

Conference Paper

Potential Lutein Extract of Broccoli (*Brassica oleracea* L. var. *italica*) as Antiradical Abts (2,2-Azinobis Acid, 3-Ethyl Benzothiazoline-6-Sulfonic Acid)

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Abstract

Broccoli (*Brassica oleracea* L. var. *italica*) is one vegetable that is often consumed by people with high nutrient content i.e protein, fat, vitamin C, fiber, potassium and calcium. Carotenoids and glutathione are contained in broccoli that are antioxidants. Lutein is a carotenoid group contained in broccoli is capable of reducing the accumulated products of free radicals in the body. The purpose of this research is to extract and identify compounds lutein from flowers of broccoli and test its potential as antiradical to the compound ABTS (2,2-Azinobis acid, 3-ethyl benzothiazolin-6-Sulfonat). Identification of lutein compounds qualitatively by TLC showed Rf value of 0.72 cm. Analysis of Lutein by HPLC showed a peak at the retention time of 3:03 min, approaching retention time of lutein standard 3:01 min. Test of potency of antiradical ABTS showed that lutein extract from flower of broccoli has antioxidant activity with IC_{50} value of $157.5278 \mu\text{g} \cdot \text{mL}^{-1}$. Fractionation of lutein extract using column chromatography with Silica gel 60 as stationary phase and mixture of methanol : chloroform : n-hexane (4:20:8) as mobile phase resulted in 4 fractions with antioxidant activity increased. Fraction 3 has the highest antiradical ABTS activity with IC_{50} $39.5842 \mu\text{g} \cdot \text{mL}^{-1}$. Vitamin E is used as a standard had antiradical ABTS activity IC_{50} $5.3036 \mu\text{g} \cdot \text{mL}^{-1}$.

Keywords: ABTS; anti-radical; Broccoli (*Brassica oleracea* L. var. *italica*); lutein.

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Received: 11 February 2017

Accepted: 08 March 2017

Published: 26 March 2017

Publishing services provided
by Knowledge E

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Selection and Peer-review under the responsibility of the ICBS Conference Committee.

1. Introduction

Fruits and vegetables are the most important source of carotenoids in the human diet, and knowledge about this is important for preventive medicine [1, 2]. *Brassica* vegetables such as cauliflower and broccoli are popular and are among the most consumed vegetables in the world. Many epidemiological studies have indicated that a diet rich in these vegetables is associated with reduced risk of a several type of cancers, type 2 diabetes, and cardiovascular diseases [3, 4]. Additionally, *Brassic*as are known to possess antioxidant activity [5]. Such beneficial health properties of these

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crops are due to the presence of health-promoting compounds such as vitamins, glucosinolates, phenols, flavonoids, minerals, and carotenoids.

Lutein is one of the most widely distributed carotenoids. The main sources of lutein are vegetables and fruit. Calvo collected data of lutein concentration in 74 species of fruit and vegetables reported by 44 authors since 1990 [6]. He observed that lutein concentration in green vegetables (lettuce, broccoli, water cress, parsley, pea, spinach) was higher than in yellowish-white vegetables. Red-orange vegetables are recognised as the weakest lutein source. Lutein compounds found in broccoli ranged from (0.41 to 1.02) mg · kg⁻¹ wet weight.

Lutein has no provitamin activity, but it displays biological activity in relation to human health. Evidence suggests that its intake is inversely related to the occurrence of eye diseases such as age-related macular degeneration and cataracts. The two major carotenoids in the human macula and retina are lutein and zeaxanthin, and they are often referred to as xanthophylls, or macular pigment. Handelman et al. found a fivefold higher content of these carotenoids in the macula compared with the peripheral retina [7]. Lutein may also serve to protect skin from UV-induced damage and may reduce the risk of cardiovascular disease [8].

Reactive oxygen species (ROS) are constantly formed in the human body by normal metabolic action and these exert oxidative damaging effects by reacting with nearly every molecule found in living cells including nucleic acids, proteins, lipids or DNA and may involve in several chronic and degenerative diseases including gastritis, reperfusion injury of many tissues, atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases and others [9]. If excess ROS and free radicals are not eliminated by endogenous antioxidant system.

ABTS is soluble in both aqueous and organic solvents, and it reacts relatively rapidly compared to DPPH, which normally takes several hours for the reaction to be completed. Color interference of the DPPH assay with samples that contain anthocyanins leads to under-estimation of antioxidant activity. However, this problem does not occur with the ABTS assay, especially when the absorbance is measured at 734 nm [10].

In this work, Lutein from broccoli (*Brassica oleracea* L. var. *italica*) identified using thin layer chromatography then fractionated by chromatography columns and analyzed using HPLC. The antioxidant activity of lutein extracts of Broccoli (*B. oleracea* var. *italica*) are reported.

2. Material and Method

2.1. Phytochemical screening of the Broccoli flower

It was carried out to test for the presence or otherwise of alkaloids, flavonoids, saponins, tannins, phenolic, anthraquinone and steroid/terpenoids derivatives using standard methods [11].

2.2. Extraction of lutein from broccoli flowers (*B. oleracea* var. *italica*)

Broccoli flowers were cleaned under tap water, chopped into small pieces and dried. Samples were allowed to equilibrate in open air and ground to pass a 0.5 mm sieve. About 20 g of broccoli powdered were macerated with 200 mL of n-hexane for 48 h. The total crude broccoli extracts were collected and evaporated to dryness by rotavapor. The broccoli oleoresin containing total carotenoids was mixed with isopropanol with stirring and heated to a temperature of 60°C. An aqueous 50% potassium hydroxide solution, equivalent to the alkali, was added slowly, and the solution was maintained at 60°C with stirring for a period of 90 min. The saponified mixture was allowed to cool to room temperature and then diluted with deionized water to reduce the solvent concentration to approximately 50% (v/v) with gentle mixing. The mixture was allowed to stand for approximately 60 min followed by addition of four times (v/v) deionized water just before centrifugation. The lutein crystalline precipitate was collected using a Sharples tubular bowl centrifuge. The precipitate was washed twice with additional water and was dried under vacuum at 40°C to less than 5% moisture content [12].

2.3. Thin layer chromatography

Mobile phase for this TLC is methanol : chloroform : n-hexane (1 : 5 : 2). Put it in the glass chamber, and let it saturate the chamber. Dissolve lutein extracts of Broccoli (*B. oleracea* var. *italica*) with methanol, Then prepare the plate Silica gel GF 254, make a spot of the sample about 1 cm from the bottom plate with a capillary tube. Moreover, do the same thing for lutein reference standard on the same plate. Then put the plate inside the chamber and close it, wait for solvent (mobile phase) to reach the top of the plate, and we will see the spot move up. Then observe it. The spots were noted and the R_f value was calculated by measuring the distance traveled by the solute and the solvent. The separated fractions were observed under UV light λ 254 nm.

The highest antioxidant activity fraction obtained from column chromatography were isolated using preparative thin layer chromatography.

2.4. Antioxidant activity assays of lutein from broccoli (*Brassica oleracea* var. *italica*)

Samples extract lutein from Broccoli obtained as described above were used to determine the antioxidant capacity by ABTS Radical Cation Decolorisation Assay. Antioxidant capacity of the extract was evaluated using a Shimadzu Spectrophotometer, by the improved ABTS method, as described by Hansraj et al. [13] with slight modification. The ABTS radical cation preparation: ABTS 2 mM (0.0548 g in 50 mL) was prepared in distilled water. Potassium per sulfate 70 mM (0.0189 g in 1 mL) was prepared in distilled water. Potassium persulphate (200 μ L) and ABTS (50 mL) were mixed and used after 2 h. This solution was used for the assay. To the 0.5 mL of various concentrations of lutein extract of broccoli/lutein standard, 0.3 mL of ABTS radical cation and 1.7 mL of phosphate buffer pH 7.4 were added and the absorbance was measured at 734 nm. Vitamin C was used as positive control. The experiment was performed in triplicate.

The inhibition was calculated in following way:

$$I (\%) = 100 \times (A_0 - A_1)/A_0 \quad (1)$$

Where A_0 is the absorbance of the control, A_1 is the absorbance of the lutein extract/standard. A percent inhibition versus concentration curve was plotted and the concentration of sample required for 50% inhibition was determined and expressed as IC_{50} value. The lower the IC_{50} value indicates high antioxidant capacity.

2.5. Analysis of lutein from broccoli by high-performance liquid chromatography (HPLC)

Lutein compounds in each fraction were determined using high-performance liquid chromatography (HPLC). Sample from preparative TLC to be analyzed is dissolved in methanol and then injected into HPLC instrument. HPLC instrument conditions were used: column C18 5 μ m, mobile phase methanol : acetonitrile (70 : 30), detector 450 nm and flow rate 1.0 mL \cdot min⁻¹.

No	Phytochemical	Inference
1.	Alkaloids	–
2.	Flavonoid	+
3.	Saponin	+
4.	Tannins	–
5.	Phenolic	–
6.	Anthraquinones	–
7.	Steroid/Terpenoid	+

Note: (+) = Present, (–) = absent.

TABLE 1: Phytochemical analysis of ethanolic leaf extract of broccoli flower (*B. oleracea* var. *italica*).

3. Results and Discussions

3.1. Results phytochemical screening of the broccoli flower (*B. oleracea* var. *italica*)

Phytochemical analysis of medicinal plants has shown that numerous compounds in plant traditionally used for medicinal purpose have chemical properties effective at treating illness. Phytochemicals are chemical compound formed during the plant normal metabolic process. These chemicals are often referred to as secondary metabolite of which there are several classes including alkaloids, flavonoids, coumarins, steroids, glycosides, gum, phenol, tannins, terpenes and terpenoids [11]. This study has revealed the presence of phytochemicals considered as active medicinal chemical constituents. Important medicinal phytochemicals such as flavonoids, saponin, and steroids/terpenoids were present in the samples. While alkaloids, tannin, phenolic and anthraquinone were not found in the broccoli flower (Table 1).

Flavonoids are a group of polyphenolic compounds with known properties of free radical scavenging, antibacterial and anti-inflammatory action [14]. Tannins are plant polyphenols, which have ability to form complexes with metal ions and with macromolecules such as proteins and polysaccharides [15]. Dietary tannins are said to reduce feed efficiency and weight gain in chicks. Saponins also have haemolytic activity against RBC [16].

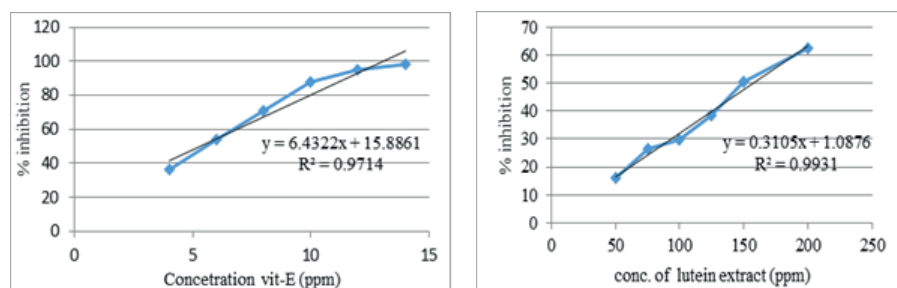


Figure 1: The relation between vitamin E concentration ($\text{mg} \cdot \text{L}^{-1}$) and lutein extract of broccoli flowers (*B. oleracea* var. *italica*) against % inhibition.

No.	Test Material	Concentrations ($\text{mg} \cdot \text{L}^{-1}$)	Absorption	% Inhibition	IC_{50} ($\mu\text{g} \cdot \text{mL}^{-1}$)
	Blank		0.579	—	—
1.	Vitamin E	4	0.370	36.096 7	5.303 6
		6	0.266	54.058 7	
		8	0.167	71.157 1	
		10	0.069	88.082 9	
		12	0.028	95.164 0	
		14	0.011	98.100 1	
		2.	Lutein Extract	50	
75	0.416 5	26.489 4			
100	0.397 5	29.801 2			
125	0.348 5	38.491 4			
150	0.284 0	50.318 3			
200	0.478 5	62.552 3			

*replications were done in *duplo*.

TABLE 2: Results of test potential antioxidant of vitamin E and lutein extract of flowers of broccoli (*B. oleracea* var. *italica*) using ABTS compound.

3.2. Antioxidant potential test of lutein extract of broccoli flowers (*B. oleracea* var. *italica*) to the ABTS compound (2,2'-Azinobis acid [3-benzothiazoline ethyl]-6-sulfonate)

The tests were carried out using the compound ABTS with UV-VIS spectrophotometer; the absorbance values were obtained and then calculated based on linear regression (Figures 1 and 2).

The test results on the antioxidant potential of vitamin E and lutein extract of flowers of broccoli (*B. oleracea* var. *italica*) using ABTS method can be seen in Table 2.

Vitamin E became a standard reference or positive control, and the antioxidant activity in it was measured. The result of vitamin E antioxidant test showed IC_{50} values

of $5.3036 \mu\text{g} \cdot \text{mL}^{-1} < 200 \mu\text{g} \cdot \text{mL}^{-1}$. Meanwhile, the result of antioxidants lutein extract test of broccoli flowers (*B. oleracea* var. *italica*) showed the IC_{50} value of $157.5278 \text{ mg} \cdot \text{mL}^{-1}$. The measurement results showed that the higher the concentration of the test solution was used, the lower the absorption was. That was caused by the fact that the flower extracts of broccoli (*B. oleracea* var. *italica*) contained lutein, which was non-polar, soluble in organic solvents, and slightly soluble in lipid or fat, so it could bind to the compound ABTS. The advantages of using ABTS method was its flexibility; it could be used to measure the radical activity of hydrophilic and lipophilic in the pure compound, extract food, and liquids.

3.3. Identification of lutein extract using thin layer chromatography (TLC)

TLC technique was aimed to identify compounds qualitatively. This method has the advantages of being very simple, sensitive, rapid in separation, and easy to reunite the separated compounds. Identification of lutein compounds by TLC using a solvent mixture of methanol eluent : chloroform : n-hexane in the ratio of (1 : 5 : 2) as mobile phase and a slab of silica gel 60 F254 as stationary phase. Selection of mobile phase is based on the nature of the polarity of a sample used. The appropriate eluent was required to separate the compound components in the sample appropriately. The results showed that the lutein extract of broccoli flowers (*B. oleracea* var. *italica*) had the same R_f value as the standard lutein had i.e. R_f 0.723 0.

3.4. Results of lutein extract fractionation of broccoli flower (*B. oleracea* var. *italica*) using column chromatography

Fractionation process used column chromatography with eluent consisting of methanol : chloroform : n-hexane (4 : 20 : 8) and the stationary phase using silica gel 60. The result of the separation of lutein compounds in extracts of broccoli flower (*B. oleracea* var. *italica*) produced nine fractions. Results of the nine fractions then were re-simplified by using thin layer chromatography resulting four fractions with different spotting patterns. The antioxidant activities of four fractions were tested using ABTS reagent using six concentrations of (50, 75, 100, 125, 150 and 200) $\text{mg} \cdot \text{L}^{-1}$ to obtain the fraction with the highest activity for further analysis by using HPLC.

3.5. ABTS antioxidant activity test of the fractions

Testing of antioxidant activity using ABTS free radical against the four fractions are listed in Table 3.

Fraction	Concentration	% inhibition	Equation	IC ₅₀
1.	50	40.951 1	Y = 0.1770 x + 33,1792	95.032 7
	75	48.612 9	R ² = 0.9212	
	100	49.801 8		
	125	65.653 8		
	150	67.767 5		
	200	72.919 4		
2.	50	44.121 5	y = 0.2192x + 32.2984	80.755 4
	75	48.745 0	R ² = 0.9609	
	100	53.385 0		
	125	55.217 9		
	150	71.070 0		
	200	74.768 8		
3.	50	53.10 4	y = 0.2850x + 38.7185	39.584 2
	75	54.95 4	R ² = 0.9610	
	100	69.08 8		
	125	75.82 6		
	150	87.97 8		
	200	90.88 5		
4.	50	36.459 7	y = 0.4349x + 16.8692	76.180 2
	75	41.6116	R ² = 0.9603	
	100	65.9180		
	125	81.5060		
	150	81.7700		
	200	98.4140		

TABLE 3: Test of the antioxidant activity of lutein broccoli sample resulted from fractionation using ABTS.

Table 3 showed that the fraction 4 was less active in preventing free radicals compared to fraction 1, fraction 2, and fraction 3 as its antioxidant activity value was at $IC_{50} < 200 \mu\text{g} \cdot \text{mL}^{-1}$. It indicated that there was the only small amount of carotenoids. Lutein compounds which is carotenoid pigments, were found in green plants and vegetables. Class of compounds that had strong antioxidant activity that carotenoids such as zeaxanthin lutein are as much as $1.403 \mu\text{g}$ and flavonoids as much as $(0.03 \text{ to } 10.85) \mu\text{g}$. Results showed that fraction 3 was the one that most actively reduced ABTS free radicals as its value was at $IC_{50} < 200 \mu\text{g} \cdot \text{mL}^{-1}$ and the value was the lowest compared to other fractions. Fraction 3 was analyzed further using HPLC.

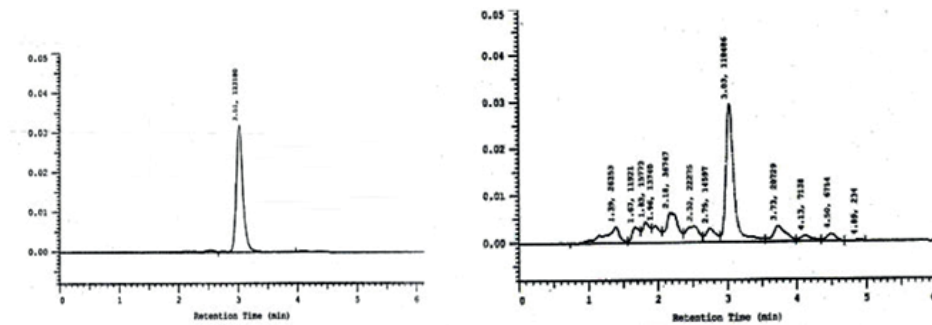


Figure 2: Chromatogram resulted from HPLC of lutein standard reference and fractioning three broccoli flower extract.

3.6. Results of identifying lutein compounds in broccoli flower extract (*B. oleracea* var. *italica*) using high-performance liquid chromatography (HPLC)

Results of the identifying lutein compounds in broccoli flowers extract (*B. oleracea* var. *italica*) can be seen in Figure 2 below.

In a qualitative analysis of standard lutein and fractions 3 broccoli flower extracts (*B. oleracea* var. *italica*) with High-Performance Liquid Chromatography method using a mobile phase of methanol, chromatogram peak Acetonitrile (70 : 30) were obtained consecutively at a retention time of 3.01 min and 3.03 min. Retention times obtained by fractions approached three broccoli flower extract of lutein standard retention times. It showed that the fraction 3 of broccoli flower extracts (*B. oleracea* var. *italica*) contained lutein compounds. The advantage of using High-Performance Liquid Chromatography method was that it had a high sensitivity and required such short time. This method could be used for qualitative and quantitative analysis, which was not destructive to the sample studied.

4. Conclusions

It can be concluded that the extraction of the broccoli flowers (*Brassica oleracea* L. var. *italica*) using method proposed by Madhavi et al. produced lutein compounds [12]. The lutein extract had antioxidant activity against ABTS radical with IC_{50} value at $157.5278 \mu\text{g} \cdot \text{mL}^{-1}$ whereas vitamin C as reference had its IC_{50} value at $5.3036 \mu\text{g} \cdot \text{mL}^{-1}$. Results of fractionation by chromatography column using silica gel 60 showed 4 fractions potential as an antioxidant with IC_{50} values of fraction 3 < fraction 4 < fraction 2 < fraction 1. Identification of broccoli flower (*B. oleracea* var. *italica*) lutein extract with Thin layer chromatography method had the same R_f value as a standard lutein, i.e R_f 0.7230. Analysis of lutein of broccoli flower (*B. oleracea* var. *italica*) with

HPLC showed the highest peak of chromatogram at the retention time of 3.03 min. The result approached the retention time of 3.01 min of standard lutein.

Acknowledgements

We would like to express our sincere thanks to S. Octarini Santoso for skilful technical assistance.

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