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Antioxidant and Antiproliferative Activities of Methanol Extracts from Leafy Vegetables Consumed in Korea

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Abstract There has been growing interest in the antioxidative and anticarcinogenic effects of vegetables. This study was aimed at evaluating the antioxidative and antiproliferative activities of 13 leafy vegetables consumed in Korea. New-beet and ornamental kale contained >1,000 mg of polyphenolics/100 g fresh weight (FW), which was the greatest amount among the test vegetable extracts. Ornamental kale also contained the greatest amount (232.84 mg/100 g FW) of flavonoids. With the exception of chicory, values of 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activities showed similar trends. New-beet was found to be the greatest antioxidant among the test vegetable extracts. New-beet exhibited the highest antiproliferative activity (>60%) against all tumor cells. No relationship was found between antiproliferative activity and antioxidant contents or antioxidant activities among samples.

Keywords: antioxidant, antiproliferation, leafy vegetable, polyphenolic, flavonoid

Introduction

During the past decade, consumption of fresh vegetables has increased due to consumers' growing concerns regarding health. There is substantial evidence that vegetable consumption decreases risks of chronic diseases such as cancer and cardiovascular disease (1). One of the beneficial constituents derived from vegetable consumption is polyphenolics, which are derivatives from mainly shikimate and acetate pathways (2). Structures of over 8,000 polyphenolics have been identified (3). Polyphenolics are classified into more than 10 classes based on the chemical structures and the largest class consists of flavonoids with a common structure of diphenylpropanes (C6-C3-C6) (2, 4).

Flavonoids are widely distributed in a variety of plants and common flavonoids include quercetin, kaempferol, myricetin, apigenin, and luteolin (4). The amount of flavonoids in foods varies depending on species, environmental circumstances, and post-harvesting treatment (2, 5, 6). Ross and Kasum (4) suggested that flavonoids have potential effects on human health such as antioxidative and antiproliferative activities. Flavonoids behave as antioxidants by scavenging free radicals and active oxygen species (7, 8). Antioxidant activities of aglycones with quercetin, luteolin, myricetin, and kaempferol are higher than the corresponding conjugated flavonoids (9). In addition to the antioxidant activity, the antiproliferative property of flavonoids has been determined in many studies (10-12). An effect of flavonoids (flavones, 2-phenyl-4H-1-benzopyran-4-one) on human colon cancer cells was determined by Wenzel *et al.* (13). The authors concluded that the flavones have protective effects against colon cancer cells. Similarly, Miranda *et al.* (12) confirmed the antiproliferative and cytotoxic effects of 6 flavonoids on human cancer cells.

Although many studies substantiate the antioxidant and anticarcinogenic activities of dietary flavonoids, the types of tested flavonoids, source of the flavonoids, and tested cell lines varied. The objectives of this study were to determine the contents of polyphenols and flavonoids in 13 leafy vegetables commonly consumed in Korea, the antioxidant activities of the vegetable extracts using 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, and the antiproliferative effects of the extracts in human cancer cell lines.

Materials and Methods

Vegetable samples Red kale (*Brassica oleracea* L.), chicory (*Cichorium endiva* L.), lettuce (*Lactuca sativa* L.), beet leaf (*Beta vulgaris* L.), red mustard (*Brassica juncea* L.), sesame leaf (*Sesamum indicum* L.), pak-choi (*Brassica camestris* L. var. *chinensis*), angelica (*Angelica utilis* L.), green mustard (*Brassica juncea* L.), tai-gu choi (*Brassica rapa* var. *rosularis*), treviso (*Chichorium intybus* L.), ornamental kale (*Brassica oleracea* L.), and new-beet leaf (*Beta vulgaris* L.) were purchased at retail stores in Cheongju in the year of 2006, Korea.

Methanol extraction Ten g of each homogenized sample were extracted with 100 mL of methanol with agitation at room temperature (24±2°C) for 24 hr. The extracts were filtered with filter papers (No 2; Toyo Roshi, Tokyo, Japan) and residues were removed. The filtrates were concentrated using a vacuum evaporator (Eyela, Tokyo, Japan) below 40°C. The extracts were redissolved with methanol for antioxidant activity assay and phosphate buffered saline (PBS) for antiproliferative activity assay and stored at -20°C prior to use.

Determination of total polyphenolic content The content of total polyphenolics in the methanolic extracts

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was determined spectrophotometrically according to the Folin-Ciocalteu colorimetric method with some modifications (14), calibrating against gallic acid and expressing the results as mg gallic acid/100 g fresh weight (FW) sample.

Determination of total flavonoid content Total flavonoid content in the extracts was determined according to the procedures described by María *et al.* (15). Total flavonoid content was calculated using quercetin as standard.

Measurement of free radical scavenging activity using DPPH radical The scavenging activity of the leafy vegetable extracts on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was measured according to the method of Kim *et al.* (16) with some modifications. The 0.2 mM DPPH radical solution (1 mL) was added to 50 μL of extract, ascorbic acid standard solution, or methanol. After 30 min, the absorbance was measured at 520 nm using spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA). The DPPH radical scavenging activity was expressed as ascorbic acid equivalent antioxidant activity (AEAC) and defined as the mg of ascorbic acid equivalents per 100 g FW of sample (17). The AEAC was calculated by the following equation and all extracts were analyzed in triplicate.

$$AEAC = (\Delta A_{sample}/\Delta A_{aa}) \times C_{aa} \times V \times (100/W_{sample})$$

Where ΔA_{sample} is the change of absorbance in presence of the sample extracts, ΔA_{aa} is the change of absorbance after addition of ascorbic acid standard solution, C_{aa} is the concentration of ascorbic acid standard solution (mg/mL), V is the volume of sample extracts (mL) and W_{sample} is the weight of sample used for extraction (g). All extracts were analyzed in triplicate.

Measurement of total antioxidant capacity using ABTS radical The scavenging activity of the leafy vegetables extracts on 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation was measured according to the method of Robert *et al.* (18) with some modifications. ABTS radical cation was generated by adding 7 mM ABTS to 2.45 mM potassium persulfate solution and the mixture was left to stand for overnight in a dark place at room temperature. The ABTS radical cation solution was diluted with distilled water to obtain an absorbance of 1.4-1.5 at 414 nm (molar extinction coefficient $\varepsilon = 3.6\times10^4$/mol·cm) (19). Diluted ABTS radical cation solution (1 mL) was added to 50 μL of extract, ascorbic acid standard solution, or methanol. After 90 min, the absorbance was measured at 414 nm using spectrophotometer (Beckman Instruments Inc.). The ABTS radical cation scavenging activity was expressed as AEAC and defined as the mg of ascorbic acid equivalents per 100 g FW of sample (19). The AEAC was calculated by the same equation in DPPH method.

Antiproliferative activity Breast (MCF7), colon (HCT 116), lung (NCI-H460), and gastric (MKN 45) tumor cells were grown in RPMI supplemented with 10% FBS, 2 mM glutamine, 100 unit/mL penicillin, 50 μg/mL streptomycin. The cultures were maintained in a humidified incubator with 5% CO_2 and seeded onto 75 cm^2 area culture flask. Antiproliferative activity of leafy vegetables on tumor cells was measured by evaluating cell viability using the MTT assay. For assay, the cell were seeded at a density of 5×10^3 cell/well for MKN 45 and 2×10^3 cell/well for the others using a brief trypsinization and then leafy vegetable extracts (20 μL) in PBS were added in a 96-well plate. After 48 hr of incubation, 20 μL of MTT reagent (5 mg/mL) was added and incubated for a further 4 hr and the absorbance of formazan was determined at 550 nm using a spectrophotometer (Beckman Instruments Inc.). The cell viability (%) was obtained by comparing the absorbance between the samples and negative control (20).

Results and Discussion

Total polyphenolic and flavonoids contents It has been reported that total phenolics were the major naturally occurring antioxidant components found in methanolic extracts from many plant foods including mushroom. Therefore, it is important to quantify total phenolic contents and to consider their contributions on antioxidant activity (21, 22). The contents of polyphenolics and flavonoids as natural antioxidants in 13 leafy vegetables were determined and showed in Table 1. New-beet and ornamental kale contained 1,194.73 and 1,166.99 mg of polyphenolics/100 g FW, respectively, which were significantly high values among the test vegetables. Chicory (194.37 mg/100 g FW), lettuce (302.85 mg/100 g FW), and pak-choi (223.87 mg/100 g FW) ranked low in polyphenolic content. The amount of polyphenolics in the other vegetables tested ranged between 454.92 and 622.06 mg/100 g FW.

The contents of total flavonoids were ranged from 11.58 to 232.84 mg per 100 g FW sample. The highest amount of flavonoids was found in ornamental kale. Similar to the contents of polyphenolics, relatively low contents of flavonoids were found in chicory, lettuce, and pak-choi. According to another study (23), quercetin contents in lettuce ranged from 1.1 to 91.1 mg/100 g FW, depending on the variety of lettuce and location of leaves. With the exception of pak-choi, the *Brassicaceae* family, including red kale, red mustard, green mustard, tai-gu choi and ornamental kale, contained polyphenolics at 519.15-1166.99 mg/100 g FW and flavonoids at 124.84-232.84 mg/100 g FW. The *Brassicaceae* family has been reported to have large amounts of total phenolics and flavonoids (24). The authors (24) reported 5.31-13.80 mg of total phenolics and 1.35-6.71 mg of flavonoids/g (dry weight) in plant samples belonging to the *Brassicaceae* family. In another study, kale (*Brassica oleracea* var. *acephala*) contained 31.8 mg flavonoids per 100 g sample (25). The differences in values between studies may be attributed in part to the variety of vegetables and different cultivars.

Antioxidant activities Free radical scavenging is one of the well-known mechanisms by which antioxidants inhibit lipid oxidation. The methods of scavenging DPPH and ABTS radicals are widely used to evaluate the antioxidant activity of specific compounds or extracts (21). Figure 1 shows the comparative values of DPPH radical scavenging activities of the methanolic extracts from 13 leafy

Table 1. Contents of total polyphenolics and flavonoids in methanolic extracts obtained from the leaf vegetables and extraction yield[1)]

Common name		Polyphenolics (mg/100 g fresh wt)	Flavonoids (mg/100 g fresh wt)	Concentration of extracts (mg/mL)
A	Red kale	613.25	149.68	7.4
B	Chicory	194.37	11.58	4.8
C	Lettuce	302.85	92.73	3.0
D	Beet	617.78	136.28	5.0
E	Red mustard	561.74	136.49	6.4
F	Sesame	589.11	214.81	5.1
G	Pak-choi	223.87	33.66	8.1
H	Angelica	560.12	187.96	9.6
I	Green mustard	519.15	124.84	8.1
J	Tai-gu choi	622.06	145.14	8.9
K	Treviso	454.92	154.65	7.0
L	Ornamental kale	1,166.99	232.84	23.5
M	New-beet	1,194.73	167.41	9.8

[1)]All samples were assayed in duplicate.

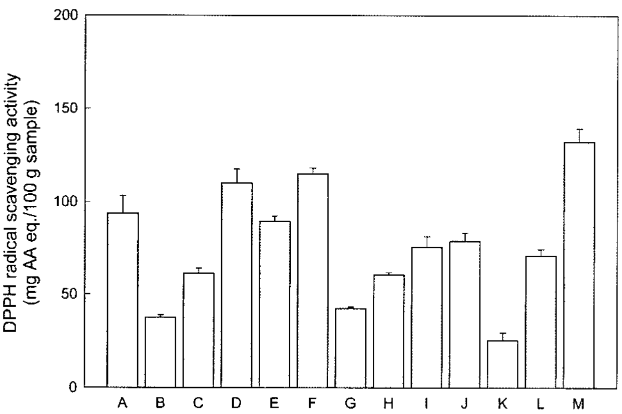

Fig. 1. DPPH radical scavenging activity of methanolic extracts form 13 leafy vegetables. A, red kale; B, chicory; C, lettuce; D, beet; E, red mustard; F, sesame; G, pak-choi; H, angelica; I, green mustard; J, tai-gu choi; K, treviso; L, ornamental kale; M, new-beet.

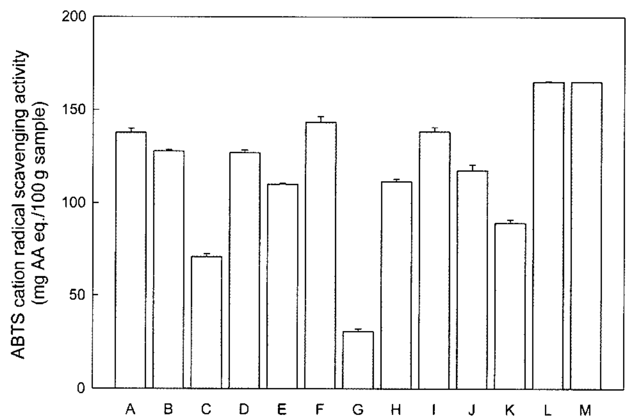

Fig. 2. ABTS cation radical scavenging activity of methanolic extracts form 13 leafy vegetables. A, red kale; B, chicory; C, lettuce; D, beet; E, red mustard; F, sesame; G, pak-choi; H, angelica; I, green mustard; J, tai-gu choi; K, treviso; L, ornamental kale; M, new-beet.

vegetables. The highest activity (132.1 mg AEAC per 100 g) was found in new-beet and treviso showed the lowest activity (25.4 mg AEAC per 100 g). ABTS radical scavenging activities of the extracts were also determined (Fig. 2). The highest antioxidant activity (165.3 mg AEAC per 100 g) was observed in new-beet extract. Overall, the values of the ABTS radical scavenging activity were relatively higher than those of the DPPH radical scavenging activity. The trends were found to be very similar except for chicory.

Antiproliferative activities The antiproliferative effects of the methanolic extracts on gastric (Fig. 3), colon (Fig. 4), lung (Fig. 5), and breast (Fig. 6) cancer cells were quantified as cytotoxicity (%). As illustrated in Fig. 3, new-beet showed the highest cytotoxicity (66.4%) against MKN45 cells, whereas red mustard, pak-choi, angelica, and tai-gu choi did not inhibit proliferation of the cells. The antiproliferative activity of the 13 vegetable extracts against HCT116 cells is shown in Fig. 4. As with MKN45 cells, new-beet showed the greatest cytotoxicity (58.4%), and significantly high cytotoxicity (>40%) against HCT 116 cells was observed in red kale, beet, sesame, and new-beet. Chicory and lettuce showed the lowest antiproliferative activity against HCT116 cells. The effects of the vegetable extracts on the proliferation of NCI-H460 cells were also determined (Fig. 5). Only red kale, treviso, ornamental kale, and new-beet expressed >40% cytotoxicity against

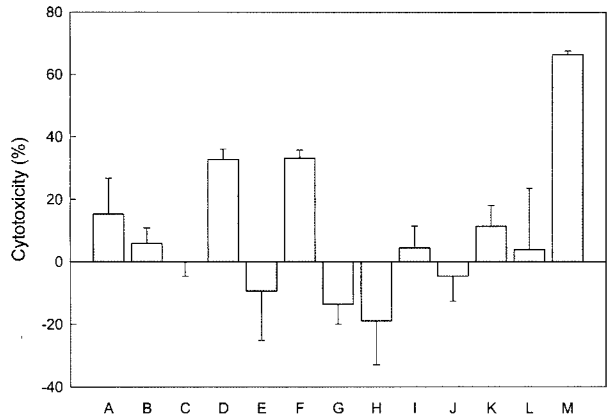

Fig. 3. Antiproliferative activity of the 13 vegetable extracts against MKN45 cell. A, red kale; B, chicory; C, lettuce; D, beet; E, red mustard; F, sesame; G, pak-choi; H, angelica; I, green mustard; J, tai-gu choi; K, treviso; L, ornamental kale; M, new-beet.

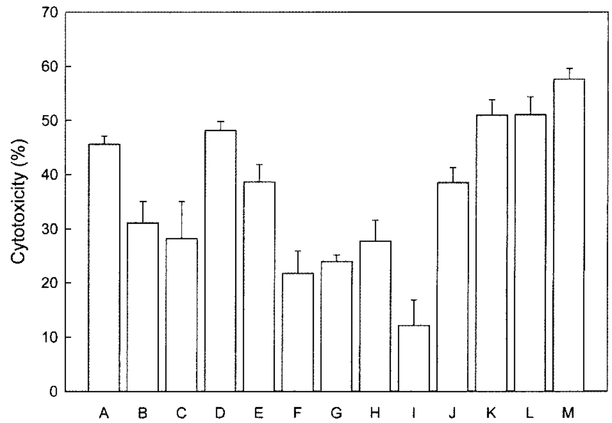

Fig. 5. Antiproliferative activity of the 13 vegetable extracts against NCI-H460 cells. A, red kale; B, chicory; C, lettuce; D, beet; E, red mustard; F, sesame; G, pak-choi; H, angelica; I, green mustard; J, tai-gu choi; K, treviso; L, ornamental kale; M, new-beet.

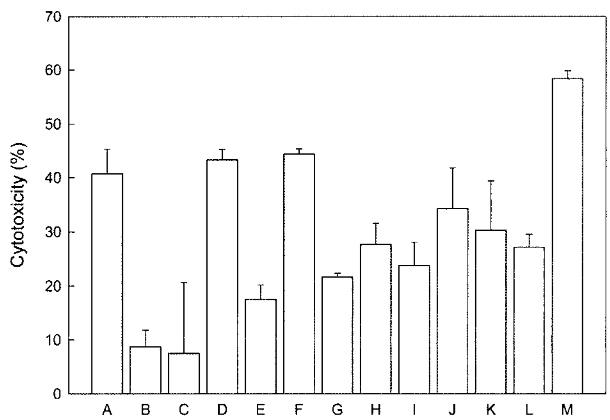

Fig. 4. Antiproliferative activity of the 13 vegetable extracts against HCT116 cells. A, red kale; B, chicory; C, lettuce; D, beet; E, red mustard; F, sesame; G, pak-choi; H, angelica; I, green mustard; J, tai-gu choi; K, treviso; L, ornamental kale; M, new-beet.

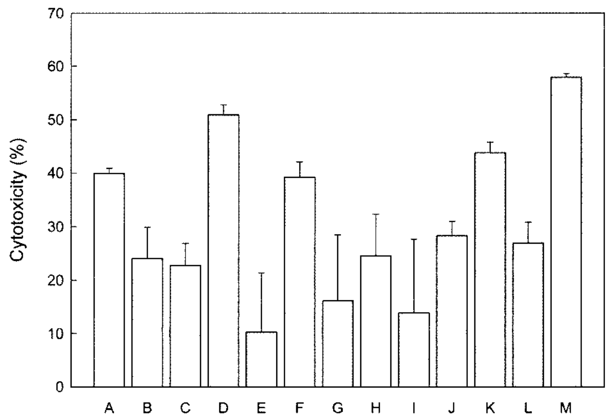

Fig. 6. Antiproliferative activity of the 13 vegetable extracts against MCF7 cells. A, red kale; B, chicory; C, lettuce; D, beet; E, red mustard; F, sesame; G, pak-choi; H, angelica; I, green mustard; J, tai-gu choi; K, treviso; L, ornamental kale; M, new-beet.

NCI-H460 cells. The greatest antiproliferative activity (57.6%) of new-beet was observed in MCF7 cells lines (Fig. 6).

Many studies have shown that flavonoids are effective in inhibiting proliferation in human cancer cell lines; however, results depended on specific compounds of flavonoids, types of flavonoids, and sources of flavonoids. Antiproliferative activities of quercetin of hydroxylated flavone aglycons against lung, colon, breast, and prostate cancer cells ranged from 40 to 86, and that of flavone glycosides showed >200 at IC_{50} (11). Kawaii *et al.* (10) also examined 27 flavonoids for their antiproliferative properties.

In summary, all test vegetable extracts showed strong antioxidant properties against ABTS and DPPH radicals. Among the test samples, the highest activities were obtained from new-beet extract. New-beet also had the greatest antiproliferative activity in gastric, colon, lung, and breast cancer cell lines. Although obvious correlations between polyphenolics/flavonoids and antioxidative/antiproliferative activity were not found (data not shown), the 13 leafy vegetables behave as antioxidants and inhibit proliferation of cancer cells with the exception of gastric cancer cells. Particularly, new-beet showed consistently high antioxidative and antiproliferative activities against cancer cells. These results will provide useful information about polyphenolic contents of vegetables and enhance consumption of leafy vegetables.

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