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Contents of Polyphenols and Limonoids in Citron (*Citrus junos* Sieb. ex Tanaka) Seed Extracts and Their Antioxidant Properties

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Abstract Contents of phytochemicals in citron (*Citrus junos* Sieb. ex Tanaka) seeds and their effects on antioxidant activities were investigated. Methanol extract of defatted citron seeds contained the highest amounts of total polyphenols followed by 70% ethanol and water extracts. Neohesperidin was the most predominant citrus flavanones in these extracts. The highest amounts of limonoids were found in methanol extract, and this extract was the most efficient in scavenging both DPPH and ABTS radicals. All 3 extracts also exhibited good antioxidant activities against attack of linoleate free radicals on β-carotene. When methanol extract was sequentially fractionated into ether, ethyl acetate, *n*-butanol, and water fractions, butanol fraction contained the highest amounts of polyphenols otherwise most of limonoids were concentrated in ethyl acetate fraction. A positive relationship between radical scavenging activities and total polyphenol contents in fractions was observed while antioxidant activity on β-carotene seemed more related with contents of limonoids and other hydrophobic polyphenols.

Keywords: citron seed, polyphenol, limonoid, radical scavenging activity

Introduction

Citron (*Citrus junos* Sieb. ex TANAKA) is a citrus fruit that is cultivated in northeast Asia. This fruit is originated in China, and introduced to Korea and Japan during the Tang dynasty. The fruit looks a bit like a very small grapefruit with an uneven skin, and can be either yellow or green depending on the degree of ripeness. Its peculiar tart flavor, which closely resembles that of grapefruit with overtones of mandarin orange, has been very attractive to Koreans. Citron is mostly consumed in Korea being thinly sliced and combined with sugar or honey to make thick marmalade-like syrup containing pieces of chopped rinds. A table spoon of this syrup (which can be either made at home or purchased in a glass jar) stirred into a cup of hot water makes a beverage called 'citron tea', which is used as a herbal remedy for the common cold and similar winter illness.

Epidemiological surveys have shown an inverse relationship between the intake of fruit and the incidence of coronary heart disease and some type of cancers (1,2). Antioxidants are also well-recognized for their potential role in reducing incidence of such diseases (3-7). Therefore, antioxidants in fruits are assumed to play major roles in such health beneficial effects. Citron is famous for containing abundant antioxidants such as vitamin C, polyphenol compounds, and limonoids (8,9). When mature citron is processed for tea or other beverage products in Korea, massive amounts of seeds are collected and discarded. Citron seeds take 14-16% of total citron fresh weight. Grapefruit closely resembles citron as mentioned previously, and its seeds possess excellent antioxidant capabilities (10,11). Therefore, we can anticipate such antioxidant activities from citron seeds too. Objectives of this study were to investigate properties of key phytochemicals, such as polyphenols and limonoids, in citron seeds and evaluate their various antioxidant capabilities

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Materials and Methods

Materials Mature citron was harvested in Gochang, Jeonbuk, Korea on November 2005. After citron was processed for 'citron tea', wasted citron seeds were collected and immediately delivered to our laboratory. Citron seeds were washed thoroughly under running tap water and dried at 50°C for 12 hr. Then, citron seed coats were removed mechanically and seeds were ground into a powder.

Extraction After defatted by hexane for 24 hr, the ground citron seed samples were extracted with refluxing 20 volumes of methanol at 70°C, 70% ethanol at 80°C and water at 90°C for 3 hr. Each solvent of the extracts was evaporated under reduced pressure (34-36 kPa) using a rotary evaporator followed by freeze drying for 72 hr. Dried methanol extract was further fractionated by dissolving it into distilled water and partitioned in a separatory funnel by adding equivalent amounts of ether, ethyl acetate, and *n*-butanol, consecutively. Each fraction was concentrated to dryness by a vacuum evaporator.

Determination of total polyphenol contents Contents of total polyphenols in each fraction were determined by the methods of Singleton *et al.* (12) with minor modification. Samples (0.125 mL) were added with distilled water (0.5 mL) and Folin-Ciocalteu's reagent (0.125 mL), and the mixture was incubated for 6 min at room temperature. Then, 1.25 mL of 7% sodium carbonate and 3.0 mL of distilled water were added and incubated for another 90 min. The absorbance at 760 nm was read and converted to total polyphenol contents according to the calibration curve of standard rutin.

HPLC analysis of vitamin C, polyphenols, and limonoids HPLC analysis of vitamin C, polyphenols, and limonoids in defatted citron seed extracts was performed using a Jasco PU-990 equipped with pump/851-AS, sampler/807-IT, integrator/LG-1580-04, gradient/DG 1580-54, and degasser (Jasco, Tokyo, Japan). Freeze-dried citron seed extracts were dissolved in water (5 mg/mL), filtered with 0.45 mm membrane and injected at ambient temperature into Eclipse XDB-C18 (4.6×250 mm, Agilent Technologies, Palo Alto, CA, USA). The mobile phase for vitamin C was 0.01% solution of sulphuric acid adjusted to pH 2.6 with a flow rate of 1.0 mL/min. The mobile phases for polyphenol compounds were: (A) 50 mM phosphoric acid and (B) methanol with a flow rate of 0.7 mL/min. Gradient elution was applied from 98% (A) as follows: 0-10 min, 98-95% (A); 10-50 min, 95-65% (A); 50-60 min, 65-60% (A); 60-70 min, 60-40% (A); 70-90 min, 40% (A). The isocratic mobile phases (acetonitrile : methanol : water, 10 : 41 : 49) were used for limonoids analysis with a flow rate of 1.0 mL/min. Vitamin C, polyphenolic compounds, and limonoids were detected at 245, 280, and 210 nm, respectively. Four citrus flavanones (naringin, neohesperidin, naringenin, and hesperitin) and 2 limonoids (limonin and nomilin) were identified and confirmed by comparing their retention time on LC with their standards (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and spiking them into the extracts. Their contents in extracts were calculated based on the calibration curve on each standard.

DPPH radical scavenging activity The free radical scavenging activity of each extract of citron seed was measured by the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method proposed by Brand-Williams *et al.* (13). Briefly, a 0.1 mM of DPPH (Sigma-Aldrich Chemical Co.) in ethanol was prepared, and 0.2 mL of this was added to 1.0 mL of samples of various concentrations. Absorbance at 525 nm was read after 20 min incubation, and $DPPH^{\cdot}$ scavenging activity was calculated according to the following equation.

$$\text{DPPH scavenging activity (\%)} = [(A_0 - A_1)/A_0] \times 100,$$

where A_0 was the absorbance in the absence of samples, and A_1 was the absorbance in the presence of samples. The percentage of remaining $DPPH^{\cdot}$ against the sample concentration was plotted to obtain the amount of sample necessary to decrease the initial concentration of $DPPH^{\cdot}$ by 50% (EC_{50}).

ABTS cation radical scavenging activity $ABTS^{+\cdot}$ radical scavenging assay was carried out by the method of Roberta *et al.* (14) with slight modification. Stock solution was prepared by reacting 7 mM 2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid] (ABTS; Sigma-Aldrich Chemical Co.) with 2.45 mM potassium persulfate to generate the ABTS cation chromophore and allowing the mixture in the dark for 12-16 hr before use. The mixture was diluted with 5 mM phosphate buffered saline (PBS; Gibco BRL, Grand Island, NY, USA) to give an absorbance of 0.700±0.02 at 734 nm. The sample solution of 10 μL was added to 990 μL of the resulting blue-green ABTS radical solution. The mixture, protected from light, was incubated in a water bath at 37°C for 10 min and $ABTS^{+\cdot}$ radical scavenging activity was calculated according to the following equation.

$$ABTS^{+\cdot} \text{ radical scavenging activity (\%)} = [(A_0 - A_1)/A_0] \times 100,$$

where A_0 was the absorbance in the absence of samples, and A_1 was the absorbance in the presence of samples.

β-Carotene bleaching assay β-Carotene bleaching assay was carried out according to Jayaprakasha *et al.* (15) with slight modification. Forty mg of linoleic acid and 400 mg of Tween 20 were added to 1.0 mL of β-carotene (1.67 mg/mL chloroform). After chloroform was evaporated by nitrogen gas, 10 mL of distilled water was added and vigorously agitated to form an emulsion followed by filling up with 10 mM hydrogen peroxide to 100 mL. Two mL aliquots of the emulsion were transferred into different test tubes containing 100 μL of samples. The mixture was then gently mixed and placed in a water bath at 50°C. The absorbance of the mixture with or without samples at 470 nm was measured every 60 min for 4 hr.

Statistical analysis The Statistical Analysis System (SAS) software ver. 6.11 was used to perform data analysis. All analysis were determined by Duncan's multiple range test at $p<0.05$.

Results and Discussion

Contents of vitamin C, polyphenols, and limonoids in citron seed extract The yield of methanol, 70% ethanol, and water extracts of defatted citron seeds were 11.4, 13.1, and 9.7%, respectively. Contents of vitamin C was the highest in water extract (1.80 mg/g d.w.) followed by 70% ethanol (1.10 mg/g d.w.) and methanol (0.72 mg/g d.w.) extracts (Table 1). Otherwise, maximum recoveries of total polyphenols were obtained in methanol extract (34.9 mg/g d.w.) followed by 70% ethanol (24.7 mg/g d.w.) and water (14.9 mg/g d.w.) extracts. When composition of polyphenol compounds in defatted citron seed extracts was compared, methanol extract was composed more of flavonoids, which were mostly appeared after 60 min under the present HPLC analysis condition (Fig. 1). There are 3 major types of flavonoids occurring in citrus fruit: flavanones, flavones, and flavonols. Among citrus flavanones, neohesperidin was the most abundant in all citron seed extracts followed by naringin, naringenin, and hesperitin (Table 1). The compositions of these 4 flavanones in total polyphenolic compounds were 8.75, 7.24, and 6.55% in methanol, 70% ethanol, and water extracts, respectively.

Limonoids are a group of chemically related triterpenoids found in Rutaceae and Meliaceae families. Limonoids are highly oxygenated with fewer hydroxyl groups than polyphenols. They are largely responsible for delayed bitterness in citrus juice and processed citrus products. Limonoids have attracted attention due to their insect antifeedant, growth regulating activities, and anti-carcinogenic activities (16,17). Limonin and nomilin are the most prevalent citrus limonoids. Contents of limonin and nomilin were 23.5 and 14.2 mg/g d.w. of methanol extract and 13.8 and 8.0 mg/g d.w. of 70% ethanol extract, respectively, whereas very small amounts of such limonoids (3.0 mg limonin and 0.8 mg nomilin) were found in water extract of citron seeds (Fig. 2).

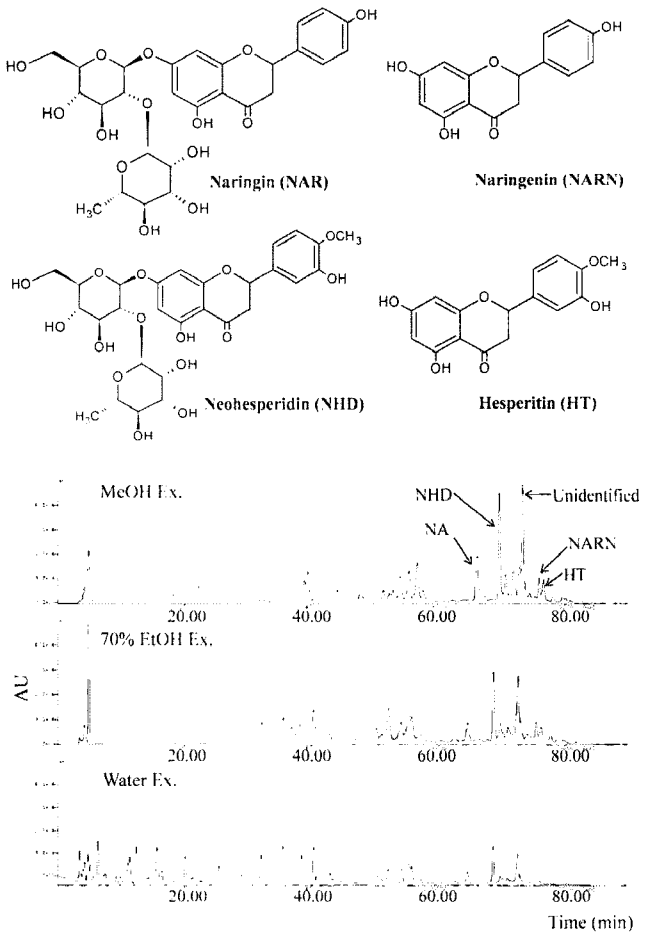

Fig. 1. Chemical structures of major citrus flavanones (upper) and HPLC chromatograms of methanol, 70% ethanol, and water extracts (bottom) of citron seed.

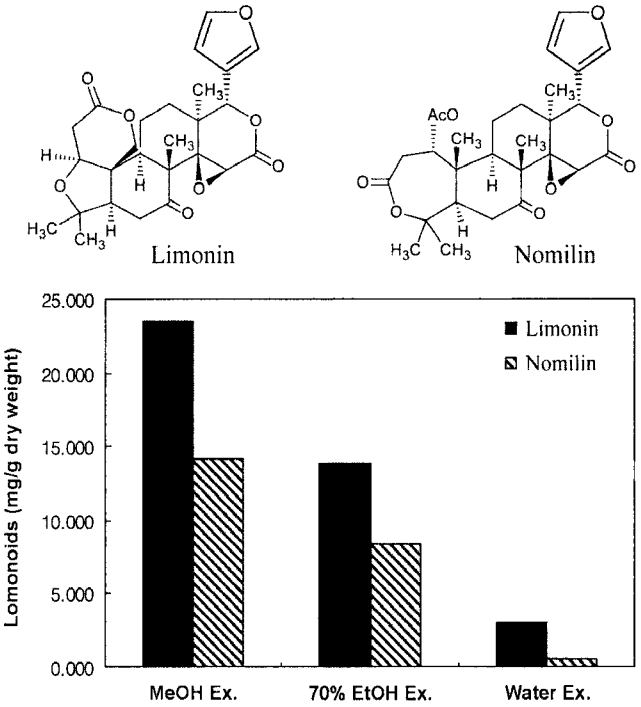

Fig. 2. Chemical structures of limonoids and their contents in citron seed extracts.

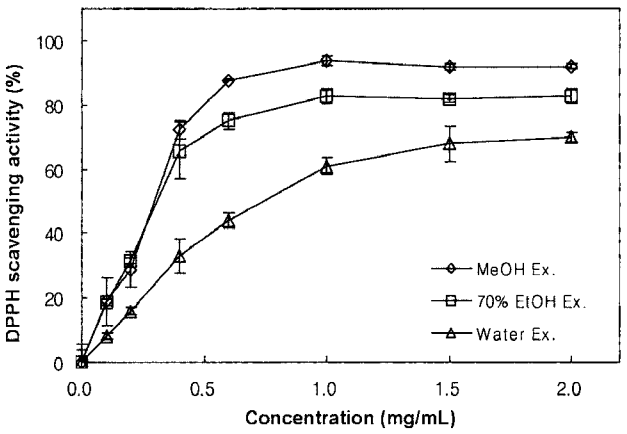

Fig. 3. DPPH radical scavenging activities of citron seed extracts at different concentrations. Values are mean±SD of 6 experiments in each concentration.

Antioxidant activities of citron seed extracts The effects of citron seed extracts on radical scavenging activities were investigated. The DPPH free radical scavenging activities of methanol, 70% ethanol, and water extracts of citron seeds were shown in Fig. 3. Methanol extract showed the best DPPH radical scavenging activity through all concentrations followed by 70% ethanol and water extracts. The EC_{50}, which is the amount of antioxidants to decrease initial DPPH radicals by 50% of the methanol, 70% ethanol, and water extracts were estimated to be 0.28, 0.31, and 0.83 mg/mL, respectively. Similar results were observed in ABTS cation scavenging activities of citron seed extracts (Fig. 4). Addition of methanol extract with various concentrations was most efficient in scavenging ABTS cation radicals. These radical scavenging activities might be related with amounts of total polyphenols and limonoids in extracts.

Effects of citron seed extracts on β-carotene bleaching were investigated. β-Carotene bleaching assay measures the degree of denatured β-carotene molecules, which are attacked by linoleic acid free radical formed upon the abstraction of a hydrogen atom in methylene groups of linoleic acid by hydrogen peroxide. As β-carotene looses their double bonds upon the attack, the system loses its characteristic orange color which can be monitored using spectrophotometer. In the absence of citron seed extracts, β-carotene in control group underwent rapid decoloration (Fig. 5). Addition of citron seed extracts effectively suppressed discoloration of β-carotene up to 4 hr. This result indicates that presence of antioxidants in each extract hinders the extent of β-carotene destruction by neutralizing the linoleate free radical and other free radicals formed in the system. It is interesting to note that, unlike radical scavenging activities, there was not a significant difference in antioxidant capabilities of 3 extracts in β-carotene-linoleate system.

Polyphenols have noticed for their attractive bio-functionalities. The interest in these compounds is mainly due to their pharmacological activity as radical scavengers (5,18-21). Antioxidative activities of polyphenol-rich extracts from various plants are of great interest to replace synthetic antioxidants by natural ones (22-25). The potential

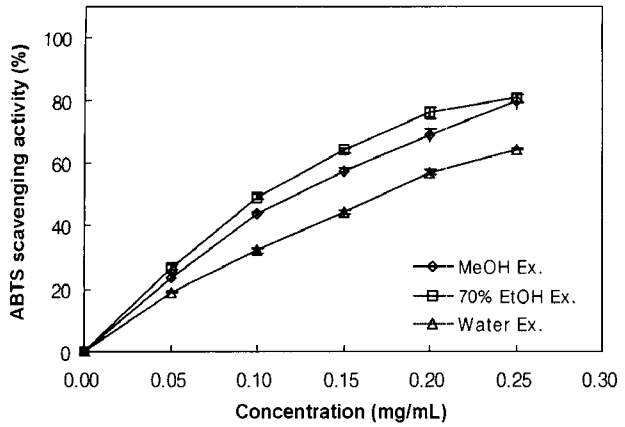

Fig. 4. ABTS cation radical scavenging activities of citron seed extracts at different concentrations. Values are mean±SD of 6 experiments in each concentration.

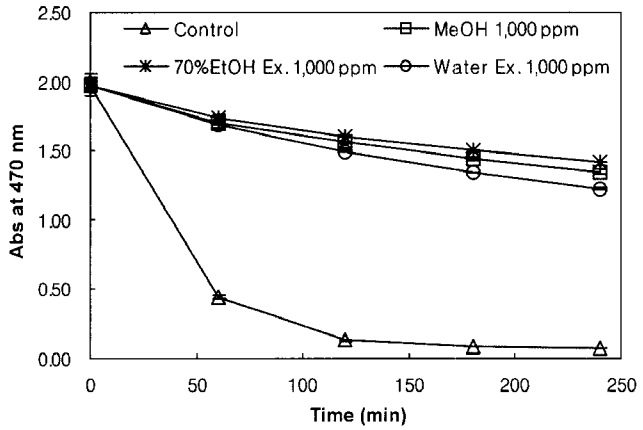

Fig. 5. Antioxidant activities of citron seed extracts in β-carotene-linoleate model system. Values are mean±SD of 6 experiments in each time period.

scavenging activities of polyphenol substances might be due to the active hydrogen donor ability of hydroxyl substitution. Limonoids are also known to have potent antioxidant activities (6,26). Their structural features probably contribute to their strong antioxidant activities in relatively hydrophobic environment. It is well accepted that hydrophilic or hydrophobic antioxidants may act synergistically and provide stronger protection to biological systems and reduce the damage caused by reactive oxygen species (ROS) including free oxygen centered radicals. Vitamin C is the major antioxidant responsible for total antioxidant activities of mature citron (9). Because contents of vitamin C were much less than polyphenols and limonoids in citron

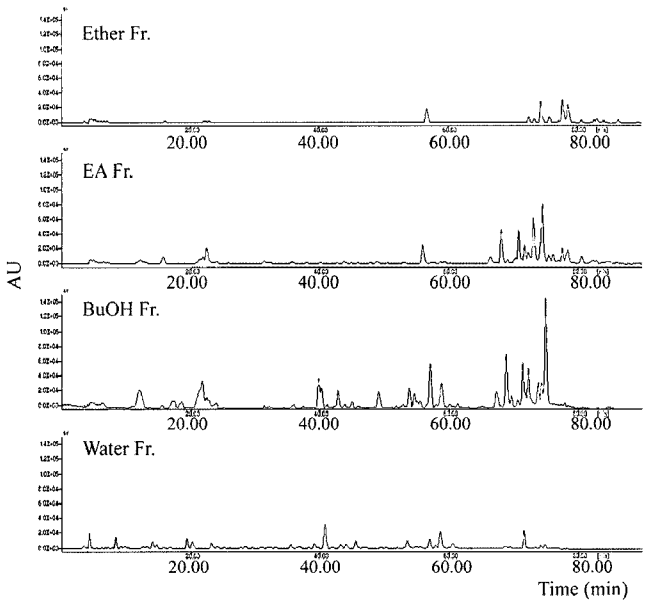

Fig. 6. HPLC chromatograms of fractions that were obtained by sequential extraction from methanol extract of citron seed.

seeds (Table 1), however, vitamin C is not considered to play a major antioxidant activity in citron seed extracts in this study.

Further fractionation of methanol extract of citron seeds Methanol extract of citron seeds, which showed the strongest antioxidant activities, was further fractionated into water, butanol, ethyl acetate, and ether fractions. Contents of polyphenols in each fraction were shown in Table 2. Butanol fraction contained the highest amounts of polyphenols (80.6 mg rutin equivalents/g d.w.) followed by ethyl acetate (45.3 mg rutin equivalents/g d.w.), water (16.3 mg rutin equivalents/g d.w.), and ether (2.7 mg rutin equivalents/g d.w.) fractions. Difference in compositions of phenolic compounds among fractions was observed by HPLC analysis (Fig. 6). Polyphenolic compounds in ether fraction were largely composed of hydrophobic naringenin (RT=75.91 min) and hesperitin (RT=76.63 min) whereas ethyl acetate fraction was mostly composed of other flavonoids including naringin (RT=66.13 min) and neohesperidin (RT=69.64 min). Butanol fraction exhibited a wide spectrum of both hydrophilic polyphenols and relatively hydrophobic flavonoids while water fraction was composed of mostly hydrophilic polyphenols.

Contents and compositions of limonoids in each fraction were also investigated (Table 2). Ethyl acetate fraction contained the highest amounts of limonin (212.0 mg/g

Table 1. Contents of vitamin C, total polyphenols, and citrus flavanones in citron seed extracts

	Vitamin C (mg/g d.w.)	Total polyphenol (mg rutin equivalents/g d.w.)	Identified citrus flavanones[1] (mg/g d.w.)				% of flavanones (in total polyphenols)
			NAR	NHD	NARN	HT	
MeOH	0.72	34.89	0.976	1.909	0.068	0.098	8.75
70% EtOH	1.10	24.75	0.447	1.268	0.054	0.023	7.24
Water	1.80	14.90	0.263	0.695	0.009	0.008	6.55

[1]NAR, Naringin; NHD, neohesperidin; NARN, naringenin; HT, hesperitin.

Table 2. Contents of total polyphenol compounds and limonoids in fractions that were obtained by sequential extraction from methanol extract of citron seed

	Total polyphenols (mg rutin equivalents/g d.w.)	Limonoids (mg/g d.w.)	
		Limonin	Nomilin
Ether Fr.	2.676	8.888	30.787
EA Fr.	45.298	212.048	56.323
BuOH Fr.	80.596	27.930	7.698
Water Fr.	16.329	0.158	0.000

d.w.) and nomilin (56.3 mg/g d.w.). Limonoids in butanol fraction was composed of 27.9 mg limonin and 7.7 mg nomilin/g d.w. Interestingly, ether fraction had higher amounts of nomilin (30.8 mg/g d.w.) than limonin (8.9 mg/g d.w.) whereas no limonoids were detected in water fraction. It is interesting to note that ethyl acetate fraction had higher contents of total limonoids (268.3 mg/g d.w.) than total polyphenols (45.3 mg rutin equivalents/g d.w.) otherwise butanol fraction contained much higher amounts of total polyphenols (80.6 mg rutin equivalents/g d.w.) than limonoids (35.6 mg/g d.w.). Such difference in compositions and contents of phytochemicals among fractions can cause different patterns in exhibiting antioxidant activities in each fraction.

Antioxidant activities of fractions of methanol extract

When each fraction was examined for DPPH radical scavenging activities, butanol fraction scavenged DPPH radicals most effectively in a dosage dependant manner. Butanol and ethyl acetate fractions exhibited even better DPPH radical scavenging activities than the reference ascorbic acid at both concentrations of 0.25 and 0.5 mg/mL (Table 3). Butanol fraction also scavenged ABTS cation radicals most effectively than other fractions while it had less ABTS cation radical scavenging activity than did the reference ascorbic acid. Moreover, the radical scavenging activities of 4 fractions are positively proportional to the concentration of total polyphenol contents. Yu *et al.* (26) reported that flavonoids showed much stronger DPPH radical scavenging activities than limonoids.

Figure 7 showed effects of addition of each fraction on the β-carotene bleaching rates. Discoloration of β-carotene occurred rapidly in the absence of citron seed fractions (control) as being observed previously. Otherwise, butanol and ethyl acetate fraction were almost identically the best

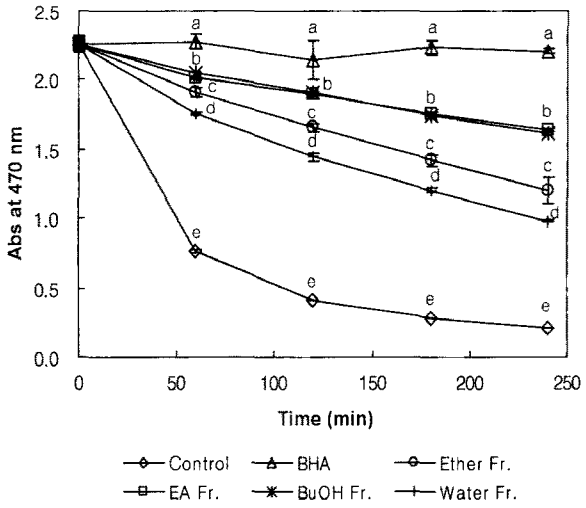

Fig. 7. Antioxidant activities of sequential fractions obtained from methanol extract of citron seed in β-carotene-linoleate model system. Values are mean±SD of 6 experiments. Different letters indicate significant difference at the level of $p<0.05$ between samples.

in suppressing β-carotene bleaching among the fractions. Butylated hydroxyanisole (BHA), as a positive reference, was stronger in suppressing β-carotene bleaching than 4 citron seed methanol fractions. Although water fraction contained more polyphenol compounds than ether fraction, its addition was less effective in suppressing oxidation of β-carotene. Because β-carotene bleaching assay is determined in more lipophilic environment than other radical scavenging activity assay systems, the extent of solubility of antioxidants in such environment is prerequisite for performing proper antioxidant activities. We found that addition of ascorbic acid had no effect on preventing oxidation of β-carotene upon the attack of radicals (data not shown). Sun *et al.* (6) reported that limonin and nomilin in citrus fruit tissues showed excellent antioxidant activities in a β-carotenelinoleic acid bleaching assay system. Therefore, contents of hydrophobic flavonoids and limonoids could be more important in determining antioxidant activity on β-carotene system than merely considering contents of total polyphenols.

Many artificial antioxidants, such as BHA, butylated hydroxytoluene (BHT), and *tert*-butyl hydroxyquinone (TBHQ), have been commonly used as food additives. However, they are reported to possibly lead to induction of cancer (27). Therefore, much effort has been invested on

Table 3. DPPH radical scavenging activities and ABTS cation radical scavenging activities of sequential fractions obtained from methanol extract of citron seed[1)]

	DPPH radical scavenging activities (%)		ABTS cation radical scavenging activities (%)	
	0.25 mg/mL	0.50 mg/mL	0.05 mg/mL	0.10 mg/mL
Ether Fr.	14.56±6.24^{d}	36.77±0.89^{d}	6.37±0.39^{e}	14.10±1.97^{e}
EA Fr.	62.55±3.39^{b}	76.86±2.67^{b}	34.46±2.84^{c}	58.52±3.80^{c}
BuOH Fr.	82.89±1.07^{a}	91.03±3.04^{a}	58.78±4.01^{b}	84.71±0.55^{b}
Water Fr.	24.61±5.32^{c}	25.06±2.24^{e}	19.74±0.52^{d}	34.62±1.04^{d}
Vit. C	64.89±0.04^{b}	66.36±3.40^{c}	100.18±0.08^{a}	100.24±0.17^{a}

[1)]Values are mean±SD of 6 experiments; different letters indicate significant difference at the level of $p<0.05$ between samples.

developing natural antioxidants from various plant resources for the purpose of health promotion. We can conclude that citron seed exhibited effective radical scavenging activities both in hydrophilic and hydrophobic environments suggesting that, as a rich source of polyphenols and limonoids, citron seeds are exploitable for antioxidant products as well as for health-promoting nutraceuticals.

Acknowledgments

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