Note

Effects of Cultivation Month and Genetic Background on Phenolic Content of *Citrus tachibana* Peel

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Received December 1, 2017; accepted May 9, 2018

Species of the *Citrus* genus are known as rich sources of phenolic compounds. Peels of *Citrus tachibana* and *Citrus unshiu* are used in herbal formulations, sometimes in similar ways. In this study, we examined the effects of plant maturity and genetic background on the total phenolic contents and quantities of specific flavonoids in *C. tachibana* peel. In addition, we compared these values in *C. tachibana* and *C. unshiu* peels. The total phenolic contents and the contents of nobiletin, tangeretin, and hesperidin were higher in the extracts of the immature peel than in those of the mature peels of *C. tachibana*; moreover, the quantities of these compounds were also influenced by the genetic background of *C. tachibana*. In the extracts of *C. unshiu* peel, the contents of total phenolics, nobiletin, and tangeretin were lower than those of *C. tachibana* peel. However, the hesperidin content was higher in extracts of *C. unshiu* peel than those of *C. tachibana* peel. This study evaluated the phenolic and flavonoid contents of *C. tachibana* and *C. unshiu* in an effort to provide new insights into herbal medicines for further study and utilization.

Key words *Citrus tachibana*; flavonoid; polyphenol

Fruits of the *Citrus* genus are widely known for their health benefits. For example, the peels of some *Citrus* genus, such as *C. tachibana* Tanaka, *C. unshiu* Marcowicz, *C. reticulata* Blanco, and *C. aurantium* Linnaeus, are common herbal medicines. *C. tachibana* Tanaka, a species endemic to Japan, is sufficiently rare to be designated as a near-threatened species. Conversely, *C. unshiu* Marcowicz grows widely in Japan and its fruit is readily available on the Japanese market. The peels of *C. tachibana* and *C. unshiu* are both used to prepare the same herbal medicine, *Tachibana Pericarpium* (Citrus Peel), despite the marked difference in their origins.[1)] A recent study reported that *C. tachibana* Tanaka species actually comprise several subspecies of different origins.[1)] Furthermore, the *Citrus* genus can be easily crossbred naturally. Thus, it appears that a variety of genetic backgrounds are found within a given species. Differences in the origin and genetic background of species from the *Citrus* genus might influence the content of medically active compounds. The *Citrus* genus is rich in vitamins, carotenoids, and phenolic compounds; in particular, nobiletin, tangeretin, and hesperidin are typical flavonoids found in members of the *Citrus* genus. It has been reported that these flavonoids exhibit antioxidant, anti-inflammatory, antidiabetic, and anti-osteoarthritic activities.[2–6)] The levels of components in herbal medicines are closely related to their quality and therefore should be specified. Although the level of any component found in *Tachibana pericarpium* is not currently specified, the level of hesperidin found in *Citri unshiu pericarpium* (*Citrus unshiu* peel) is specified as more than 4% by the 17th Edition of the Japanese Pharmacopoeia. In this study, we evaluated the total phenolic content, and contents of nobiletin, tangeretin, and hesperidin in *C. tachibana* peel, assessed the impact of plant maturity and genetic background on these compounds, and compared the levels with those in *C. unshiu* peel.

MATERIALS AND METHODS

Materials Nobiletin and tangeretin were purchased from Indofine Chemical Company, Inc. (Hillsborough, NJ, U.S.A.). Hesperidin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Folin and Ciocalteu's phenol reagent was purchased from MP Biomedicals, LLC. (Santa Ana, CA, U.S.A.). All other chemicals used were purchased from Wako Pure Chemical Industries, Ltd.

Preparation of *Citrus* Peel Extracts *C. tachibana* peels (T1–T5) were collected from plants in five different locations in Asuka Village (Nara, Japan), and *C. tachibana* peels (T6) were collected from one location in Nara City (Nara, Japan). The plants that provided peels T1–T4 were cultivated every month between October 2014 and February 2015, whereas those corresponding to peels T1–T3, T5, and T6 were cultivated in January 2016. *C. unshiu* peels harvested from plants in Wakayama (U1) and Kagawa (U2) prefectures, Japan, were obtained from Maetyu Co., Ltd. (Nara, Japan). All peels were dried, powdered, and subjected to extraction. In accordance with the method for dilute ethanol-soluble extracts in the 17th Edition of the Japanese Pharmacopoeia,[7)] we used an extraction time of 5 h, but used water at 100°C or EtOH at 80°C as the solvent. The resulting extracts were filtered and lyophilized or evaporated to remove solvent.

Total Phenolic Content The total phenolic content was determined in accordance with the method of Giorgi,[8)] with minor modifications, which uses the Folin–Ciocalteu reagent. The reaction mixture contained the test sample, 0.5 N Folin–Ciocalteu reagent, and 0.29 M sodium carbonate. After the

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Table 1. Total Phenolic Content and Contents of Flavonoids of *C. tachibana* Peel Extracts

Code no.	Harvest date	Content (mg/g water extract)				Content (mg/g EtOH extract)			
		Total phenolics	Nobiletin	Tangeretin	Hesperidin	Total phenolics	Nobiletin	Tangeretin	Hesperidin
T1	2014.10.9	106.5±6.4	15.5±3.0	10.4±0.9	61.7±0.4	136.8±6.6	30.0±0.2	19.2±0.7	144.6±7.4
	2014.11.11	89.3±3.5	10.8±0.6	7.8±0.5	44.0±6.0	102.0±11.5	19.1±1.4	12.7±1.9	78.3±8.4
	2014.12.9	80.4±4.8	9.7±1.7	7.2±0.5	30.5±2.5	90.6±14.5	15.5±2.0	10.6±2.1	47.1±2.9
	2015.1.14	77.2±9.3	6.0±1.9	4.5±1.0	24.2±3.7	69.4±12.8	8.4±1.5	5.8±1.4	34.6±4.6
	2015.2.12	75.7±4.0	6.6±1.5	4.8±0.7	28.5±2.0	81.3±15.2	10.0±0.3	6.8±0.6	41.0±3.6
T2	2014.10.9	108.0±3.2	22.7±3.2	7.5±0.8	41.3±1.2	150.3±11.5	40.3±2.4	12.8±1.3	105.7±17.8
	2014.11.11	90.7±3.7	15.6±2.5	5.6±0.5	29.3±2.3	117.0±10.8	28.9±2.9	9.8±1.6	53.5±6.8
	2014.12.9	89.4±3.6	13.3±3.7	4.9±1.0	22.3±2.5	112.8±12.1	24.4±2.7	8.2±1.3	38.3±3.4
	2015.1.14	86.4±3.2	11.2±3.7	4.1±1.1	24.7±2.8	101.3±13.6	19.3±0.3	6.6±0.7	37.2±1.2
	2015.2.12	84.0±4.8	11.3±1.3	4.3±0.2	20.9±1.4	98.5±14.3	21.2±0.1	7.2±0.6	39.0±4.7
T3	2014.10.9	109.7±5.8	19.3±3.2	11.6±1.2	69.8±4.7	138.5±7.2	32.6±3.4	18.6±2.6	183.4±28.5
	2014.11.11	84.7±3.3	12.7±1.9	8.4±0.5	33.1±3.2	112.0±13.9	19.1±0.2	10.9±1.0	106.6±9.3
	2014.12.9	85.0±4.9	11.0±2.1	7.3±0.7	45.7±0.9	93.7±10.3	16.2±0.4	10.0±0.8	78.4±13.3
	2015.1.14	77.4±4.4	8.6±1.9	5.7±0.7	38.7±2.0	81.3±12.0	12.5±0.7	7.6±1.0	63.1±2.9
	2015.2.12	75.8±4.3	8.9±0.8	5.9±0.0	42.4±4.2	85.8±12.6	15.0±0.8	9.0±1.1	76.4±9.2
T4	2014.10.9	95.0±3.5	17.6±2.6	6.0±0.3	35.7±3.3	132.7±11.9	37.5±3.0	11.6±1.6	84.6±10.5
	2014.11.11	89.4±3.9	14.2±1.6	4.9±0.0	26.9±2.6	118.0±11.9	32.9±1.8	10.7±1.7	55.5±6.4
	2014.12.9	82.8±2.5	12.5±1.5	4.3±0.1	21.2±2.7	102.1±12.5	24.1±0.3	7.7±0.6	39.2±3.0
	2015.1.14	82.1±3.9	13.6±3.8	4.4±0.9	16.3±2.1	93.1±13.1	25.3±0.8	7.7±1.0	25.4±2.0
	2015.2.12	84.6±4.0	11.0±2.9	3.7±0.7	21.9±4.0	98.4±13.3	25.1±0.5	7.9±0.8	40.9±3.5

The total phenolic content is expressed as gallic acid monohydrate equivalent. The results are shown as the mean±standard deviation (S.D.) ($n=3$).

mixture was incubated at 50°C for 5 min, the absorbance at 765 nm was measured by using a U-3010 UV/vis spectrophotometer (Hitachi High-Technologies Corporation, Japan). The total phenolic content was calculated from standard curves of absorbance against the concentration of standard gallic acid monohydrate solutions and expressed as gallic acid monohydrate equivalents.

HPLC Analysis The quantification of nobiletin, tangeretin, and hesperidin was performed by HPLC analysis using a Waters 2690 separation module (Waters Co., Milford, MA, U.S.A.) coupled to a Waters 996 photodiode array detector (Waters Co.). Separation was conducted using Inspire™ C18 and Luster™ C18 (both 5 μm, 4.6×150 mm) columns. For the quantification of nobiletin and tangeretin, the mobile phase comprised 50% (v/v) acetonitrile in water, with a flow rate of 1 mL/min and a column temperature of 35°C. UV detection was performed at 333 nm. For the quantification of hesperidin, the mobile phase comprised a mixture of water/acetonitrile/acetic acid at a ratio of 82/18/1, with a flow rate of 0.9 mL/min and a column temperature of 40°C. UV detection was performed at 285 nm.

Statistical Analysis The experimental data were evaluated for statistical significance by using Student's *t*-test. A value of $p<0.05$ was considered statistically significant.

RESULTS

Changes in the Total Phenolics and Main Flavonoids in *C. tachibana* Peel during Maturation To investigate the influence of harvest month on the composition of *C. tachibana* peels, T1–T4 samples, collected each month between October 2014 and February 2015, were tested. The total phenolic content expressed as gallic acid monohydrate equivalents and the contents of nobiletin, tangeretin, and hesperidin in each sample are shown in Table 1. Compared with water extracts, EtOH extracts contained higher quantities of phenolic compounds and flavonoids. In addition, the contents of phenolic compounds and flavonoids in T1–T4 extracts gradually decreased with cultivation month from October to January.

Difference in the Total Phenolic Content and Main Flavonoids in *C. tachibana* Peel between Genetic Backgrounds Matsumoto *et al.* previously reported a DNA analysis of *C. tachibana* used in this study.[9] By using the nucleotide sequence data of the internal transcribed spacer (ITS) region, the resulting phylogenetic tree classified *C. tachibana* samples (T1–T6) into two groups. T1–T4 samples belonged to one group (group A), whereas T5–T6 samples belonged to a distinct group (group B). In the current study, the phenolic and flavonoid contents of *C. tachibana* groups A and B were compared. The total phenolic content in group A samples was higher than in group B samples (Fig. 1). Furthermore, group A samples had markedly higher levels of nobiletin, tangeretin, and hesperidin than group B samples, showing a clear distinction between the two groups (Fig. 1).

Comparison of the Total Phenolics and Main Flavonoids in *C. tachibana* and *C. unshiu* Peels Twenty samples of *C. tachibana* (T1–T4) cultivated between October 2014 and February 2015 and two samples of *C. unshiu* (U1 and U2), obtained from Maetyu Co., Ltd., were compared. The total phenolic content in EtOH extracts of T1–T4 peels was equal to or greater than that of U1 and U2 peels (Fig. 2). Nobiletin and tangeretin contents in the extracts of U1 and U2 peels were low, whereas hesperidin content was equal to or greater than that in extracts of T1–T4 peels (Fig. 2).

DISCUSSION

Citrus peels are known as rich sources of flavonoids, such

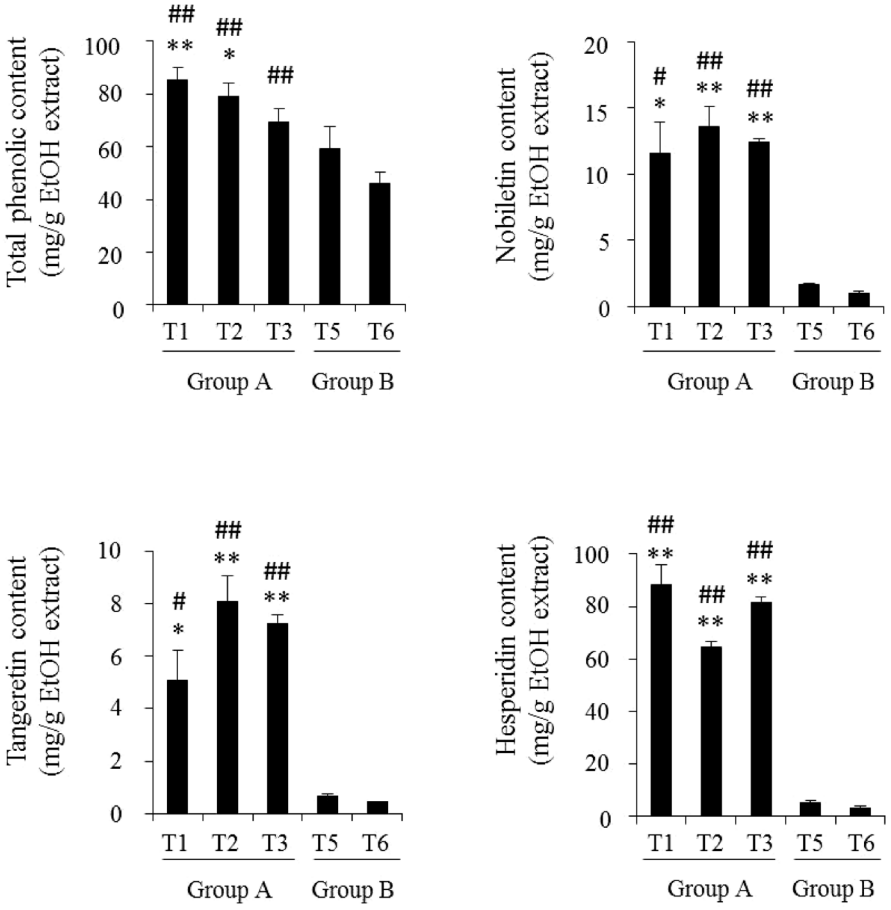

Fig. 1. The Phenolic Content of *C. tachibana* Peel Extracts of Different Genetic Backgrounds

C. tachibana (T1, T2, T3, T5, and T6) were harvested in January 2016 and EtOH extracts were prepared. The total phenolic content is expressed as gallic acid monohydrate equivalents. The results are shown as the mean±S.D. (n=3). $^{*}p<0.05$ and $^{**}p<0.01$ compared with T5. $^{\#}p<0.05$ and $^{\#\#}p<0.01$ compared with T6.

as nobiletin, tangeretin, hesperidin, and other compounds. In this study, we evaluated the total phenolic contents, and contents of nobiletin, tangeretin, and hesperidin in *C. tachibana* peels and studied the effect of their maturity on the levels of these compounds. Total phenolic content, nobiletin, tangeretin, and hesperidin were higher in EtOH extracts than in water extracts (Table 1). This may be attributable to the higher content of saccharides and other polar compounds in water extracts than that in EtOH extracts, which leads to a relatively decreased proportion of flavonoids. In addition, the extracts of immature peel contained more of each compound than those of mature peel (Table 1). Some studies have reported that the saccharide content of various *Citrus* genus fruits, such as those of *C. unshiu* and *C. hassaku*, increases with maturity.[10,11] The proportion of saccharides in the peels of *C. tachibana* also increased with maturity (data not shown) and was accompanied by a corresponding decrease in phenolic content.

The *Citrus* genus contains a wide range of varieties owing to the repeated crossing of hybrids. A recent study determined the parentage of more than 60 citrus varieties, and *C. tachibana* Tanaka was found to include three types from different origins.[1] Furthermore, Matsumoto *et al.* performed DNA analysis of thirteen *C. tachibana* samples collected in Japan and the resulting phylogenetic tree classified *C. tachibana* samples into six groups.[9] The *C. tachibana* samples used in this study (T1–T6) were classified into two of these six groups; group A (T1–T4 samples) and group B (T5–T6 samples). Within group A, ITS sequence of T2 perfectly matched with that of T4; 648 of 651 nucleotides matched those of T1; and 650 of 651 nucleotides matched those of T3. In group B, 646 of 651 nucleotides of T5 matched those of T6. The sequence of *C. tachibana* in group A differed from that of group B by seven nucleotides at the same position. The quantitative analysis reported in this study demonstrated that the content of total phenolics, nobiletin, tangeretin, and hesperidin in the extracts from group A were higher and markedly different from those of group B (Fig. 1). Thus, the genetic background of *C. tachibana* appeared to affect these quantities. Within group A, the content of nobiletin in the EtOH extracts from T2 and T4 samples collected from October to December tended to be higher than those reported for the T1 and T3 extracts (Table 1). In contrast, the contents of tangeretin and hesperidin from T2 and T4 extracts tended to be lower than those reported for the T1 and T3 extracts (Table 1). The ITS sequence of T2 was a perfect match to that of T4, but differed from that of T1 and T3 by a few nucleotides. A slight difference in ITS sequence might be related to the content of phenolic and flavonoid compounds in the immature peel of *C. tachibana*.

C. tachibana and *C. unshiu* peels are sometimes used in the same herbal medicine. The genetic characteristics of *C. tachibana* and *C. unshiu* have been previously character-

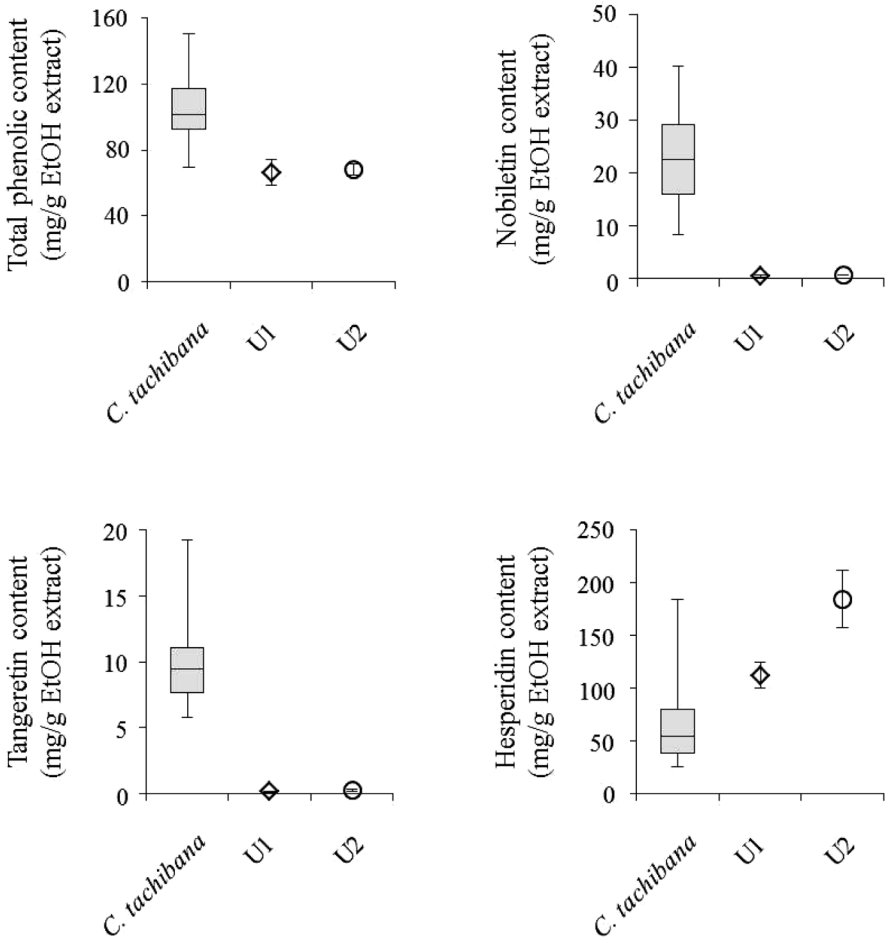

Fig. 2. Total Phenolic and Flavonoid Contents of *C. tachibana* and *C. unshiu* Peel Extracts

Box plot: extracts of *C. tachibana* (T1–T4) harvested between October 2014 and February 2015 (20 samples). The top bar is the maximum value, the lower bar is the minimum value, the top of the box is the upper or third quartile, the bottom of the box is the lower or first quartile, and the middle bar is the median value. Symbols: extracts of *C. unshiu* (◊, U1 and ○, U2). The total phenolic content is expressed as gallic acid monohydrate equivalents. The results for *C. unshiu* are shown as the mean±S.D. (n=3).

ized.[1] The phylogenetic analysis revealed that the origin of *C. tachibana* differed from that of *C. unshiu*. In addition, genotyping identified very few sequence variations in 21 samples of *C. unshiu*. The quantitative analyses reported herein showed low concentrations of nobiletin and tangeretin in *C. unshiu* peels (<0.7 mg/g and <0.3 mg/g EtOH extract, respectively), whereas hesperidin content in *C. unshiu* peels (95–155 mg/g EtOH extract) was comparable with that found in *C. tachibana* peels cultivated in October 2014 (72–151 mg/g EtOH extract). Thus, the composition of *C. tachibana* peel appeared to differ significantly from that of *C. unshiu* peel. Furthermore, the hesperidin content of T1 samples collected in January 2016 was higher than that of the T1 samples collected in January 2015 (Table 1 and Fig. 1). Thus, the content of phenolic and flavonoid compounds in *C. tachibana* peel may be modulated by the cultivation year. However, the nobiletin and tangeretin contents in *C. tachibana* peels collected from October 2014 to February 2015 were at least ten times higher than those in *C. unshiu* peels (Fig. 2). This suggested that the difference in nobiletin and hesperidin content between *C. tachibana* and *C. unshiu* was likely to be a result of interspecies difference. A previous study reported that nobiletin was more abundant in *C. tachibana* peel than in other varieties of citrus fruit.[12] The activities of nobiletin include the inhibition of tumor promotion, inflammation, and oxidative stress.[2,13] *C. tachibana* peel may therefore be a more potent raw material for these pharmacologic activities than other citrus fruits.

In summary, we have demonstrated that the composition of citrus peel differs according to its maturity, genetic background, and *Citrus* species. Our findings indicate that a consideration of the maturity and genetic background of *C. tachibana* is important for the production of effective herbal medicines.

Conflict of Interest The authors declare no conflict of interest.

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