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Antioxidant activity of different parts of Dolsan Leaf Mustard

SunKyung Oh, KiWoong Kim[1], and MyeongRak Choi*

Department of Biomedical and Electronic Engineering, Chonnam National University, Yeosu, Jeonnam 59626, Korea
[1]Department of Marine Bio Food Science, Chonnam National University, Yeosu, Jeonnam 59626, Korea

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*Corresponding Author
Tel: +82-61-659-7303
Fax: +82-61-659-7309
E-mail: mrchoe@chonnam.ac.kr

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Abstract This study aimed to investigate the sinigrin content and antioxidant properties of Dolsan mustard seeds (DMS) and Dolsan leaf mustard (DLM) extracted with 50% acetonitrile. The extracted substances were fractionated by HPLC using a preparative ODS column and then applied to measure sinigirn, total polyphenol (TP), and total flavonoid (TF) contents and antioxidant activities using 2,2-azino-bis(3-ethyl-benzothizoline-6-sulfonic acid) (ABTS), electron donating ability (EDA), and ferric reducing antioxidant power (FRAP) assays. The contents of sinigrin (53.77 mg/g), TP (404.33 mg gallic acid equivalents/g extract), TF (395.33 mg quercetin equivalents/g extract) were higher content in DMS than in DLM. Antioxidant activities of DMS were a slightly higher than DLM. ABTS, EDA, and FRAP assays showed that there was a linear correlation between TP and TF contents and antioxidant capacity. These results suggested that the antioxidant effects of DMS and DLM might be mediated through the TP and TF contents, providing potential source of natural antioxidants.

Keywords: Dolsan leaf mustard, Dolsan mustard seed, sinigrin, antioxidant capacity

Introduction

Free radicals are important causes of aging and disease in the body. There are different kinds of free radicals, including superoxide radicals, and hydroxyl radicals (1,2). These free radicals cause diseases in adults, damage cell membranes, inhibit proteolysis or fat oxidation, and inhibit DNA synthesis. Owing to the increase in longevity and associated health concerns, functional materials of plants have been extensively studied to prevent aging and maintain health (3,4). Leaf mustard (*Brassica juncea*) is leafy vegetables that belong to the Cruciferae family. The leafy parts of this plant are widely used as the main ingredients of leaf mustard kimchi or as minor ingredients of various other types of kimchi. Moreover, the seeds of this plant are used as acrid spices because of their hot taste and unique flavor (5). Myrosinase functions to produce sinigrin, a type of glucosinolate. Leaf mustard contains large amounts of thiosulfates and organosulfur compounds, which are known to hinder the development of chemically-derived tumors (6). Reports have shown that indoles, as degradation products of glucosinolates, including sinigrin, inhibit tumor formation in animal experiments; similar effects have also been reported in humans (7). The leaf mustard contains glucosinolates such as *sec*-butyl isothiocyanates or *p*-hydroxybenzyl glucosinolates, flavonoids such as kaempferol, isorhamnetin, and their glycosides, and carotenoids, including β-carotene, lutein, epoxide, or cryptoxanthin (8). In particular, leaf mustard contains high levels of ascorbic acid, chlorophyll, and β-carotene and has more antioxidant activity than other types of leaf and stem vegetables (9,10). Furthermore, a previous study on separation of bioactive substances from leaf mustard showed that leaf mustard contains antioxidant components such as sinapine, isorhamnetin 3,7-di-O-β-D-glucopyranosides, and antimutagenic components, including decan-4-ol. Leaf mustard native to Korea contains antimicrobial substances, such as isorhamnetin 3-O-β-D-glucopyranosides. In addition, an aliphatic higher alcohol and three types of flavonoids were separated from the above ground part of the leaf mustard. Furthermore, leaf mustard contains 3,5-dimethoxy-4-hydroxycinnamic acid, which also has peroxynitrite scavenging activity (11-16). Previous studies have mostly evaluated the activity of crude extracts; analyses of single substances isolated from crude extracts of Dolsan mustard seeds (DMS) and Dolsan leaf mustard (DLM) have not yet been performed. Therefore, in this study, we aimed to investigate bioactive substances and physiological effects of DMS and DLM and to re-examine the value of leaf mustard as a functional food.

Materials and Methods

Materials and reagents DMS and DLM used in this study were harvested in July 2015 by the Dolsan Leaf Mustard Farming Association located in Dolsan-eup, City of Yeosu, Southern Jeolla Province. Acetonitrile and methanol are products of J. T. Baker

(Phillipsburg, NJ, USA). Sinigrin, Folin-Ciocalteu's phenol reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethyl-benzothizoline-6-sulfonic acid) (ABTS), sodium carbonate, aluminum chloride, gallic acid, phosphate buffered saline (PBS), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), iron(III) chloride hexahydrate, iron(II) sulfate heptahydrate ($FeSO_4 \cdot H_2O$), potassium persulfate, and ascorbic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Extraction of DMS and DLM To measure the sinigrin content in DMS and different parts of DLM, we placed 10 g of roots, leaves, and stems in thimble filters and 200 mL of 50% acetonitrile (CH_3CN) in round flasks. The flasks were connected to a Soxhlet extractor and extraction was performed in a water bath at 90°C for 24 h. The extracted contents were then cooled at room temperature and were filtered with Whatman No. 4 filters (Whatman International Ltd., Maidstone, England) (17). The filtrates were concentrated using a vacuum rotary evaporator (N-1000; Eyela, Tokyo, Japan) to a final volume of 100 mL. The final extracts were stored at −18°C for use in subsequent experiments.

Sinigrin standard curve and high-performance liquid chromatography (HPLC) operating conditions The standard reagent sinigrin was prepared at 125, 250, 500, and 1000 ppm; the measurements were repeated three times, and the mean of the three values was used to create a standard curve, which was used to measure the level of sinigrin in DMS and different parts of DLM. Analytical HPLC and preparative ODS-HPLC were used for the separation and purification of substances from DMS and different parts of DLM. The operating conditions are shown in Table 1. In all experiments, single substances were separated by preparative ODS-HPLC.

Measurement of total polyphenol content The total polyphenol content in DMS and different parts of DLM was measured using colorimetry by using Folin-Ciocalteu's phenol reagent (18). We added 2.6 mL of distilled water and 200 μL of Folin-Ciocalteu's phenol reagent to 200 μL of the sample and mixed together; the mixture was allowed to react for 6 min at room temperature, and then, 2 mL of 7% (w/v) Na_2CO_3 solution was added. The mixture was then allowed to react for 90 min, and the absorbance was measured using a spectrophotometer (Ultraspec 2000; Pharmacia Biotech, Cambridge, England) at 750 nm. A standard curve was constructed using gallic acid as the standard, and the polyphenol content was reported in mg gallic acid equivalents (GAE)/g extract.

Measurement of total flavonoid content The total flavonoid content in DMS and different parts of DLM was measured as by the method of Jia *et al*. (19). 1 mL of the sample, 3.2 mL of distilled water, and 150 μL of 5% (w/v) $NaNO_2$ were mixed together and were allowed to react for 5 min. Then, 10% $AlCl_3$ solution was added and the mixture was reacted for 1 min, and 1 M NaOH was added; subsequently, the absorbance was measured at 510 nm. A standard curve was constructed using quercetin as a standard substance, and the flavonoid content was reported in mg quercetin equivalents (QE)/ g extract.

ABTS radical scavenging activity The ABTS radical scavenging activity was measured by a slight modification to the method reported by Kriengsak *et al*. (20). Equal volumes of 1.8 mM ABTS solution and 0.63 mM potassium persulfate were combined and were reacted in dark for 24 h at 37°C to create an ABTS solution with ABTS free radicals; the solution was modified so that the absorbance at 735 nm was 1.4±0.1. We mixed 5 mL of the ABTS solution with ABTS radicals and 0.1 mL of the sample, and the mixture was allowed to react for 7 min; subsequently, the absorbance was measured at 735 nm. The results were calculated according to the following equation:

ABTS radical scavenging activity (%)
=(1−absorbance of the solution with the sample added/
absorbance of the solution without sample)x100

Electron donating ability The electron donating ability (EDA), which is the radical scavenging effect of DPPH, was shown as the reducing power of the DMS and different parts of DLM extracts. We added 0.5 mL of 0.5 mM DPPH solution to a test tube containing 1 mL of the sample, 1 mL of methanol, and 0.99 mL of 100 mM sodium acetate buffer (pH 5.5), and the mixture was agitated. Then, the mixture was reacted in dark for 5 min; the concentration of the remaining radicals was measured at 517 nm and was reported according to the following equation (21).

Table 1. High-performance liquid chromatography (HPLC) apparatus and operating conditions

Items	Conditions	
	Analytical-HPLC	Preparative ODS-HPLC
HPLC	Shimadzu JP/LC-10A, System/CDD-6A detector	Shimadzu JP/LC-10A, System/CDD-6A detector
Column	Shim-pack VP ODS (250x4.6 mm, Shimadzu, Tokyo, Japan)	Shim-pack PREP-ODS (20x250 mm, 5 μm)
Mobile phase	Acetonitrile:double distilled water (DDW)=40:60 (v/v)	Acetonitrile:DDW=40:60 (v/v)
Flow rate	1.0 mL/min	8.0 mL/min
Detector	UV 228 nm	UV 254 nm
Oven temp.	40°C	40°C
Injection volume	10 μL	200 μL

EDA (%)
=(1−absorbance of the solution with the sample added/ absorbance of the solution without sample)x100

Measurement of ferric reducing antioxidant power The ferric reducing antioxidant power (FRAP) was measured using a slight modification of the method reported by Benzie (22). We mixed 30 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyls-triazine (TPTZ) dissolved in 40 mM HCl, and 20 mM iron (III) chloride hexahydrate in a 10:1:1 ratio. We mixed 150 μL of the sample and 2850 μL of the reaction mixture and allowed them to react for 30 min; then, the absorbance was measured at 593 nm. A standard curve was constructed using $FeSO_4 \cdot H_2O$, and FRAP content was quantified in mg $FeSO_4$ equivalents/g extract.

Statistical analysis All tests and analyses were repeated at least three times. The results are expressed as mean±standard deviation (SD). One way analysis of variance (ANOVA) and Duncan's test were used for multiple comparisons using the SPSS version 21.0 (SPSS Institute, Chicago, IL, USA). Differences were considered statistically significant in all experiment means at $p<0.05$.

Results and Discussion

Total content of sinigrin Sinigrin was used as the standard reagent for quantitative analysis, and a standard curve was obtained from the sinigrin reagent manufactured at different concentrations. The relationship with the peak area of HPLC chromatogram was shown as $r^2=1.000$. The contents of sinigrin in the DMS and different parts of DLM extracts are shown in Table 2. Sinigrin content in the DMS 53.77±0.62 mg/g was significantly higher than that in the roots, leaves, and stems of DLM. Also sinigrin contents of cabbage, cauliflower, and Brussels sprouts seed were reported to be 23.79, 23.69, and 17.71 μmol/g, respectively, and mustard green are reported 122.5 μmol/g (23,24).

Total polyphenol content Phenolic compounds are one of the secondary metabolites widely distributed in plants. They have various structures and molecular weights. The phenolic hydroxyl (OH) groups of these compounds enable them to be easily combined with proteins or other giant molecules. They exhibit many physiological activities such as antioxidant or anticancer activities. The total polyphenol contents in the DMS and different parts of DLM extracts are shown in Table 3. The total polyphenol content in the seeds was indicate 404.33±2.52 mg GAE/g extract and that in the roots, stems, and leaves 371.33±0.58, 370.67±2.08 and 368.67±1.15 mg GAE/g extract, respectively. Turkmen *et al*. (25) reported total polyphenol content of fresh vegetable was 183.28-1344.77 mg GAE/g extract and it gradually decreased by cooking.

Total flavonoid content Flavonoids have a diphenylpropane (C6-C3-C6) skeletal structure and are widely distributed in vegetables, fruits, seeds, and nuts (26). The total flavonoid content is shown in Table 3. Unlike the total polyphenol content, the total flavonoid content in the seeds is the highest (395.33±2.89 mg QE/g extract), followed by that in the leaves as 391.00±1.76 mg QE/g extract, which is slightly higher than that in the roots and stems, 388.67±1.15 and 387.33±1.53 mg QE/g extract, respectively. Hot water extract (1560.8 mg CE/g extract) and ethanol extract (778.2 CE/g extract) of red cabbage showed a higher content than present study (27).

ABTS radical scavenging activity When ABTS and potassium persulfate are placed in a dark place, ABTS+· is generated. The extract scavenges ABTS+· and results in a blue-green color, unique to that of radicals. The ABTS+· decoloring reaction shows the degree of scavenging of free radicals already generated as optical density to measure ABTS+· scavenging activity. The ABTS+· decoloring reaction ends within one minute, which ensures prompt measurement, and it can be applied in hydrophobic and hydrophilic conditions. The results of ABTS+· scavenging activities of DMS and different parts of DLM are shown in Table 4. The ABTS radical scavenging activity was not significantly different between the DMS ($p<0.05$). Ethanol extract of fresh cabbage was showed to dose-dependently highly 18.58-77.99% ABTS radical scavenging activity (28).

Table 2. The concentration of sinigrin in different parts of the Dolsan Leaf Mustard

	Seeds	Roots	Leaves	Stems
Sinigrin (mg/g)	53.77±0.62$^{a1)}$	5.75±0.06^{bc}	5.17±0.140^{c}	6.35±0.09^{b}

[1)]Data were the mean±SD of triplicate experiment. The different lower-case letter superscripts in the same row indicate the significant difference by Duncan's multiple range test ($p<0.05$).

Table 3. The total polyphenol and flavonoid contents of the different parts of the Dolsan Leaf Mustard

	Seeds	Roots	Leaves	Stems
Total polyphenol (mg GAE/100 g)	404.33±2.52$^{a1)}$	371.33±0.58^{b}	368.67±1.15^{b}	370.67±2.08^{b}
Total flavonoid (mg QE/100 g)	395.33±2.89^{a}	388.67±1.15^{a}	391.00±1.73^{a}	387.33±1.53^{a}

[1)]Data were the mean±SD of triplicate experiment. The different lower-case letter superscripts in the same row indicate the significant difference by Duncan's multiple range test ($p<0.05$).

Table 4. 2,2-Azino-bis(3-ethyl-benzothizoline-6-sulfonic acid) (ABTS) radical scavenging activity, electron donating ability (EDA), and ferric reducing antioxidant power (FRAP) of different parts of Dolsan Leaf Mustard

	Seeds	Roots	Leaves	Stems
ABTS (%)[1)]	48.67±0.58[b 3)]	48.27±0.95[b]	50.07±0.90[a]	48.53±1.51[b]
EDA (%)	16.67±0.58[b]	17.30±1.05[a]	16.67±1.31[b]	17.40±0.89[a]
FRAP[2)]	114.63±0.99[a]	108.80±0.95[b]	110.50±1.01[a]	108.97±0.38[b]

[1)]The concentration of all test samples was 200 μg/mL.
[2)]mg $FeSO_4$ equivalents/g extract
[3)]Data were the mean±SD of triplicate experiment. The different lower-case letter superscripts in the same row indicate the significant difference by Duncan's multiple range test (p<0.05).

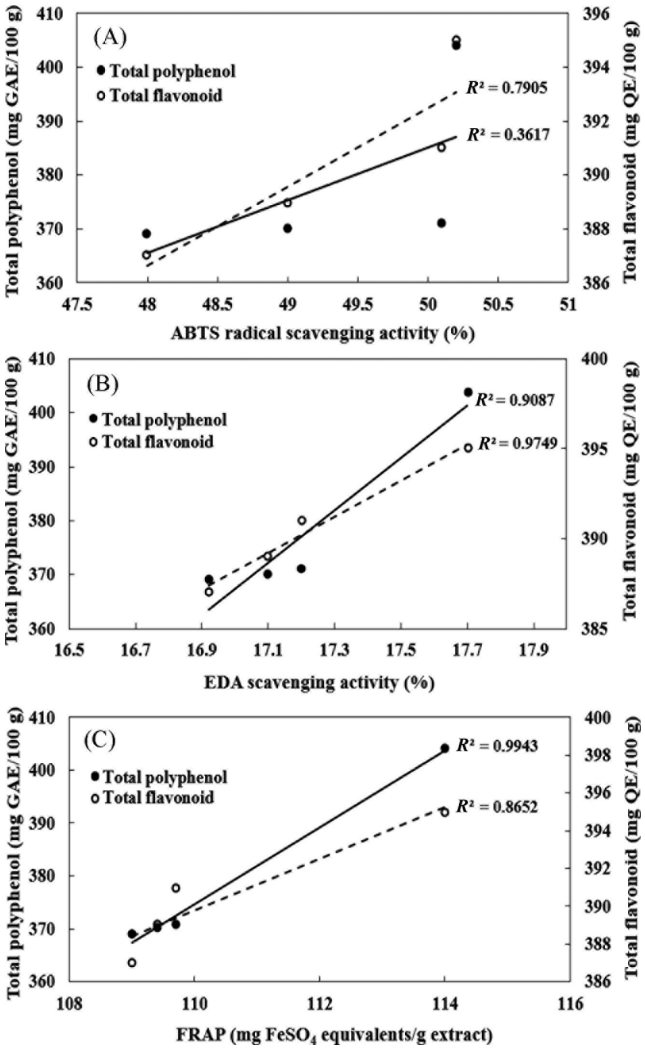

Fig. 1. Correlations between the antioxidant capacity measured using 2,2-Azino-bis(3-ethyl-benzothizoline-6-sulfonic acid) (ABTS) (A), electron donating ability (EDA) (B), and ferric reducing antioxidant power (FRAP) (C) assays and the total polyphenols and total flavonoids of Dolsan Leaf Mustard.

EDA EDA is one of the typical indices to measure antioxidant activity. We used, DPPH as a substrate to measure antioxidant activity. We used DPPH as a substrate to measure antioxidant activity. DPPH is used to measure the antioxidant activity of phenolic substances such as phenols or flavonoids. This activity hinders fat oxidation in food and prevents aging in the human body by donating electrons to active radicals (29). The results of DPPH scavenging activity of the extracts of DMS and different parts of DLM are shown in Table 4. The extracts of DMS and different parts of DLM had DPPH scavenging activity as low as 16-17% (p<0.05).

Measurement of FRAP Measurement of FRAP is one of the ways to measure antioxidant activity. Unlike the method of measuring radical scavenging activity using DPPH, the method of measuring FRAP is based on ferric reduction. The FRAP of the extracts of DMS and different parts of DLM are shown in Table 4. The roots, stems, and leaves had similar FRAP, and FRAP of the seeds was 114.63±0.99 mg $FeSO_4$ eq./g extract. DMS and different parts of DLM have high antioxidant activity because they contain a large amount of phenolic compounds.

Correlation of total phenolic and flavonoid content and antioxidant capacity Correlation between the antioxidant capacity in the extracts of DMS and different parts of DLM and the total levels of phenols and flavonoids was examined through ABTS scavenging activity, EDA, and measurement of FRAP, and the results of these experiments are shown in Fig 1. The correlation coefficient (R^2) between the total phenolic content in the extracts of DMS and different parts of DLM and their antioxidant capacity measured by ABTS, EDA, and FRAP was 0.3617, 0.9087, and 0.9943, respectively, while that between the total flavonoid content and the antioxidant capacity was 0.7905, 0.9749, and 0.8652, respectively. This results showed the extracts of DMS and different parts of DLM effectively scavenged the free radicals and had a high antioxidant capacity. This correlation was observed with a straight line having a positive grade.

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Disclosure The authors declare no conflict of interest.

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