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Article

Inhibition of Browning on Fresh Apple Juices by Natural Phytochemicals from *Rumex crispus* L. Seed

Hwa-Jin Suh[1†], Sanggyu Park[2], and Shin Park[2*]

[1]*Department of Biotechnology,* [2]*Division of Life and Environment, Daegu University, Gyeongsan, Gyeongbuk 712-714, Republic of Korea*

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Efficacy of phytochemicals of *Rumex crispus* L. seeds as an anti-browning agent was examined. Anti-browning candidates were screened using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Antioxidant activities of DPPH radical scavengers increased with increasing amount of the extracts. Butanol (BE) and ethyl acetate extracts (EE) displayed significantly higher DPPH radical-scavenging activity as compared to that of ascorbic acid. Anti-browning activity was evaluated by monitoring the change of *L, *a**, and *b** values as well as total color differences (ΔE). BE and EE effectively inhibited browning in apple juice at a concentration below 0.3 mg mL^{-1}, whereas other extracts did not show anti-browning effects in apple juice. *R. crispus* L. seed extracts could be of good resources as anti-browning agents. Results indicate possible contribution to the development of new and natural anti-browning agents with potential applications to reduce oxidative damage.**

Key words: anti-browning, antioxidant, phytochemical compounds, *Rumex crispus* L.

Consumption of food or food products requires minimally processed fruit products such as fresh-cut fruits and fruit juices. Moreover, consumers have placed a high premium on foods retaining their natural nutritional and flavor qualities. In order to meet these demands, the food industry has focused on the development of new processing techniques. The browning, catalyzed by polyphenol oxidase (PPO), significantly increases upon exposure of these products to air. Therefore, development of effective procedures to control browning has been a critical part in food processing. Both chemical and physical approaches have been extensively investigated and applied. For the physical approach, several processing methods such as thermal and high-pressure treatments, application of edible coatings, and supercritical CO_2 treatment have been proposed to prevent browning in fruit products [Prestamo and Arroyo, 1999; Mchugh and Senesi, 2000; Gui *et al*., 2006; Krapfenbauer *et al*., 2006; Li *et al*., 2007]. Generally, addition of PPO inhibitors to suppress enzymatic browning and reductants to counteract non-enzymatic browning are preferred to chemical methods [Kubo *et al*., 2000]. A number of natural compounds such as ascorbic acid, thiol-containing amino acid, kojic acid, oxalic acid, 4-hexylresorcinol, oxyresveratrol, lincorice extract, and mulberry twig extract were reported to demonstrate sufficient inhibitory activities when added to fruit products [Gacche *et al*., 2004; Jeon and Zhao, 2005; Nerya *et al*., 2006; Oms-Oliu *et al*., 2006; Rojas-Grau *et al*., 2006; Li *et al*., 2007].

Rumex crispus L. is a perennial wild plant which exists abundantly in Korea and other Asian countries. It is widely used in traditional medicine to control the fungal disorders and is considered as a potent peptic agent. The extracts of *R. crispus* L. have been shown to possess an antioxidant, antimicrobial and antifungal activities [Yildirim *et al*., 2001]. Previously the antioxidant activities of ether extracts of leaf and seed from this plant were evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH), reducing power and thiocyanate method in ether, ethanol, and water extracts. Whereas, leaf and root extracts of *R. crispus* have been shown to possess antimicrobial and antifungal activities [Choi *et al*., 2005]. Based on the dual properties of these extracts, one might conjecture as a promising anti-browning agent for their possible application in fruit

†Present address: R&D Team Gyeongbuk Natural Color Industry Institute, Yeongchen, 770-906, Korea

*Corresponding author
Phone: +82-53-850-6751; Fax: +82-53-850-6759
E-mail: spark@daegu.ac.kr

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and vegetable products. The objective of the present study was to determine the efficacy of various extracts of *R. crispus* L. as natural potent anti-browning photochemicals to suppress the browning in fresh apple juice.

Materials and Methods

Apples. Fresh Fuji apple were purchased from a local traditional market (Gyeongsan, Korea) and immediately used for analysis. For the juice preparation, apples were peeled, cored, sliced, and homogenized in a homogenizer for 1 min at 6°C. The homogenate was pressed through a piece of cheesecloth to remove large particles and centrifuged at 1,000 g for 5 min at 6°C. The supernatant was used directly for analysis.

Preparation of extracts. The seeds of *R. crispus* L. were harvested at the end of June, in the Gimje (Jeonbuk, Korea) and were left on a bench to dry. The dried sample (517 g) was extracted with 70% ethanol for 10 h at 20°C. Extraction was followed by filtration (Whatman No. 2 filter) and evaporation by vacuum rotary evaporator (Büchi B-480, Flawil, Switzerland) at 40°C to discard ethanol. From this aqueous solution (1 L), four different solvent-water partitioning systems were progressively used including hexane (H), chloroform (C), ethyl acetate (E), and butanol (B). Final remaining aqueous solution was lyophilized by freeze dryer and dissolved in methanol (100 mL) and centrifuged at 8,000 rpm for 10 min at 4°C. The supernatant was used as methanol extract (ME), and the pellet was dissolved in water (WE) (Fig. 1). Six different extracts were completely dried, and residual parts of all the extracts were weighed and prepared at 500 mg mL^{-1} in their respective solvents. The extract solutions were diluted at 50 mg mL^{-1} in dimethylsulfoxide (DMSO), and were kept in a refrigerator before use. The process of extraction yielded hexane (HE, 0.89 g), chloroform (CE, 0.79 g), ethyl acetate (EE, 1.79 g), butanol (BE, 1.10 g), ME (1.02 g), and WE (0.91 g) extracts. Solvents (analytical grade) for extraction were obtained from Sigma-Aldrich (St Louis, MO).

The screening of anti-browning candidates. Total phenolic contents of various seed extracts (HE, CE, EE, BE, ME, and WE) of *R. crispus* L. were determined by Folin-Ciocalteu reagent in alkaline medium and were expressed as gallic acid equivalents (mg GA g^{-1}) [Osawa and Namiki, 1981]. The reaction was conducted in triplicate.

The antioxidant activity of *R. crispus* L. seed extracts was measured on the basis of the scavenging activities of the stable DPPH free radical [Molyneux, 2004]. DPPH radical-scavenging activity was measured against a blank at A_{517} using spectrophotometer. The values of SC_{50}, concentrations of samples required to scavenge 50% of free radicals, were calculated from the regression equation prepared from the concentration of various extracts, and percentage inhibition of free radical formation was assayed. Synthetic antioxidant reagent, ascorbic acid (AA), was used as a positive control, and all tests were carried out in triplicate.

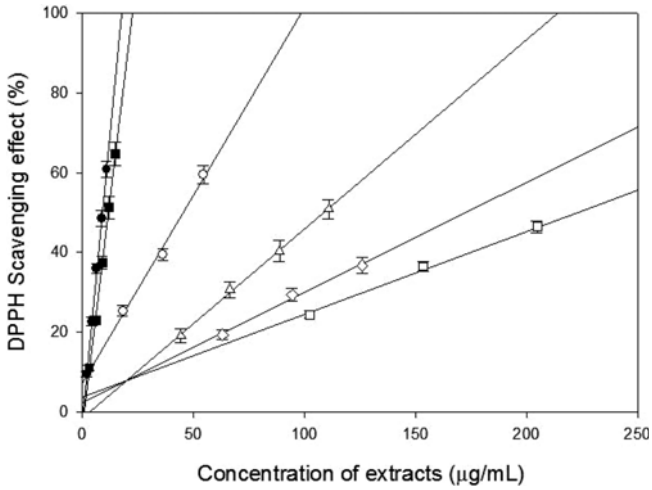

Fig. 1. Changes in DPPH scavenging activity depending on concentration.

Color measurements. Visual assessment of color development in the samples was performed with a digital camera, and the relative extents of browning were measured with a tristimulus reflectance colorimeter (Minolta CR-400 Chroma Meter, Osaka, Japan) [Li *et al*., 2007]. For juices, 5 mL of thoroughly mixed juice was centrifuged at 1000 g for 5 min to discard cell debris. Apple juice was exposed to white light (10 W m^{-2}) for 0, 1.5, 3, and 6 h and then transferred into the liquid tester of Minolta CR-400 Chroma Meter for measurement of L^* (changes of darkness), a^* (changes of redness), and b^* values (changes of yellowness). Measurements were made immediately after the tested materials were added and at predetermined time intervals thereafter. Total color difference (E) was used for evaluation as follows: $E=[(L^*_t-L^*_{initial})^2+(a^*_t-a^*_{initial})^2+(b^*_t-b^*_{initial})^2]^{0.5}$. All tests were performed at 6°C and carried out in triplicate.

Measurement of mass spectrometry analysis. The gas chromatography-mass spectrometry (GC-MS) analysis of the BE and EE was performed using a GC-MS (Model QP 2010, Shimadzu, Japan) equipped with a DB-1 MS-fused silica capillary column (30 m×0.25 i.d., film thickness 0.25 μm). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas was used as a carrier gas at a constant flow rate of 1 mL min^{-1}. Injector and mass transfer line temperatures were set at 220 and 290°C, respectively. The oven

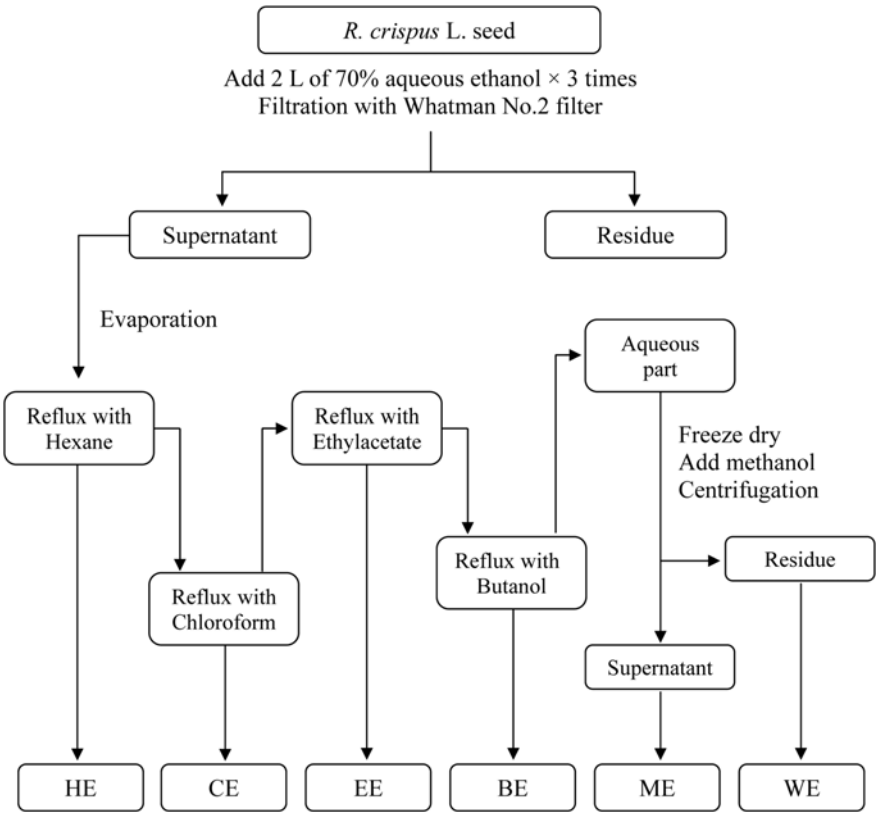

Fig. 1. Schematic diagram of *R. crispus* L. seed extracts. Averaged results from triplicated experiments are given, with error bars representing SD (open circle, WE; open square, ME; open square, BE; closed square, EE; open triangle, CE; open diamond, HE).

temperature was programmed from 50 to 210°C at 10°C min^{-1}, held isothermal for 1 min, and finally raised to 300°C at 10°C min^{-1}. Diluted samples (1/100, v/v, in methanol) of 1 μL was manually injected at the split less mode. The relative percentage of the each extract constituents was expressed as percentage by peak area normalization. The identity of the components of the extracts was assigned by comparison of their retention indices, relative to a series of *n*-alkane indices on the DB-1 capillary column and GC-MS spectra from the Wiley 6.0 MS data and literature data.

Results and Discussion

In the present study, the effect on DPPH radical scarvenging and the contents of total phenolics of *R. crispus* L. were determined for the screening of anti-browning candidates. Although standard and quantitative methods to monitor browning reaction in foods have not yet been set, the most applied parameters are *E*, *L**, *a**, and *b** values. Generally, a decrease in *L** and an increased in *a**, *b** or *E* reveal the occurrence of browning in food products [Luo and Barbosa-Canovas, 1997; Rojas-Grau *et al*., 2006], in agreement with our preliminary observation; thus, *L** and *b** were chosen as screening parameters for estimating the anti-browning activity in the present study.

DPPH radical scavenging activity and total phenolics. DPPH, a stable free radical, produces a violet solution in ethanol. It is reduced and decolorized in the presence of an antioxidant molecule in a relatively short time compared to other methods, and is often used in evaluating the radical scavenging activity of antioxidants such as natural and synthetic pure compounds as well as plant extracts [Brand-williams *et al*., 1995; Ko *et al.*, 1998]. The scavenging activity of the extracts differed depending on the concentration of extracts added (Fig. 2). The scavenging effects of six different seed extracts on the DPPH radical were found in the following order: EE >BE>WE>HE>CE>ME, which were 91.8, 91.5, 53.8, 21.9, 16.1, and 14.8% at 0.05 mg mL^{-1}, respectively as compared to the ascorbic acid (92.6%). Prior to testing, HE was dissolved in 2:2:1 part solution (methanol+ethyl acetate+hexane), and then added with 1 volume DMSO, because non-polar solvent extracts could not be detected due to their low solubility in aqueous assay system. The values of 50% radical scavenging (SC_{50}) for DPPH were calculated from the regression equation, prepared from the various concentrations of each extracts (Table 1). Interestingly, EE (SC_{50}=12 μg mL^{-1}) and BE (SC_{50}=9 μg

Table 1. Antioxidant activity and total phenol content of various extracts of *R. crispus* L. seed

	+ Control	Various solvent					
	AA	WE	ME	BE	EE	CE	HE
DPPH [a]	0.010	0.046	0.223	0.009	0.012	0.172	0.109
TPC [b]		0.101	0.037	0.261	0.328	0.069	0.043
Yield [c]		0.910	1.020	2.100	1.790	0.790	0.890
(%)		(0.18%)	(0.20%)	(0.41%)	(0.35%)	(0.15%)	(0.17%)

AA, WE, ME, BE, EE, CE and HE is ascorbic acid, water, methanol, buthanol, ethyl acetate, chloroform and hexane extract, respectively. [a]SC_{50} the concentration of sample required to scavenge 50% of radicals, [b]total phenolic contents (mg GA/mg extract) [c]extraction yield (g). The values are given as means (n=3) from linear regression curve and rounded off decimal point 4th.

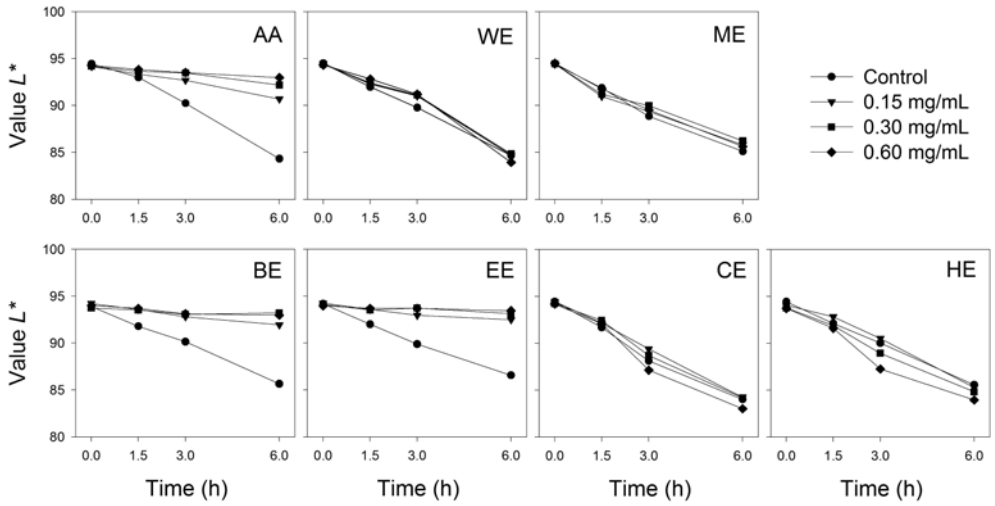

Fig. 3. Reflectance measurement of L* of apple juices with ascorbic acid and various extract ranging from 0 to 0.6 mg mL^{-1}. The value L* represent the means of three determinations. (AA, ascorbic acid; WE, water; ME, methanol; BE, butanol; EE, ethylacetate; CE, chloroform; HE, hexane extracts).

mL^{-1}) extracts had remarkable efficacy on radical scavenging effect as compared to AA (SC_{50}=10 μg mL^{-1}), a well-known synthetic antioxidant. Based on the findings that these extracts showed DPPH activities, it could be conjectured that some of extracts could be used as promising anti-browning agents in fruit and vegetable products.

The total phenolic contents (TPC) of various seed extracts were determined following a modified Folin-Ciocalteu reagent method, and results were expressed as gallic acid equivalents (Table 1). TPC of various extracts of *R. crispus* L. were found in the range of 0.037-0.328 (mg GA/g extract). Higher levels of TPC were observed for EE and BE, whereas lower levels were observed for ME and HE. TPC may contribute directly to the antioxidative action. Significant differences were observed for TPC among the various extracts.

Anti-browning activity of *R. crispus* seed extracts. The anti-browning activities of various seed extracts of *R. crispus* were first evaluated by direct addition of the extracts into fresh prepared Fuji apple juice samples as follows. Various extracts (final concentration: 0.15, 0.3, and 0.6 mg mL^{-1}) were added to freshly prepared fine juice, which formed the reference point for latter evaluation of the differential progression of browning in the control and in samples intervened with the tested chemicals or extracts. On the basis of the changes in *L**, *a**, *b**, and *E* values (Figs. 3 and 4), the ascorbic acid and six different seed extracts demonstrated a dose-dependent anti-browning activity. In our previous work (data not shown) BE and EE showed significant anti-browning effect during the 6 h observation period and also were proved to be excellent singlet oxygen quenchers with low cytotoxicity. BE and EE at different concentrations highly suppressed the *L** value of browning as compared to that of AA (Fig. 3). The other extracts did not show any suppression capacity of browning in *value L**. Moreover, decrease of value *L** (changes in darkness), increase of value *a** (changes in redness), and increase of value *b** (changes in yellowness) were suppressed by treatments of

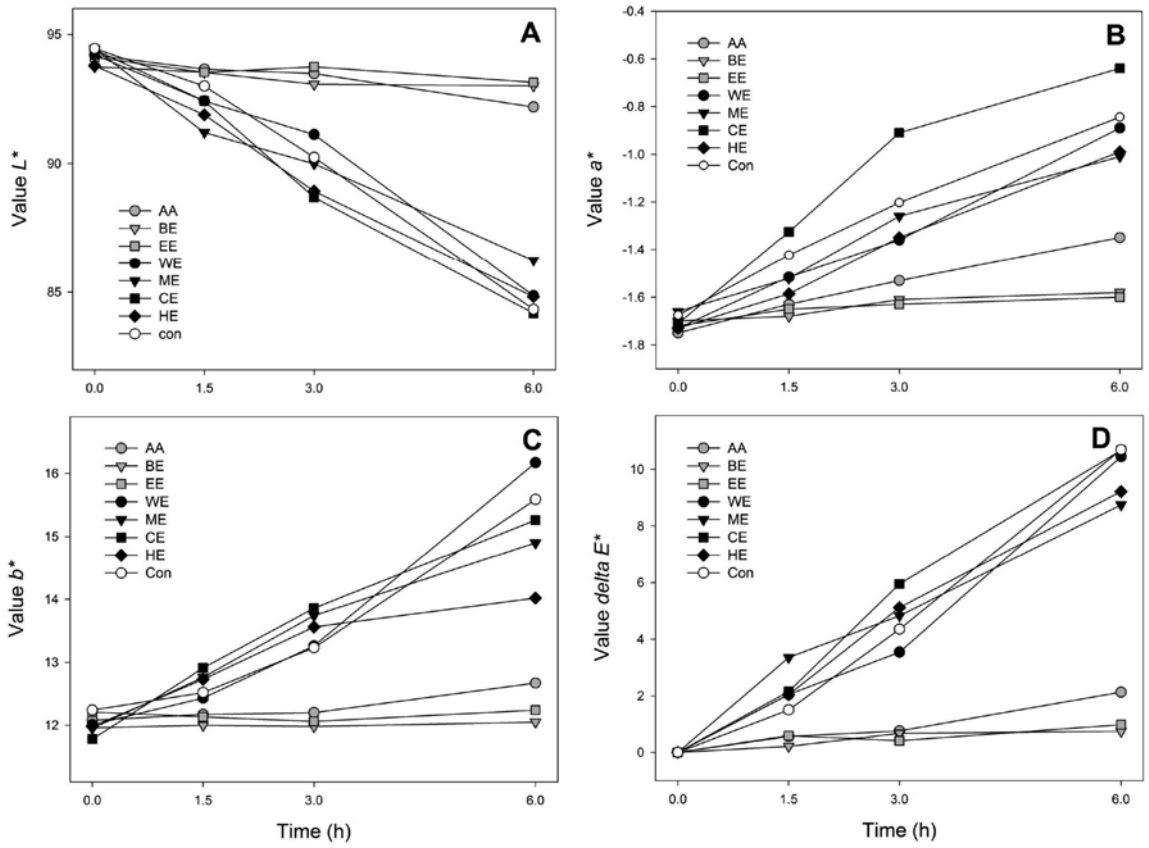

Fig. 4. Reflectance measurement of color change of apple juices with AA and sample extracts at 0.3 mg mL^{-1}. The values of color change represent the means of three determinations.

BE and EE (Fig. 4). Total color difference (value *E*) revealed that the closed symbols (WE, ME, CE, and HE) increased in a time-dependent manner, whereas those of the gray symbols (BE, EE, and AA) were not affected (Fig. 4D).

On the basis of changes in color values, EE and BE showed more effective activities than those of the other extracts with significant statistical difference at 0.6 mg mL^{-1} at two different testing periods (Table 2). Among the six different seed extracts, EE and BE were found to be much more potent in suppressing apple juice browning based on visual observation and measured values; however, suppression capacity of the other extracts was not observed after 6 h of treatment. The noticeable difference between various seed extracts may be related to their different contents of chemical composition as supported by subsequent quantitative GC-MS analysis, which revealed that the EE and BE contained phthalic acid, 1,2,3-benzenetriol, and 2,4-di-*tert*-butylphenol. However, it should be noted that the two extracts at 0.03 mg mL^{-1} in apple juice showed much stronger anti-browning activity than ascorbic acid at the same concentration. These results indicate the existence of the anti-browning activity in these extracts. They could be PPO inhibitors and/or reductants, among others, which might produce a synergistic effect and result in a very strong inhibition of apple juice browning.

Chemical composition of the BE and EE. Because BE and EE exhibited the strongest anti-browning activity in apple juice among all extracts, they were further subjected to analysis by GC-MS. GC-MS analyses of the BE and EE led to the identification of five different compounds, representing 66.8 and 60.1% of the total extracts, respectively. The solvent and base peaks take up 33.2 and 39.9%. The identified compounds are listed in Table 3 according to their elution order on a ZB-1 capillary column. The detected compounds were 2,2,5-trimethylhexane-3,4 dione (8.2%), 1,2,3-benzenetriol (5.1%), 2,4-di-*tert*-butylphenol (4.9%), diethyl Phthalate (53.7%), 1,2,3-benzenetriol (3.7%), 2,2,5,5 tetramethyl-hexane (11.9%), and Phthalic acid ethyl isopropyl ester (48.3%). The phytochemical (particularly 1,2,3-benzentriol and 2,4-di-*tert*-butylphenol) are phenolics in nature. Thus, the above results suggest these phenolic compounds might contribute to the antioxidant activity of the extracts [Leston, 2000; Suh *et al.*, 2011]. Therefore, the antioxidant activity of phenolics is important factors contributing to the anti-browning activity of these

Table 2. Color change values of apple juices treated with various extracts and ascorbic acid at concentration of 0.6 mg mL^{-1}

Antibrowning agent	L*		a*		b*	
	0 h	6 h	0 h	6 h	0 h	6 h
Control	94.45±0.86	84.33±1.21	-1.68±0.05	-0.84±0.06	12.15±0.29	16.15±0.66
AA	94.27±1.02	92.89±0.82	-1.75±0.03	-1.35±0.10	12.14±0.61	12.97±0.46
WE	94.32±0.59	83.95±0.73	-1.73±0.13	-0.77±0.03	11.94±0.34	15.89±0.31
ME	94.51±0.81	85.64±0.67	-1.66±0.08	-0.89±0.05	11.87±0.57	14.24±0.72
BE	93.01±1.08	92.98±1.31	-1.70±0.06	-1.46±0.09	12.02±0.61	12.16±0.91
EE	93.78±0.86	93.45±1.26	-1.725±0.08	-1.54±0.07	11.98±0.71	12.15±1.06
CE	94.18±1.12	81.03±1.64	-1.71±0.04	-0.81±0.07	11.75±0.92	15.69±1.17
HE	93.72±0.95	83.969±1.82	-1.78±0.10	-1.06±0.11	11.77±1.01	14.26±1.06

AA: ascorbic acid, WE: water extract, ME: methanol extract, BE: butanol extract, EE: ethyl-acetate extract, CE: chloroform extract, HE: hexane extract. Each value is expressed as the mean±standard deviation (n=3).

Table 3. Major chemical composition of BE and EE of *R. crispus* L. seed

	Compound	RI	RT	Composition (%)
BE	2,2,5-Trimethylhexane-3,4dione	1039	7.200	8.2
	1,2,3-Benzenetriol	1243	9.400	5.1
	2,4-di-*tert*-butylphenol	1555	12.817	4.9
	Diethyl Phthalate	1639	19.567	53.7
EE	2,2,5,5-Tetramethylhexane	846	7.192	11.9
	1,2,3-Benzenetriol	1243	9.390	3.7
	Phthalic acid, ethyl isoporpyl ester	1674	13.583	48.3

BE: butanol extract, EE: ethyl-acetate extract, RT: retention time.

extracts.

In conclusion, *R. crispus* seed extracts may play an important role in the inhibition of apple juice browning without affecting the sugar contents (data not shown) and can protect cells against oxidative stress with no cytotoxicity. Furthermore, the ability of EE and BE to scavenge radicals could significantly affect total phenolic contents and compositions, an important factor for the potency of anti-browning capacity. Considering the high consumer demand for natural anti-browning agents due to the beneficial health effects, *R. crispus* seed extracts (particularly BE and EE), with a very strong anti-browning activity and good antioxidant capacity, can be utilized as vital candidates for food processing, and the development of functional foods. Moreover, with widespread sources and relatively easy extraction from these resources, ethyl-acetate and butanol extractions could be the reliable steps for obtaining *R. crispus* seed extracts with reasonable yields of important phenolic compounds. The main advantage of adding natural anti-browning agents to the fresh fruits and vegetables products is that they are derived from edible plant tissues and thus free of regulatory constraints. Because no texture and sensory data were collected in the present study, whether the nutritional values, aroma, and flavor of apple juices were changed or not by the addition of *R. crispus* seed extracts is yet unknown. Therefore, further studies are essentially required in this regard.

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