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Contributed Paper

Antioxidant Capacity of Broccoli Seeds Grown in Thailand

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ABSTRACT

Five broccoli seed cultivars grown in Thailand were assayed to determine variation in phenolics, flavonoids, flavonols, ascorbic acid, carotenoids and tocopherols within and between each genotype. The variability in hydrophilic and lipophilic antioxidant capacity found among these genotypes suggests that potential efficiency from antioxidants vary considerably from genotype to genotype. Results indicated that there was a substantial variation between cultivars. Total antioxidants capacity were significantly higher in 'Top Green #067' (4.871 mg/100mg DW) cultivar than in 'Green Queen' (3.855 mg/100mg DW), 'Packman' (3.876 mg/100mg DW), 'Pak Ging' (3.934 mg/100mg DW) cultivars which were not significantly different, while 'Rod Fai' cultivar demonstrated significantly lower concentration (3.292 mg/100mg DW).

Keywords: antioxidant, broccoli seeds, carotenoids, flavonoids, flavonols, phenolics.

1. INTRODUCTION

It is well known that fruits and vegetables contained several classes of compounds that can potentially contribute to antioxidant capacity, including flavonoids, water-soluble vitamin (ascorbic acid) as well as the fat-soluble vitamins (vitamin E) and carotenoids (vitamin A precursor). The antioxidant capacity of fruits and vegetables has been determined with a variety of methods. Many researches have indicated that broccoli (*Brassica oleracea* var. *italica*) possessed high antioxidant capacity [1-5]. The present investigation evaluated the levels of antioxidant compounds, such as total phenolics, flavonoids and flavonols, ascorbic acid, carotenoids and tocopherols in different genotypes of broccoli seeds grown in Thailand.

2. MATERIALS AND METHODS

2.1 Seed Materials

Broccoli were grown in the winter growing season in October, 2002, in the Phu Ruea Highland Cultivation Experimental Station, Loei province, Thailand. Five broccoli seeds cultivars composed of, 'Green Queen', 'Packman', 'Pak Ging', 'Rod Fai', and 'Top Green #067' were selected. The seeds were harvested in April-May, 2003 and were packed then sealed in aluminium foil-laminated sachets and stored under refrigeration at 4 °C until use.

2.2 Reagents and Standards

The following reagents were used: 2.0 M Folin-Ciocalteu phenol reagent, n-hexane,

aluminium trichloride hydrate, sodium acetate (Labscan Asia, Thailand), ethanol, methanol and potassium hydroxide (Merck, Germany), HPLC-grade solvents acetonitrile, chloroform, methanol, m-phosphoric acid (Merck, Germany), glacial acetic acid and sodium acetate (J.T. Baker, NJ, USA). The following standards were used: gallic acid, rutin (hydrate, min 95%), anhydrous sodium carbonate, α-tocopherol, β-carotene and L-ascorbic acid (all from Sigma, St. Louis, USA).

2.3 Extraction

Broccoli seeds were ground with a mortar and pestle and then extracted with 99.5 % methanol. Two-step extraction was applied by the shaking flasks with 10 g (± 0.01 g) of seeds and 100 mL (50+50) of solvent in a shaking machine (Shaking Water Bath™, model SB-200-10, Thailand). Each extraction step was completed within 3 h. The extracts were filtered, concentrated in a rotary evaporator apparatus (Büchi, Flawil, Switzerland) at approximately 40 ˚C. A BioRad spectrophotometer was used for measurements of total phenolics, flavonoids and flavonols.

2.3.1 Total Phenolics Assay

The content of total phenolic compounds in broccoli seeds methanolic extracts was determined by Folin–Ciocalteu method [6-7]. For the preparation of calibration curve 1 mL aliquots of 0.024, 0.075, 0.105 and 0.3 mg/mL ethanolic gallic acid solutions were mixed with 5 mL Folin–Ciocalteu reagent (diluted ten-fold) and 4 mL (75 g/L) sodium carbonate. The absorption was read after 30 min at 20 ˚C at 765 nm and the calibration curve was drawn. One mL methanolic seed extract was mixed with the same reagents as described above, and after 1 h the absorption was measured for the determination of seed phenolics. All determinations were performed in triplicate. Total content of phenolic compounds in seed methanol extracts in gallic acid equivalents (GAE) was calculated by the following formula:

$$C = c\ (V/m') \quad (1)$$

where: C-total content of phenolic compounds, mg/g seed extract, in GAE; c-the concentration of gallic acid established from the calibration curve, mg/mL; V-the volume of extract, mL; m′-the weight of pure seed methanolic extract, g.

2.3.2 Flavonoids and Flavonols Assay

The content of flavonoids was determined by a US pharmacopeia method, 1989 [8] using rutin as a reference standard compound. One mL of seed extract in methanol was mixed with 1 mL aluminium trichloride in ethanol (20 g/L) and diluted with ethanol to 25 mL. The absorption at 415 nm was read after 40 min at 20 ˚C. Blank sample was prepared from 1 mL of the seed extract and 1 drop of acetic acid was added, and diluted to 25 mL. The absorption of rutin solutions was measured under the same conditions. All determinations were carried out in triplicate. The amount of flavonoids in seed extracts in rutin equivalents (RE) was calculated by the following formula:

$$X = C\ (V/m) \quad (2)$$

where: X-flavonoids content, mg/g seed extract in RE; C-the concentration of rutin solution, established from the calibration curve, mg/mL; V, m-the volume and the weight of seed extract, mL, g.

The content of flavonols was determined by the Yermakov method, 1987 [9]. The rutin calibration curve was prepared by mixing 2 mL of 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL rutin ethanolic solutions with 2 mL (20 g/L) aluminium trichloride and 6 mL (50 g/L) sodium acetate. The absorption at 440 nm was read after 2.5 h at 20 ˚C. The same procedure was carried out with 1 mL of broccoli seeds extracts instead of rutin solution. All determinations were carried out in triplicate. The content of flavonols, in rutin equivalents (RE) was calculated by the following formula:

$$X = C\ (V/m) \quad (3)$$

where: X-flavonols content, mg/g seed extract in RE; C-the concentration of rutin solution,

established from the calibration curve, mg/mL; V, m-the volume and the weight of seed extract, mL, g.

2.4 Ascorbic Acid Extraction and Assay

10 g of seeds were homogenized with 15 mL of 5% m-phosphoric acid. The homogenate was filtered with three layers of cheesecloth and the residue was treated with 10 mL of 5% m-phosphoric acid for two successive extractions. The filtrates were combined and centrifuged at 4000g for 10 min. The supernatant was collected and made up to 25 mL and then filtered through a 0.45 μm Sartolon polyamide (Sartorius, Germany) membrane filter for HPLC analysis on an analytical 5 μm LiChrosorb RP-18 column, 250x4.6 mm (Merck, Darmstat, Germany). HPLC analysis was performed using a Hewlett Packard Model HP 1090 photodiode array detector, and an automated injector. Ultra-pure water generated by the Milli-Q system (Millipore, Bedford, MA, USA). Data were processed with HP ChemStation Software. The mobile phase consisted of acidified distilled water (0.1% phosphoric acid) (solvent A) and acetonitrile (solvent B), at a ratio of 95:5. The flow rate was 1.0 mL/min. L-ascorbic acid was detected at 254 nm.

2.5 Carotenoids Extraction and Assay

The broccoli seeds sample (10 g) was extracted successively (usually three times) with 40 mL ethanol-hexane (1:1), followed by filtration in a glass sintered funnel. The combined filtrates were transferred to a separatory funnel containing 25 mL hexane and 20 mL water. After gentle shaking for 30 to 60s, the phases were allowed to separate. The aqueous phase was drawn into a second separatory funnel and re-extracted two or three times with 40 mL portions of ethanol-hexane (1:1). The combined hexane extracts were washed three times with water, collected, dried over anhydrous Na_2SO_4 and concentrated in a rotary evaporator (40 ˚C), the volume being adjusted to 25 mL. Samples were separated on a Novapak 5mm C18 column (150x3.9 mm) by an HPLC HP 1090 equipped with a photodiode array detector. The mobile phase consisted of acetonitrile-chloroform (9:1). The flow rate was 1.0 mL/min. The content of carotenoids was expressed as milligramm β-carotene equivalents per 100g DW at 450 nm.

2.6 Tocopherols Extraction and Assay

The broccoli seeds were ground (10 g), saponified and protected from light for over 2 h with stirring in an alcoholic solution of potassium hydroxide plus ascorbic acid to avoid oxidation of liposoluble vitamins. The composition of reagent was as follows: 50 mL of ethanol, 10 mL of aqueous 0.1% ascorbic acid, 10 mL of aqueous 40% KOH and 25 mL of water. The solution was then extracted with n-hexane (2x25 mL) and the extracts were washed with water (2x10 mL). The organic phase was removed by evaporation in a rotary evaporator under vacuum at 50 ˚C and the residue was dissolved in methanol (50 mL) and filtered through Sartolon polyamide (Sartorius, Germany) membranes filter with a pore size of 0.45 μm to clean the extracts before their injection into the chromatographic system. Chromatography was carried out with a Hewlett Packard liquid chromatograph equip model HP 1090. A 290 nm wavelength kit was fitted into the detector. A 250 x 4.6 mm i.d. LiChrosorb RP-18 (5 μm) column was used with 2.5 mM acetic acid-sodium acetate in methanol-water (97:3) as mobile phase. The flow rate was 1.0 mL/min.

2.7 Data Analysis

Analysis of variance was performed on the data to identify significant differences in antioxidant capacity among genotypes. Least significant differences (Fisher's LSD) were determined among genotypes at $p = 0.05$. Pearson's correlation coefficients for antioxidant capacity were calculated using means of each assayed in triplicate measurements from each genotype.

3. Results and Discussion

3.1 Content of Phenolics, Flavonoids and Flavonols

The major constituents consist of phenolic groups which act as free radical terminators and/or primary consideration as antioxidants, it was reasonable to determine the total amount of phenolics in the seed extracts. Flavonoids comprise the most widespread and diverse group of polyphenolic plant secondary metabolites. These compounds play an important role in biological and chemical activities including free radical scavenging properties. Such properties are especially distinct for flavonols [10-11]. Based on such concept, the content of both phenolics was also evaluated in the extracts. The total antioxidants values presented in Table 1 is the sum of all these antioxidants detected in each cultivar. The content of phenolic compounds (mg/100g DW) in methanolic extracts, determined from regression equation of calibration curve ($y = 9.6581x+0.0796$, $R^2 = 0.99$) and expressed in gallic acid equivalents (GAE), varied between 0.88 and 1.58. It is also noteworthy that the highest amount of phenolics was found in 'Top Green # 067' cultivar, whereas in 'Packman' and 'Pak Ging' cultivars were no significant differences among the other cultivars. It can be obtained that the content of phenolics in the extracts correlates with their antiradical activity in our previous data [12]. The correlation coefficient between data of total phenolic compounds and the scavenging of radical cation 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), (ABTS) assay is 0.65, and in ferric reducing antioxidant power (FRAP) assay, R is 0.87, confirming that phenolic compounds are likely to contribute to both free radical-scavenging and metal chelation activity of these seed extracts [13-14].

The content of flavonoids (mg/100g DW), in rutin equivalents varied from 0.86 to 1.51. The highest amount of flavonoids was found in the extracts of 'Top Green # 067' cultivar, while 'Green Queen', 'Packman' and 'Pak Ging' cultivars contained remarkably no significant amounts of these compounds. Relatively low amounts of flavonoids was also detected in 'Rod Fai' cultivar which contained the lowest amount of phenolics. It can be reported that the amount of flavonoids in the analysed seed extracts showed high correlation with the total amount of phenolics (R=0.95). It is known that only flavonoids of a certain structure and particularly hydroxyl position on the aromatic ring of the phenolic molecules demonstrate antioxidant properties, in general these properties depend on the ability to donate hydrogen or electron to a free radical [11, 14]. The concentration of flavonols, expressed in rutin equivalents (regression

Table 1. Total amount of phenolics, flavonoids, flavonols, ascorbic acid, β-carotene and tocopherols in broccoli seeds cultivars available in Thailand.[a]

Compounds	Cultivar				
	Green Queen	Packman	Pak Ging	Rod Fai	Top Green #067
Total phenolics (in GAE)	1.226aA	1.170bA	1.166bA	0.896cA	1.573dA
Total flavonoids (in RE)	1.027aB	1.017aB	1.063aB	0.898bA	1.467cB
Total flavonols (in RE)	1.024aB	1.070bC	1.060cB	0.962dB	1.128eC
Ascorbic acid	0.034aC	0.038bD	0.032aC	0.021cC	0.045dD
β-carotene	0.526aD	0.566bE	0.596cD	0.502dD	0.636eE
Tocopherols	0.018aE	0.015bD	0.017cE	0.013dC	0.022eD

[a] Data are means of triplicate experiments. All values are expressed in mg/100g of DW. Small letters (a-e) compare means between cultivar in each row, capital letters (A-E) compare means between antioxidant capacity (the same genotype) at 5% level according to ANOVA test.

equation of calibration curve y = 5.042x+ 0.30, R^2 = 0.99) in mg/100g of seed extract, varied in a same wide range as compared with total phenolics and flavonoids, from 0.96 to 1.13. Total flavonols were significant differences among the cultivars. Indeed, some correlation between total phenolics and flavonols can be observed, as well as the highest amount was found in 'Top Green # 067'. Flavonols are known as important compounds in terms of free radical scavenging properties. In our study flavonols contents had higher correlation with flavonoids contents (R=0.84). Detailed examination of phenolics, flavonoids and flavonols composition in seed extracts is required for the comprehensive assessment of individual compounds exhibiting antioxidant activity.

3.2 Content of Ascorbic Acid, β-carotene and Tocopherols

To investigate the amount of these vitamins, analysis of variance were generated using means of individual compounds among the broccoli cultivar accessions (Table 1). Results also show no significant differences in concentrations of ascorbic acid among 'Green Queen' and 'Pak Ging' cultivars tested. Ascorbic acid concentrations in broccoli seeds ranged from 0.020 to 0.047 mg/100g DW. These results do, however, indicate that significant differences of β-carotene and tocopherols levels were found in 'Pak Ging', 'Rod Fai' and 'Top Green #067' cultivars surveyed here. The β-carotene concentrations ranged from 0.490 to 0.640 mg/100g DW, although tocopherols concentrations ranged from 0.013 to 0.023 mg/100g DW. At this point, β-carotene and tocopherols were low correlated with ABTS assay (R = 0.28 and 0.58, respectively). This is not surprising, in general, because these are associated with the lipid phase in biological systems, whereas ascorbic acid is found in the aqueous phase.

4. Conclusion

Significant differences in total antioxidant concentrations were also observed within each broccoli seed cultivar grown in Thailand. The mean total antioxidant concentration was the highest in 'Top Green #067' (4.871 mg/ 100mg DW) cultivar, whereas the lowest was 'Rod Fai' cultivar (3.292 mg/100mg DW). Total phenolics compounds concentration was higher in 'Top Green #067' cultivar than in 'Green Queen', 'Packman', 'Pak Ging' and 'Rod Fai' cultivars, respectively, while in 'Green Queen', 'Packman', 'Pak Ging' cultivars tested in this study the flavonoids concentrations did not differ significantly. These findings indicated that 'Green Queen' and 'Pak Ging' cultivars had similar concentrations of ascorbic acid at p = 0.05.

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