, barley seeds were planted at a field of Experimental Farm on November 15, 2004. The plant samples were collected from the barley field outside when plant height reached 15cm high (winter-barley), corresponding with 4-5 leaf stage. The collected plant tissues were separated into shoot and root parts. The separated plants were washed in tap water with 4 times, and directly freeze-dried at - 40°C for 5 days. The dried samples were ground with a Wiley mill to pass a 1-mm screen and then stored in a refrigerator at 2°C until used. Whole plant samples were used for a bioassay of phytotoxic effect of methanol extracts, and the 1st and 4th leaves for investigation of antioxidant activity

Phytotoxic effects of methanol extracts by plant part

Spring barley with 4 - 5 leaf stage grown in greenhouse were carefully collected from the pots, and separated into shoot and root parts. The separated plants were washed in tap water with 4 times, and directly freeze-dried at - 40°C for 5 days. 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). 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). Four milliliters of each of the solutions at 25, 50, 75 and 100 g l^{-1} were placed in a 9-cm plastic Petri dish lined with one Whatman No. 1 filter paper and evaporated to dryness for 24 h at 24 °C. For the distilled water control, 4 ml of methanol applied to Petri dishes. After evaporation, four milliliters of distilled water was added onto the filter paper and then 15 imbibed seeds of alfalfa were placed on the paper and grown for 6 days. Root length was measured for all seedlings in each Petri dish.

Phytotoxic effects of methanol extracts from winter- and spring-barley

To compare phytotoxicities of plant extracts from different growing conditions, field condition for winter-barley and greenhouse condition for spring-barley, ground leaf samples from spring- and winter-barley seedlings were extracted with 95% methanol at room temperature, as mentioned previous experiment. Four milliliters of each of the extracts at 25, 50, 75 and 100 g l^{-1} were poured in a 9-cm plastic Petri dish lined with one Whatman No. 1 filter paper and evaporated to dryness for 24 h at 24°C. For the distilled water control, 4 ml of methanol applied to Petri dishes. After evaporation, four milliliters of distilled water was added onto the filter paper and then 15 imbibed seeds of alfalfa were placed on the paper and grown for 6 days. Root length was measured for all seedlings in each Petri dish.

DPPH radical scavenging activity of methanol extracts and the fractions

For fractionation, crude methanol extracts were diluted with distilled water and 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°C, and transferred into vacuum freeze dryer to obtain dry matters.

DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging assay was carried out according to the procedure described by Blosi (1958). Methanol extracts from different plant part of spring- and winter barley, and 5 different fractions from shoot parts of spring barleys at various concentrations (0. 250, 500, 1000, 2000, and 4000 μg/ml) 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 IC_{50}, which was defined as the concentration (in ppm or ìg 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 (SAS Institute, 2000).

Identification and quantification of causative substances

Leaf samples of spring- and winter-barley were separately dissolved in 80% HPLC grade methanol for analysis. The standard phenol compounds used for HPLC analysis were coumarin, *o*-coumaric acid, *p*-coumaric acid, ferulic acid, and chlorogenic acid (Aldrich Chemical Co). All of chemicals were purchased as high purity standards. The used solvents ratios were HPLC spectral grade. All solvents and distilled water were degassed before use. All solvent ratios were based on volume.

The compounds were identified by a high-performance liquid chromatography (HPLC) using a HP 1100 system, flow rate of 1ml/min, wavelength at 280 nm, a column of HP Eclipse XDB-C_{18} (4.6×250 mm), and an autoinjector

with a 20 μl sample loop. The mobile phase consisted of water and acetonitrile. Analyses of extracts and standard compounds were based on the method of Banwart *et al.* (1985). Retention times for the standard compounds and the major peaks in the extract were recorded. Phenolic compounds were identified by retention times or standard addition, and their contents were calculated comparing peak area with those of standards.

RESULTS AND DISCUSSION

Phytotoxic effects of methanol extracts by plant part

Methanol extracts of barley leaf and root extracts were assayed against Chinese milkvetch seedlings to determine their phytotoxicity, and the results showed significant inhibition in the both extracts. Leaf extracts from 25 g L^{-1} inhibited root length of Chinese milkvetch (90% inhibition) more than did root extracts (85% inhibition). The degree of inhibition increased with increasing the extract concentration (Fig. 1). Kwak and Kim (1984) studied allelopathic effects of barley residues including straws and roots, and the results showed the high inhibition in germination and shoot growth of rice and paddy weeds. Recently, Chon and Kim (2004) also reported that extracts from 60-day-old barley plants significantly reduced root growth of alfalfa, barnyard grass, and eclipta.

However, hypocotyls length was less sensitive to the extracts than was root length. No difference in phytotoxicities was observed among extract concentrations, even between shoot and root extracts. This result corroborates earlier reports that root growth is more sensitive to alfalfa extracts than is seed germination or hypocotyls growth of alfalfa (Hedge and Miller, 1990; Chung and Miller, 1995; Chon *et al.*, 2000).

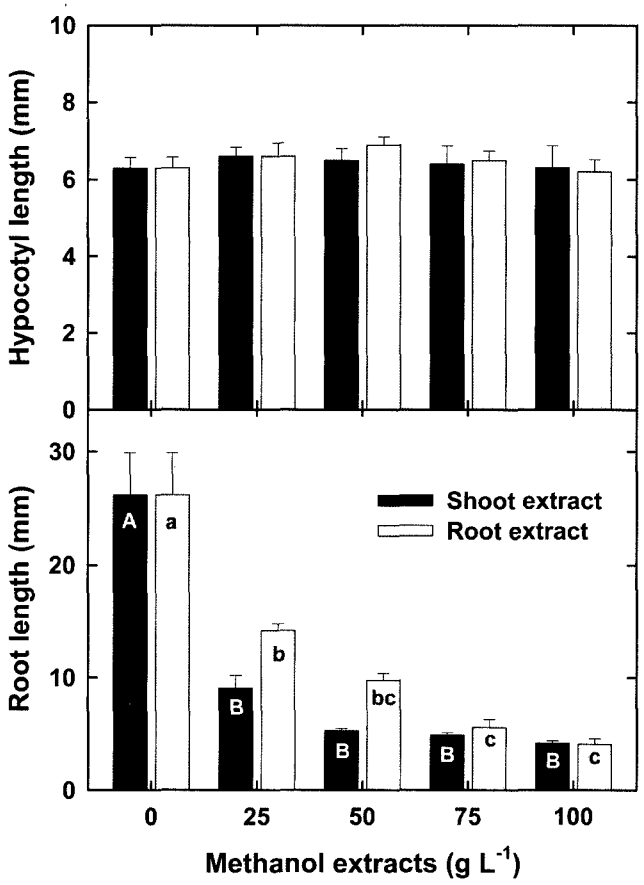

Fig. 1. Effects of methanol extracts from barley leaves and roots on root length of Chinese milkvetch. The root length was determined 6 days after seeding on the filter paper wetted with the extracts. Within a plant part, means of leaf (upper case) and root (lower case) extracts followed by the same letter are not significantly different at $p<0.05$. Each bar represents standard error of the mean.

Phytotoxic effects of methanol extracts from winter- and spring-barley

With root growth of Chinese milkvetch, both extracts exhibited significant phytotoxicity compared with the control. Methanol extracts at 50 g L^{-1} reduced Chinese milkvetch by 95%. The degree of inhibition increased with increasing the extract concentration. However, no significant difference in phytotoxicity between two plant materials was

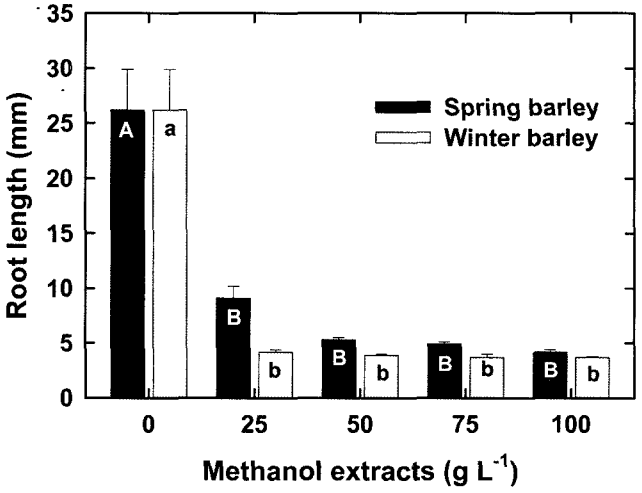

Fig. 2. Effects of methanol extracts from spring- and winter barley leaves on root length of Chinese milkvetch. The root length was determined 6 days after seeding on the filter paper wetted with the various extracts. Within a growing season, means of spring- (upper case) and winter-barley (lower case) extracts followed by the same letter are not significantly different at $p<0.05$. Each bar represents standard error of the mean.

observed, even though the extracts from winter barley were little more inhibitory than those from spring barley (Fig. 2).

DPPH radical scavenging activity of methanol extracts of winter- and spring-barley

All methanol extracts showed DPPH radical scavenging activity in a dose-dependent manner. Winter barley shoot extracts showed the highest DPPH radical scavenging activity, with an IC_{50} value of 1,640 μg ml^{-1}, and followed by spring barley leaves (IC_{50}=2,650 μg ml^{-1}). However, extracts from spring barley roots had lowest DPPH radical scavenging activity (Fig. 3). The results show that causative antioxidant components were more present in winter-barley than spring-barley, and shoot part than root part.

DPPH radical scavenging activity of the fractions from winter- and spring-barley

After fractionation, the antioxidant potential of the individual fraction was in order of *n*-butanol > ethyl acetate > water > chloroform > *n*-hexane fraction. Butanol fraction showed the highest DPPH radical scavenging activity, with the lowest IC_{50} value of 397 μg ml^{-1}, and followed by ethylacetate (IC_{50} = 1,525 μg ml^{-1}) and water fractions (IC_{50} = 1,810 μg ml^{-1}). However, IC_{50} value from hexane fraction was the highest (>4,000 μg ml^{-1}), meaning the lowest DPPH radical scavenging activity (Fig. 4). Fractions of barley seedlings dose-dependently increased DPPH free radical scavenging activity at *in vitro* test. The results show that causative antioxidant components were present as the highest amount in the BuOH fraction, resulting in most inhibitory effects on DPPH radicals. 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.

Identification and quantification of causative substances

The responsible allelopathic or antioxidant substances present in fractions from methanol extracts of barley were identified and quantified by HPLC, using standard com-

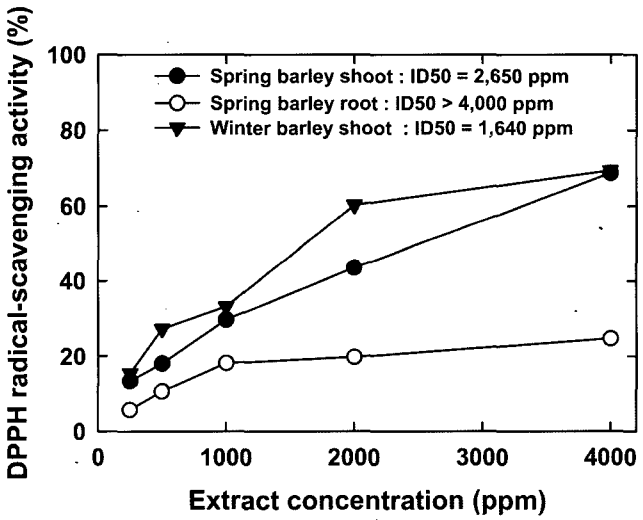

Fig. 3. The scavenging effect of methanol extracts from different plant parts and growing conditions on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical. Each bar represents standard error of the mean.

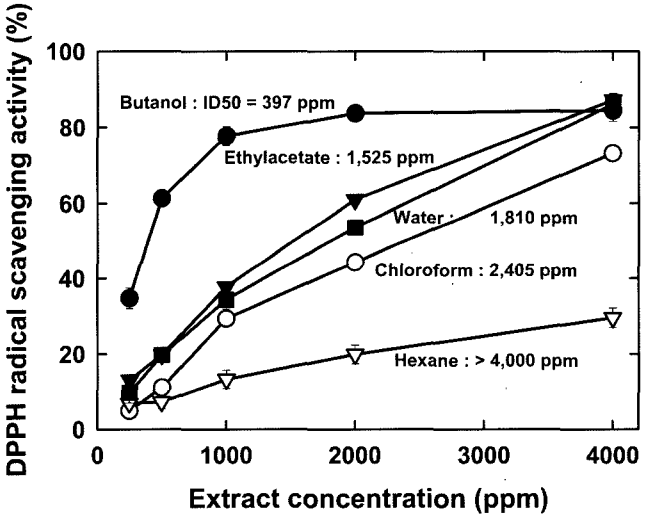

Fig. 4. The scavenging effect of different fractions from methanol extracts winter barley seedlings on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical. Each bar represents standard error of the mean.

Table 1. Quantitative determination of HPLC analysis on phenolic compounds present in methanol extracts from spring- and winter-barley seedlings.

Growing time	Phenolic compounds (mg/100g sample)					
	Coumarin	*o*-coumaric acid	*p*-coumaric acid	Ferulic acid	Chlorogenic acid	Total
Spring barley	0.14	15.23	1.79	183.46	0.00	200.62 (176)*
Winter barley	2.10	1.65	0.59	101.91	7.83	114.08 (100)

*Values in parenthesis represent % of winter barley

pounds. The compounds identified were coumarin, *o*-coumaric acid, *p*-coumaric acid, ferulic acid, and chlorogenic acid. The phenol compounds were more detected in spring-barley (201 mg/100g) than in winter-barley (114 mg/100g), showing 76% more than in winter-barley. Of them, ferulic acid was present as the highest amounts in methanol extracts from spring-barley and winter barley seedlings with 184 and 102 mg/100 g, respectively (Table 1). Kwak and Kim (1984), by means of paper chromatography, phenolic compounds identified from residues were *p*-coumaric acid, *p*-hydroxybenzoic acid, ferulic acid, vanillic acid and salicylic acid, they showed allelopathic effects on test plants. Chon and Kim (2004) also reported that in their high-performance liquid chromatography (HPLC) analysis, caffeic acid, hydro-cinnamic acid, ferulic acid, *m*-coumaric acid, *p*-coumaric acid and coumarin were present in all the crop plant species, and hydro-cinnamic acid were detected as the highest amount.

In conclusion, the present study demonstrates allelopathic effects of methanol extracts from barley on Chinese milkvetch, regardless of plant part and growing season. Methanol extracts and the fractions from barley seedlings also showed potent antioxidant activity, showing inhibitory effects of their fractions on formation of DPPH radicals. Methanol extracts from winter barley shoot extracts showed the higher DPPH radical scavenging activity than those from spring barley leaves, and from leaves than from roots. A HPLC analysis with several standard phenolic compounds showed that the concentration and composition of compounds detected depend on plant growing season. Different compounds that cause allelopathy or antioxidative effect were more detected in spring-barley than winter-barley. Such differences in types and amounts of phenolic substances also might be related to specific allelopathic or antioxidant compounds being produced in larger quantities in specific season, imparting a higher level of biologically-active component. Our results suggest that the allelopathy and antioxidant activity from early seedlings of barley may be a valuable alternative natural materials based on plant material.

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