International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 9, Issue 12, 2017

Original Article

PHYTOCHEMICAL AND ANTIOXIDANT CHARACTERIZATION OF THINNED IMMATURE *CITRUS UNSHIU* FRUITS

JI HYE KIM, MIN YOUNG KIM

Toxicology Laboratory, Faculty of Biotechnology (Biomaterials), College of Applied Life Science, SARI, Jeju National University, Jeju, Republic of Korea
Email: jeffmkim@jejunu.ac.kr

Received: 07 Oct 2017 Revised and Accepted: 02 Nov 2017

ABSTRACT

Objective: We aimed to evaluate the characterization of thinned immature *Citrus unshiu* fruits with regard to their phytochemical profile and antioxidant capacity.

Methods: Determination of total phenolic, flavonoid, and carotenoid and ascorbic acid contents was done by UV-Visible spectrophotometry, whereas UPLC-mass detection was used for the analysis of individual flavanone (naringin, hesperidin, hesperetin, neohesperedine and narirutin) and flavonol (rutin). In addition, free radicals (DPPH, O_2^-, H_2O_2 and NO) scavenging assays were used to determine the antioxidant capacity.

Results: Naringin, hesperidine, neohesperedine and narirutin were the main flavanones in all thinned immature *Citrus unshiu* fruits. The contents of total phenolic, flavonoid and carotenoid were more prevalent in immature fruits than the level found in mature fruits. All thinned immature *Citrus unshiu* fruits possess an evident antioxidant capacity. The immature *Citrus* extract concentrations providing 50% inhibition (IC_{50}) for free radicals; 1.2-1.49 mg/ml for DPPH, 1.03-1.46 mg/ml for superoxide, 1.95-3.43 mg/ml for hydrogen peroxide and 1.64-3.45 mg/ml for nitric oxide was lower than those of mature *Citrus* extracts.

Conclusion: Thinned immature *Citrus unshiu* fruits could be an economic and readily accessible source of natural antioxidants and as a possible food and pharmaceutical supplement.

Keywords: Thinned immature *Citrus*, Phytochemical composition, Antioxidant activity

DOI: http://dx.doi.org/10.22159/ijpps.2017v9i12.22971

INTRODUCTION

Citrus fruits have an important role in the world economy, with a total production of 122.5 million tons in 2010 [1]. In the last 30 **y**, there has been a steady increase of *Citrus* consumption worldwide [1]. *Citrus*, belonging to the *Rutaceae* family, grows widely and major fruit crop in Jeju Island, Korea and amount of production is about 600 thousand tons every year. It also has been mainly used in Korean folk medicine for its wide range of medicinal benefits. *Citrus* fruits are rich sources of vitamins, minerals, fibre, and phytochemicals such as carotenoids and flavonoids, which can potentially protect health. These bioactive compounds in *Citrus* fruits have been shown to be protective against chronic diseases such as cancer and heart disease [2].

Thinning fruit on *Citrus* trees is a technique intended to produce better fruit. In Jeju Island, thinning normally commences in late June to early September fruit size is about 30-40 mm in diameter, and more than 50,000 tons of *Citrus* wastes including thinned immature fruits in orchard annually have been incinerated in disposal yards and dumped into the ocean [3]. Immature *Citrus* fruits are currently of highly considerable interest to both pharmaceutical and food industries because they fruits usually have higher contents of phytochemical compounds than mature fruits [4-5]. However, there is little information is available about the utilization of thinned immature *Citrus* fruit and few systematic studies of the phytochemical profile and antioxidant activity of different *Citrus* species immature fruit. Therefore, the aim of the present study was to investigate the composition and distribution of flavonoids and phenolic acids, and their antioxidant activity of thinned immature fruit in different *Citrus* species grown in Jeju Island, which will be useful for the production of functional nutraceutical and pharmaceutical sources. We report here a comparative analysis of the bioactive compounds and *in vitro* antioxidant properties of the mature *Citrus* extract and thinned immature *Citrus* fruits.

MATERIALS AND METHODS

Chemicals and reagents

Rutin, aluminum chloride, metaphosphoric acid, L-ascorbic acid, xanthine oxidase, sodium nitrite, 1,1-diphenyl-2-picrylhydrazyl (DPPH), sodium nitroprusside, griess reagent and 2,6-dichloro-indophenol were purchased from Sigma-Aldrich (MO, USA). All other solvents were ACS grade.

Plant material and extraction procedure

Three cultivars of thinned immature *Citrus* (*Citrus unshiu* Marc.) fruits (table 1) were collected from the orchard of the Jeju-do Agricultural Technology Institute located Seogwipo-si, Jeju-do Province, South Korea, under the coordinates 33 ° 17' 30" N, 126 ° 29' 59" E during July and August 2011 and the voucher specimens (KC-IM-201101, KC-IM-201102 and KC-IM-201103) have been deposited in the herbarium of College of Applied Life Science, Jeju National University. Mature *Citrus* fruits collected at the same place during November 2011 (voucher specimens, KC-M-201101) also selected for this research (table 1). Samples were extracted twice according to the method described previously [6]. In Brief, the thinned immature whole *Citrus* fruit and, peel and pulp of mature *Citrus* fruit were separated, cut finely and successively extracted with 100% MeOH. The solvent was soaked three times at room temperatures over a period of 3 d.

Analysis of total phenolic and flavonoid contents

Contents of total phenol and flavonoid were determined according to the method described previously [6]. For total phenol quantitation, 30 µl of the samples was added to 30 µl of 95% ethanol, 150 µl of distilled water, 15 µl of Folin-Ciocalteu reagent and 30 µl of 5% saturated Na_2CO_3 solution. After 60 min of incubation, the absorbance was measured at 725 nm using a Spectra MR microplate reader (Dynex Technologies, Inc., Chantilly, VA, US). The blank consisted of all reagents and solvents without the sample. The total phenolic content was determined using the standard gallic acid calibration curve. To determine total flavonoids, an aliquot (15 µl) of the sample was mixed with 4.5 µl of 5% $NaNO_2$, 60 µl of distilled water and 4.5 µl of 10% $AlCl_3$. Following 6 min, 2 ml of 1 M sodium hydroxide were added to the mixture. The final volume of the reaction

mixture was made up to 150 µl with distilled water. Absorbance was measured at 510 nm against a blank. The total flavonoid content was determined using a standard curve of rutin and the results were expressed as rutin equivalents.

Table 1: Scientific and Korean names, type, harvest dates and group code of the thinned immature and mature *Citrus* fruits analyzed

Scientific name (Variety)	Korean name	Type	Harvest month	Group code
Citrus unshiu (Miyagawa wase)	Gungcheonjosaeng	Peel of mature fruit	November	1A
		Pulp of mature fruit	November	1B
Citrus unshiu (Okitsu wase)	Heungjinjosaeng	Immature fruit	July	2A
		Immature fruit	August	2B
Citrus unshiu (Sangdojosaeng)	Sangdojosaeng	Immature fruit	July	3A
		Immature fruit	August	3B
C. unshiu×C. sinensis×C. reticulatia	Hallabong	Immature fruit	July	4A
		Immature fruit	August	4B

Total carotenoid content

An aliquot of the extracts was used for quantification of total carotenoid content using a Spectra MR microplate reader. Total carotenoid content was calculated by measuring the absorbance at 470, 653 and 666 nm according to the equations reported previously, and expressed as mg/100 g [7].

Ascorbic acid content

Ascorbic acid content of the methanol extracts of immature and mature *Citrus* fruits was determined as described earlier [8] with some modifications. The results are expressed as mg ascorbic acid per 100 g dry matter (mg AA/100 g).

UPLC-MS analysis of flavonoids

Chromatographic separation of five major flavonoids (naringin, hesperidin, hesperetin, narirutin and rutin) in the methanol extracts of immature and mature *Citrus* fruits was performed on the Acquity UPLC system (Waters, Milford, MA, US) equipped with a binary solvent system and an autosampler according to the method described previously [9]. The extracts were performed on a UPLC BEH C18 column (50 mm x 2.1 mm, 1.7 µm). Water with 0.1% (v/v) formic acid and acetonitrile were used as Mobile Phases A and B, respectively, for chromatographic elution: from 0-0.63 min, Phase B was linearly increased from 2%, then linearly increased to 2-60% at 3.5 min and maintained for 3.0 min and then decreased 60-2% for 4-4.5 min; Phase B was adjusted to 2% at 4.5-5 min for re-equilibration. The injection volume was 2 µL and the UV spectra by PDA were recorded between 210 and 410 nm. MS detection was performed directly after an Acquity photodiode array (PDA) detector coupled with a triple quadrupole tandem mass spectrometer (MS) (Micromass® Quattro microTM API, Waters, Milford, MA, US) measurements. The major operating parameters for the electrospray ionization source (ESI) were set as follows: scan spectra from m/z 100 to 400, capillary voltage 3.30 kV, cone voltage 20 V, source temperature 120 °C and desolvation temperature 360 °C, collision gas-argon, desolvation gas (nitrogen). The data were collected using Mass-Lynx V 4.1 software.

Antioxidant assay

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay was conducted by the method described before [6]. An aliquot of 100 µl of a solution of DPPH radicals (0.4 mmol in methanol) was mixed with methanol extracts of immature and mature *Citrus* fruits and shaken. The reaction was allowed to proceed at room temperature for 10min, and the absorbance was measured at 517 nm using a Spectra MR microplate reader. A control reaction mixture was prepared without any extract. The same procedure was repeated to obtain the antioxidant capacity of L-ascorbic acid which was used as positive control. The superoxide anion scavenging capacity was analyzed by the reduction of nitroblue tetrazolium (NBT), as described previously [9]. The reaction mixture contained 50 mmol Na_2CO buffer, 3 mmol xanthine, 3 mmol ethylenediaminetetraacetic acid, 0.5 mmol NBT and bovine serum albumin solution. Test extracts were added to the reaction mixture and incubated at 25 °C for 10 min. The reaction was started with the addition of xanthine oxidase (XO) (0.25 units/ml). After further incubation at 25 °C for 25 min, the absorbance at 560 nm was measured against an appropriate blank to determine the quantity of formazan generated. The hydrogen peroxide scavenging assay of the methanol extracts of immature and mature *Citrus* fruits was performed as previously reported [6]. The inhibition of nitric oxide was quantified by the use of Griess Illosvoy reaction [6]. The reaction mixture (100 µl) containing 10 mmol sodium nitroprusside in phosphate-buffered saline (pH 7.0), with or without methanol extracts at concentrations of 0.125, 0.25, 0.5, 1 and 2 mg/ml, was incubated at 25 °C for 3 h. Following incubation, reaction mixture was mixed with an equal amount of Greiss reagent (1% sulfanilamide and 0.1% N-1-naphthylethylene diamine dihydrochloride in 2.5% polyphosphoric acid), which was allowed to stand for 5 min, then absorbance of assay mixture was determined at 540 nm. A dose-response curve was plotted to determine the IC_{50} values which are defined as the concentration sufficient to obtain 50% of a maximum scavenging capacity. All tests were performed in triplicate.

Statistical analysis

Results are presented as means ± standard deviation. Statistical comparisons were made by analysis of variance (ANOVA) procedure followed by Duncan's multiple range tests (SPSS 12.0). $p<0.05$ was considered significantly different. After multiple comparisons, the means in the following table and fig. were followed with different small letter "a-d" based on their values and statistical differences.

RESULTS AND DISCUSSION

Phenolic and flavonoid contents

Phenolic compounds, including flavonoids and phenolic acids, are known to be responsible for antioxidant activity in fruit; fruits with higher total phenolic contents generally show stronger antioxidant activity [10]. The total phenolic composition of the methanol extracts of thinned immature and mature *Citrus* fruits is presented in fig. 1. The amount of total phenolics varied widely and ranged from 22762±259.9 mg/100 g in 4B (*C. unshiu×C. sinensis×C. reticulatia*, August) to 28272±464.5 mg/100 g in 2A (*Citrus unshiu* cv. *Okitsu wase*, July). The total phenolic contents in thinned immature *Citrus* extracts (2A-4B) was significantly higher ($p<0.05$) than in the peel and pulp of mature *Citrus* extracts (fig. 1). Significant differences ($p<0.05$) were observed in the phenolic levels of 3A and 3B (*Citrus unshiu* cv. *Sangdojosaeng*, July and August), ranking second on the list. 4A and 4B (*C. unshiu×C. sinensis×C. reticulatia*, July and August) ranked third in terms of total phenolic content ($p>0.05$). The differences may be caused by differences among the plant species and maturity period. The same observation is made in the flavonoid content (fig. 1). Highest levels of total flavonoids ($p<0.05$) were obtained in extracts of 3A (*Citrus unshiu* cv. *Sangdojosaeng*, July) (12369±1456.9 mg/100 g), followed by 2A (*Citrus unshiu* cv. *Okitsu wase*, July) and 1A (*Citrus unshiu* cv. Miyagawa wase, Peel) (12182±995.5 mg/100 g and 10679±1400.7 mg/100 g, respectively) (fig. 1). Thinned immature *Citrus* extracts of the *Citrus unshiu* variety, which had the highest total phenols, contained similar levels of total flavonoids compared with mature *Citrus* extracts. Flavonoids are a large class of benzopyrone derivatives, ubiquitous in plants exhibit antioxidant activity. The antiradical property of flavonoids is directed mostly toward hydroxyl, superoxide as well as peroxyl and alkoxyl radicals [11].

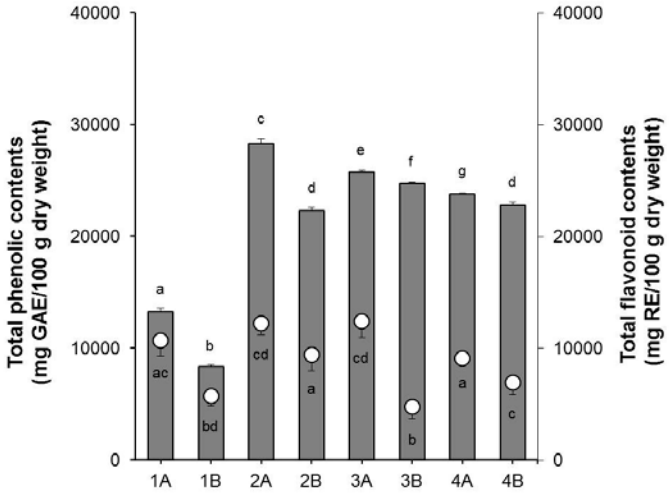

Fig. 1: Total phenolic and flavonoid contents of the thinned immature and mature *Citrus* fruits. Each value is expressed as mean±SD [a-i] Values with different superscripts in a column are significantly different (p<0.05). In the case that a mean was followed with "[ab]", this mean was not significantly different from a mean with "[a]", and was not significantly different from another mean with "[b]"

Total carotenoid and L-ascorbic acid contents

Carotenoids, vegetable dyes present in the chloroplasts and chromophores, play an auxiliary role in the process of photosynthesis [12]. In addition, ascorbic acid, as well as carotenoids, also have a protective function against photo-oxidation processes. Carotenoids and ascorbic acid present in the tissues of studied plants may also act as scavengers of free radicals. Effectively inactivate singlet oxygen, and also react with organic free radicals produced during the process of lipid peroxidation [12]. The total carotenoid composition of the methanol extracts of thinned immature and mature *Citrus* fruits is presented in table 2. The number of total carotenoids varied widely and ranged from 12.83±0.226 µg/g in 1A (*Citrus unshiu* cv. Miyagawa wase, Peel) to 44.24 ±1.180 µg/g in 2A (*Citrus unshiu* cv. *Okitsu wase*, July). The total carotenoid contents in the immature *Citrus* extracts (2A-4B) were significantly higher (p<0.05) than in than the peel extracts (1A) of mature *Citrus* fruit. No significant differences (p>0.05) were observed in the carotenoid levels of 2A, 3B and 4A, all ranking second on the list. 2B and 4B ranked third in terms of total phenolic content (p>0.05). The L-ascorbic acid composition of the immature and mature Citrus extracts is shown in table 3. Highest levels of L-ascorbic acid (p<0.05) were obtained in extracts of 1B (110.3±1.79 mg AA/100 g) followed by 4A (p<0.05) and 3A (p<0.05) (107.0±0.72 mg AA/100 g and 106.1±1.44 mg AA/100 g, respectively) (table 2). Extracts of 1A, peel extracts (1A) of mature Citrus fruit, whose L-ascorbic acid contents ranked last on the list. The immature Citrus extracts (2A-4B) had significantly higher total carotenoid and L-ascorbic acid contents than the peel extracts (1A) of mature Citrus fruit (p<0.05) (table 2). These results indicate that the maturity of Citrus may influence the concentration of carotenoids and ascorbic acids in the fruit.

Table 2: Total carotenoid and L-ascorbic acid contents of the thinned immature and mature *Citrus* fruits

Test group	Total carotenoids (µg/g dry weight)	L-Ascorbic acid (mg AA/100 g)
1A	12.83±0.226[a]	58.6±1.89[a]
1B	42.04±0.306[b]	110.3±1.79[b]
2A	44.24±1.180[c]	102.5±2.50[c]
2B	39.48±0.995[d]	99.9±3.03[c]
3A	42.03±0.963[b]	106.1±1.44[d]
3B	43.50±0.589[c]	99.5±1.36[c]
4A	43.42±0.764[c]	107.0±0.72[d]
4B	40.19±0.465[d]	92.6±1.25[e]

Abbreviations: AA = L-ascorbic acid, mean±SD for n=3, [a-e] Values with different superscripts in a column are significantly different (p<0.05).

Flavonoid distribution

Flavonoids are of interest for their various pharmacological potentials, the most important of which are antitumor, anti-inflammatory, antimutagenic, and antiallergic properties [13]. Four types of flavonoids occur in *Citrus*: flavanones, flavones, flavonols and anthocyanidins [14]. *Citrus* fruit contains high levels of the flavanones, as well as flavonol, which are very rare in other plants [15]. In this study, the quantitative determination of the components found in thinned immature (2A-4B) and mature (1A and 1B) *Citrus* extracts. Table 3 summarizes the results obtained. The main components were identified as naringin (95-145 mg/100 g), neohesperedin (76-94 mg/100 g) and narirutin (73-112 mg/100 g) in thinned immature *Citrus* extracts. Small amounts of hesperidin and rutin were also found (77.7-95.3 and 0.8-12.9 mg/100g, respectively). Significant amounts of naringin and neohesperedine were present only in thinned immature *Citrus* extracts (table 3). From this result, it can be seen that the content of polyphenolic compounds varied during fruit development. Secondary metabolites may play a role in plant protection against photooxidative stress, in mediating thermotolerance and indirect defence against microbes and insects [13-15]. Similar results were documented by Choi, SY *et al.* [16], they have analyzed 7 flavonoids from 20 citrus species. Hesperidin was widely distributed in large amounts in the peels of both immature and mature citrus fruit, whereas naringin and rutin were present at high levels in only specific citrus fruits among the 20 citrus species. Other literature reported that there were no naringin or neohesperidin detected in all mature mandarin cultivars [17]. This phenomenon should be studied in more detail. In general, the contents and distributions of flavonoids in different *Citrus* species are highly variable and depend on genetic and environmental factors such as geographical region, climate, soil conditions, harvest date, storage and low-dose irradiation [18].

Table 3: Flavonoid content (mg/100 g dry weight) in the thinned immature and mature *Citrus* fruits

Test group	Flavanone					Flavonol
	Naringin	Hesperidin	Hesperetin	Neo-hesperedine	Narirutin	Rutin
1A	nd*	305.7±3.03[a]	nd	nd	83.1±3.42[a]	62.3±2.76[a]
1B	nd	178.8±0.43[b]	nd	nd	92.4±0.59[b]	57.7±0.93[b]
2A	124.9±3.63[a]	87.1±1.21[c]	nd	85.1±3.14[a]	91.5±0.20[b]	12.9±0.55[c]
2B	95.5±0.64[b]	77.7±0.25[d]	nd	76.6±0.95[b]	73.3±0.48[c]	8.3±0.16[d]
3A	144.7±1.17[c]	90.4±0.64[e]	nd	90.8±1.65[c]	108.8±3.26[d]	9.6±0.85[d]
3B	104.0±0.59[d]	79.5±0.37[d]	nd	80.0±1.30[d]	76.0±0.75[c]	7.9±0.45[d]
4A	145.9±0.76[c]	95.3±0.85[f]	nd	94.5±1.68[e]	112.2±1.34[e]	1.3±0.04[e]
4B	112.9±1.17[e]	77.8±0.25[d]	nd	77.9±0.78[bd]	87.0±1.55[f]	0.8±0.08[e]

mean±SD for n=3, [a-f] Values with different superscripts in a column are significantly different (p<0.05). In the case that a mean was followed with "[ab]", this mean was not significantly different from a mean with "a", and was not significantly different from another mean with "b", *Not detected.

Flavonoids have been associated with the health benefits derived from their antioxidant activity [19] and could be a natural source of antioxidants as well as a major determinant of antioxidant potentials of foods [20]. Therefore, our results may provide important information on utilization of thinned immature *Citrus* fruits as a primary antioxidant source. Since the key role of phenolic compounds to scavenge free radicals has been emphasized in the literature [21], antioxidant properties of immature *Citrus* extracts were investigated in the following experiments.

Antioxidant activities

Free radicals are known to play a definite role in a wide variety of pathological manifestations of pain, inflammation, cancer, diabetes, alzheimer, hepatic damage etc. antioxidants fight free radicals and protect us from various diseases [22]. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defence mechanisms [22]. In the present study, the methanol extracts of the thinned immature and mature *Citrus* fruits were evaluated for their antioxidant capacity by free radical scavenging (DPPH, superoxide, hydrogen peroxide and nitric oxide) assay, as shown in table 4. Scavenging activity for free radicals of DPPH has been widely used to evaluate the antioxidant activity of natural products from plant sources [23]. In this study, dose-response curves for the DPPH radical scavenging activity were observed in all immature and mature *Citrus* extracts (data not shown). The most potent scavenging DPPH radical activity was observed in immature *Citrus* extract 4A (*C. unshiu*×*C. sinensis*×*C. reticulatia*, July) (1.03 mg/ml IC_{50}), which possess significantly higher DPPH free radical scavenging activity than those of mature *Citrus* extracts 1A and 1B (1.66 mg/ml and 2.24 mg/ml IC_{50}) (p<0.05), indicating that immature *Citrus* extracts were more effective in scavenging effects than mature *Citrus* extract (table 4). Scavenging effect on superoxide radical of immature and mature *Citrus* extracts were 73-83% and 65% at 12.5 µg/ml, respectively (data not shown). The IC_{50} values for the immature and mature *Citrus* extracts were 1.03-1.30 mg/ml and 0.84-1.59 mg/ml, respectively (table 4), indicating that maturity affected the scavenging effect of *Citrus* fruits. Same trend was observed for the level of hydrogen peroxide and nitric oxide scavenging effects in tested the thinned immature and mature *Citrus* extracts; both extracts scavenged nitric oxide in a dose-dependent manner, and immature *Citrus* extract had slightly higher hydrogen peroxide and nitric oxide scavenging ability than mature *Citrus* extracts (table 4). These free radical scavenging activities may be attributed to the presence of high amount of phenolic compound and flavonoid shown in fig. 1, indicating that the immature fruits from most of the local *Citrus unshiu* cultivars may be good resources of medicinal agents.

Table 4: IC_{50} value in free radical scavenging capacities of the thinned immature and mature *Citrus* fruits

Test group	IC_{50} value (mg/ml)*			
	DPPH radical	Superoxide radical	Hydrogen peroxide radical	Nitric oxide radical
1A	1.66±0.026[a]	0.84±0.022[a]	2.58±0.153[a]	1.88±0.034[a]
1B	2.24±0.064[b]	1.59±0.087[b]	2.57±0.237[a]	2.27±0.130[b]
2A	1.49±0.010[c]	1.24±0.021[cd]	2.17±0.117[b]	1.86±0.129[c]
2B	1.62±0.006[a]	1.21±0.007[cd]	3.43±0.247[c]	1.64±0.034[d]
3A	1.35±0.037[c]	1.03±0.036[e]	2.05±0.040[b]	2.30±0.104[b]
3B	1.23±0.044[d]	1.30±0.011[d]	2.90±0.214[a]	3.45±0.180[c]
4A	1.03±0.016[e]	1.12±0.017[ce]	1.95±0.153[b]	2.40±0.262[c]
4B	1.22±0.061[d]	1.46±0.010[b]	2.69±0.315[c]	3.04±0.106[d]

*IC_{50}, the concentration of premature citrus fruits that inhibited 50% of radicals. IC_{50} was obtained by interpolation from linear regression analysis. mean±SD for n=3, [a-g] Values with different superscripts in a column are significantly different (p<0.05). In the case that a mean was followed with "[ab]", this mean was not significantly different from a mean with "a", and was not significantly different from another mean with "b".

CONCLUSION

The thinned immature *Citrus* fruit is better than the mature fruit in terms of nutritional and antioxidant profiles. This study indicated that immature *Citrus* extracts might be helpful in preventing various oxidative stress-related diseases. Further studies on the biological activities of phytochemicals in thinned immature *Citrus* fruits are warranted.

ACKNOWLEDGEMENT

This work was supported by the research grant of Jeju National University in 2017.

AUTHORS CONTRIBUTION

Ji Hye Kim contributed in performing the experiment and data compilation. Experiment design and writing of the manuscript was done by Prof. Min Young Kim.

CONFLICT OF INTERESTS

We declare that there is no conflict of interest

REFERENCES

1. Turner T, Burri BJ. Potential nutritional benefits of current *Citrus* consumption. Agriculture 2013;3:170-87.

2. Molina EG, Domínguez RP, Moreno DA, Garcia CG. Natural bioactive compounds of *Citrus* limon for food and health. J Pharm Biomed Anal 2010;51:327-45.
3. Yang EJ, Kim SS, Oh JS, Baik JS, Lee NH, Hyun CG. Essential oil of citrus fruit waste attenuates LPS-induced nitric oxide production and inhibits the growth of skin pathogens. Int J Agric Biol 2009;11:791-4.
4. Barreca D, Bellocco E, Caristi C, Leuzzi U, Gattuso G. Flavonoid composition and antioxidant activity of juices from *Chinotto* (*Citrus x myrtifolia Raf*.) fruits at different ripening stages. J Agric Food Chem 2010;58:3031-6.
5. Inoue T, Tsubaki S, Ogawa K, Onishi K, Azuma J. Isolation of hesperidin from peels of thinned *Citrus unshiu* fruits by microwave-assisted extraction. Food Chem 2010;12:542-7.
6. Im SJ, Kim JH, Kim MY. Evaluation of bioactive components and antioxidant and anticancer properties of *Citrus* wastes generated during bioethanol production. Nat Prod Commum 2014;9:483-6.
7. Rainha N, Lima E, Baptista J, Rodrigues C. Antioxidant properties, total phenolic, total carotenoid and chlorophyll content of anatomical parts of *Hypericum foliosum*. J Med Plants Res 2011;5:1930-40.
8. Klein BP, Perry AK. Ascorbic acid and vitamin an activity in selected vegetables from different geographical areas of the United States. J Food Sci 1982;47:941-5.
9. Kim JH, Kim MY. The potential use of *Citrus* juice waste as sources of natural phenolic antioxidants. J Appl Pharm Sci 2016;6:202-5.
10. RiceEvans CA, Miller NJ. Antioxidant activities of flavonoids as bioactive components of food. Biochem Soc Trans 1996;24:790-5.
11. Aharmas S, Saxena J. Phytochemical screening and quantitative estimation of total phenolic content and total flavonoid content of grains of *Paspalum scrobiculatum*. Asian J Pharm Clin Res 2016;9:73-6.
12. Sikora E, Cieślik E, Topolska K. The sources of natural antioxidants. Acta Sci Pol Technol Aliment 2008;7:5-17.
13. Wang YC, Chuang YC, Hsu HW. The flavonoid, carotenoid and pectin content in peels of *Citrus* cultivated in Taiwan. Food Chem 2008;106:277-84.
14. Benavente Garcia O, Castillo J, Marin FR, Ortuno A, Del Rio JA. Uses and properties of *Citrus* flavonoids. J Agric Food Chem 1997;45:4505-15.
15. Gattuso G, Barreca D, Gargiulli C, Leuzzi U, Caristi C. Flavonoid composition of *Citrus* juices. Molecules 2007;12:1641-73.
16. Choi SY, Ko HC, Ko SY, Hwang JH, Park JG, Kang SH, *et al*. Correlation between flavonoid content and the NO production inhibitory activity of peel extracts from various *Citrus* fruits. Biol Pharm Bull 2007;30:772-8.
17. Ye XQ, Chen JC, Liu DH, Jiang P, Shi J, Xue S. Identification of bioactive composition and antioxidant activity in young mandarin fruits. Food Chem 2011;124:1561-6.
18. Nogata Y, Sakamoto K, Shiratsuchi H, Ishii T, Yano M, Ohta H. Flavonoid composition of fruit tissues of *Citrus* species. Biosci Biotechnol Biochem 2006;70:178-92.
19. Heim KE, Tagliaferro AR, Bobilya DJ. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationship. J Nutr Biochem 2002;13:572-84.
20. Parr A, Bolwell GP. Phenols in the plant and in man: The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. J Sci Food Agric 2000;80:985-1012.
21. Archana B, Dasgupta N, De B. *In vitro* study of antioxidant activity of Syzygium cumini fruit. Food Chem 2005;90:727-33.
22. Atina RC, Irda F, Komar R. Comparison of five antioxidant assays for estimating antioxidant capacity from three *Solanum SP*. extracts. Asian J Pharm Clin Res 2016;9:123-8.
23. Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. J Agric Food Chem 2005;53:1841-56.