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Soluble and insoluble phenolic compounds and antioxidant activity of immature calamondin affected by solvents and heat treatment

Shyi-Neng Lou [a,*], Yu-Shan Lin [a], Ya-Siou Hsu [a], E-Ming Chiu [a], Chi-Tang Ho [b]

[a] Department of Food Science, National Ilan University, 260 Ilan, Taiwan
[b] Department of Food Science, Rutgers University, New Brunswick, NJ 08901-8520, USA

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ABSTRACT

Hot water extract of immature calamondin peel contains high total phenolic content, which shows significant correlation to DPPH scavenging potency. By heat treatment, the extraction yields of naringin, tangeretin, ferulic acid, *p*-coumaric acid and gallic acid increased, but the amount of 3′,5′-di-C-β-glucopyranosylphloretin (DGPP) decreased drastically. The major soluble phenolic compounds in the nonpolar extract are nobiletin and tangeretin, while DGPP and hesperidin are in the hot water extract. For insoluble phenolic compounds, ferulic acid, *p*-coumaric acid and sinapic acid are mainly in ester linkage form. After heat treatment, gallic acid and *p*-coumaric acid are the major increased soluble and insoluble phenolic acids, respectively. This indicates that high temperature heating (150 °C) probably produces two major effects: (1) degradation of flavonoids, such as DGPP and hesperidin; (2) destruction of the cell wall structure, leading to an increase in soluble nobiletin, tangeretin and gallic acid, as well as insoluble ferulic and *p*-coumaric acids.

1. Introduction

Calamondin (*Citrus mitis* Blanco) is a hybrid of *Citrus reticulate* Blanco and *Fortunella* species (Moshonas & Shaw, 1996; Myrna, Baldwin, Moshonas, & Philip, 1992). It bears small sized fruits and is used in Taiwan to make a hot drink due in part to its potential health beneficial properties. The flavonoid composition of *Fortunella* species differs from those of the *Citrus* species (Ogawa, Kawasaki, Omura, & Yoshida, 2001; Sadek, Makris, & Kefalas, 2009). A large quantity of 3′,5′-di-C-β-glucopyranosylphloretin (DGPP) was observed in calamondin's peel, juice sac and leaf (Ogawa et al., 2001), especially in immature calamondin peel (Lou, Yu, & Ho, 2012; Yu, Lou, & Ho, 2013). In addition, eight polymethoxyflavones including nobiletin and tangeretin were isolated and identified from the peel of calamondin (Tatum & Berry, 1978). Flavonoids, such as, hesperidin, neohesperidin, narirutin and diosmin have also been extracted from dried calamondin pulp powder (Ramful, Tarnus, Aruoma, Bourdon, & Bahorun, 2011). In our previous study, DGPP, naringin, and hesperidin were found in water extract of immature calamondin peel, while nobiletin and tangeretin were only observed in an ethyl acetate extract (Yu et al., 2013). This suggests that different solvents used for extraction can lead to different compositions of phenolic compounds in extracts, because the solubility of each phenolic compound in a giving solvent could be quite different. Consequently, the antioxidant activity of an extract might also be affected.

Dried citrus fruit peel has been widely used as traditional medicine in Asia countries, such as China, Japan, Korea, and Taiwan (Choi et al., 2011; Zang, 2005). Several studies reported that heat treatment might change the amount of extractable phenolic compounds and antioxidant activity of citrus peel (Chen, Yang, & Liu, 2011; Choi et al., 2011; Ho & Lin, 2008; Jeong et al., 2004; Xu, Ye, Chen, & Liu, 2007). The antioxidant activity of citrus peel extract increased as heating temperature increased (Jeong et al., 2004). The free phenolic acids of Huyou (*Citrus paradisi*) extract increased after heat treatment, whereas ester, glycoside, and ester-bound fractions decreased. Zeong et al. also reported that flavanone glycosides might be destroyed when heated to 120 °C for 90 min or 150 °C for 30 min (Xu et al., 2007). Another study reported that total phenolic content of orange peel was low during low temperature heating (50–60 °C) and increased by a higher drying temperature (70–100 °C) (Chen et al., 2011). Naturally existing phenolic compounds in fruits and vegetables are usually covalently bound to insoluble polymers (Choi et al., 2011; Jeong et al., 2004; Peleg, Naim, Rouseff, & Zehavi, 1991). Therefore, heat treatment may be used to release bound phenolic compounds from citrus as well as increasing their antioxidant activity (Choi et al., 2011;

* Corresponding author. Tel.: +886 3 9317769; fax: +886 3 9313809.
E-mail address: snlou@niu.edu.tw (S.-N. Lou).

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Gil-Izquierdo, Gil, & Ferreres, 2002; Xu et al., 2007). Flavonoifd of calamondin are quite different from other citrus fruits. Literature about profiles of phenolic compounds and antioxidant activity of calamondin, especially immature fruit, after heat treatment is still lacking. In this study, we investigated the soluble and insoluble phenolic compounds of calamondin after heat treatment. The effect of extraction solvents on phenolic compounds of calamondin was also evaluated.

2. Materials and methods

2.1. Materials

Calamondin (*C. mitis* Blanco) was collected from a calamondin estate in the Jao-Si region, Ilan, Taiwan in June 2008. Calamondin with whole green appearance was collected and sorted as immature calamondin and had average weight of 16.60 ± 2.96 g. After manual peeling, the separated peels and pulps were lyophilized for 48 h. Prior to extraction, the peels and pulps were pulverized in a blender and passed through a 60 mesh sieve. The obtained powders of immature calamondin peel and pulp were stored in a suitable brown bottle with screw cap at −18 °C.

2.2. Chemicals

Methanol, ethanol, ethyl acetate, and acetonitrile were LC grade from Merck Chemical Co. (Darmstadt, Germany). Acetic acid, Na_2CO_3, $AlCl_3{\cdot}6H_2O$, DPPH (α,α-diphenyl-β-picrylhydrazyl), and Folin–Ciocalteu's phenol reagent were analytical grade. Gallic acid, quercetin, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid, 97% (Trolox), 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH), disodium fluorescein (FL), standards of flavonoids (including diosmin, diosmetin, hesperetin, hesperidin, kaempferol, luteolin, naringin, naringenin, neohesperidin, nobiletin, rutin, tangeretin, sinensetin, and neoeriocitrin) and standards of phenolic acids, including caffeic acid, syringic acid, gentisic acid, ferulic acid, ellagic acid, *p*-coumaric acid, vanillic acid, protocatechuic, acid and gallic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3′,5′-Di-C-β-glucopyranosylphloretin (DGPP) was purified from hot water extract of immature calamondin peel in our laboratory. The extract was separated by a semi-preparative HPLC and the compound collected. It was then subjected to LC/MS/MS and NMR for identification.

2.3. Heat treatments

The separated peel of immature calamondin was subjected to hot air drying in an oven at 70 °C for 11 h, 85 °C for 9 h, 100 °C for 4 h, or 150 °C for 1.5 h to obtain dried products with ca. 5% moisture content. All the obtained dried peels were then lyophilized for 48 h. Prior to extraction, the peels were pulverized in a blender and passed through a 60 mesh sieve. The obtained powders of hot air dried immature calamondin peel were stored in a suitable brown bottle with screw cap at −18 °C.

2.4. Extraction procedure

Three grams of dried and powdered immature calamondin peels and pulps were extracted with (A) 50 mL deionized hot water (80, 90, and 100 °C) for 1 h in a shaking water bath for each temperature, or extracted with (B) 30 mL ethanol (50%, 60%, 70%, 80%, and 95%), or (C) 30 mL methanol, or (D) 30 mL ethyl acetate in a shaker (100 rpm) at room temperature for 1 h. The extract was filtered with Whatman No. 1 filter paper. The obtained residue was extracted by the same procedure two more times. The three resulting filtrates were transferred into a 250 mL flask and dried by rotary vacuum evaporator at 50 °C. To dissolve the filtrate, a suitable volume of deionized water, ethanol, methanol and ethyl acetate was added to the flask for each extract. The obtained solutions were poured into brown bottles with screw cap and stored at −18 °C until further use. Three grams of the powders of hot air dried immature calamondin peel were extracted with 50 mL 90 °C deionized water for 1 h in a shaking water bath at 90 °C. Triplicate determinations ($n = 3$) were carried out during the study. The yield of hot water extraction from immature calamondin peel was 64.0 ± 6.3% dry basis.

2.5. Successive extraction of soluble and insoluble phenolic compounds

The extraction procedure in Section 2.4 was used for soluble phenolic compounds. The first extraction solvent used was hexane. The residue of the extraction was collected and extracted by the second solvent, ethyl acetate. Similarly, the residue of ethyl acetate extraction was collected and then extracted by hot water. The extracts of these three solvents were analysed by HPLC as soluble phenolic compounds.

For the insoluble phenolic compounds (bound form), the method of Mattila and Kumpulainen (2002) was modified. A 10 M NaOH solution was added into the residue of soluble phenolic extraction (5:1, v/w), and stirred at room temperature for 16 h using a magnetic stirrer. The solution was then adjusted to a pH of 2.5, and liberated phenolic compounds were extracted three times with 15 mL of a mixture of cold diethyl ether (DE) and ethyl acetate (EA) (DE/EA, 1:1, v/v) by 15 min shaking and centrifuging. DE/EA layers were combined, evaporated to dryness, and dissolved into methanol. After samples were filtered through a membrane filter, the HPLC analyses were performed. The results indicated insoluble ester linkage phenolic compounds.

After the above alkaline hydrolysis was completed, an acid hydrolysis was performed by adding 12 mL of 6 M HCl into the dried residue (alkaline hydrolysis residue was dried by a rotary vacuum evaporator at 50 °C) and incubating in a water bath (85 °C) for 30 min. After acid hydrolysis, the sample was allowed to cool, and the pH was adjusted to 2.5. The DE/EA extraction performed is similar to that for alkaline hydrolysis. Evaporated extract was then dissolved into methanol, filtered through a membrane filter, and analysed by HPLC. The results indicated insoluble glycoside linkage phenolic compounds. The yields of extractions from immature calamondin peel were 1.37 ± 0.03% for hexane, 1.85 ± 0.05% for ethyl acetate, 67.21 ± 2.79% for hot water, 1.24 ± 0.02% for alkaline hydrolysis, and 2.08 ± 0.04% for acid hydrolysis. All of these data were based on dry basis.

2.6. Determination of total phenolic content

Two hundred and fifty microliters of immature calamondin extract, or standard solution, were mixed with 250 μL of Folin–Ciocalteu's phenol reagent for 3 min (Taga, Miller, & Pratt, 1984). The mixture was added to 2.5 mL of 20% Na_2CO_3 solution and incubated in the dark for 30 min at room temperature. After incubation, the absorbance was measured at 750 nm against the blank. The standard curve was determined with gallic acid, and the total phenolic content was expressed as mg gallic acid equivalent (GAE) per 100 g dry extract using the standard curve. All samples were analysed in triplicate.

2.7. Determination of total flavonoids content

Five hundred microliters of immature calamondin extract or standard solution was mixed with five hundred microliters of 2% methanolic $AlCl_3{\cdot}6H_2O$ (Christel et al., 2000). The mixture was then

incubated for 10 min at room temperature. After incubation, the absorbance of the mixture was measured at 430 nm. Six calibration solutions of quercetin (2, 4, 6, 8, 10, and 20 ppm final concentration) were tested to establish a standard curve. All samples were analysed in triplicate. The total flavonoid content was expressed as mg quercetin equivalents (QE) per 100 g dry extract using the standard curve established previously.

2.8. HPLC analysis of phenolic compounds

The extracts of different solvents were subjected to a HPLC analysis with a reverse phase column (Lichrospher C18e, 250 mm × 4.6 mm, 5 μm, Merck, Darmstadt, Germany) using a gradient with 2% (v/v) acetic acid in water as solvent A and 0.5% acetic acid in water/acetonitrile (1:1, v/v) as solvent B (Schieber, Keller, & Carle, 2001). The gradient was carried out as 0–10 min, 5–10% B; 10–55 min, 10–55% B; 55–60 min, 55–80% B; 60–65 min, 80–100% B; 65–70 min, 100% B; and 70–75 min, 100–5% B. The flow rate was 1 mL/min. Photodiode array (PDA) detection was performed between 220 nm and 350 nm, with a resolution of 2 nm. Sixteen flavonoids and nine phenolic acids used as standards were available in our laboratory as follows: neoeriocitrin, rutin, diosmin, diosmetin, naringin, hesperidin, neohesperidin, naringenin, hesperetin, sinensetin, luteolin, quercetin, kaempferol, nobiletin, tangeretin, 3′,5′-di-C-β-glucopyranosylphloretin (DGPP), and caffeic acid, syringic acid, gentisic acid, ferulic acid, ellagic acid, *p*-coumaric acid, vanillic acid, protocatechuic, acid and gallic acid. The phenolic compounds were identified by their retention times and the UV spectra of standards. They were quantified from peak area at 280 nm by an external standard method, using calibration curves. Their concentrations were expressed as milligramme per 100 g dry weight.

2.9. DPPH radical scavenging activity

The DPPH radical scavenging activity of immature calamondin extracts was estimated according to a slightly modified method of Yamaguchi, Takamura, Matoba, and Terao (1998). After 0.5 mL of immature calamondin extract was mixed with 0.5 mL of 0.5 mM DPPH in methanol for 30 min, the mixture was subjected to HPLC analysis with reverse phase column (Thermo ODS-2 Hypersil, 250 mm × 4.6 mm, 5 μm) under photodiode array (PDA) detection at 517 nm. The mobile phase was methanol/water (7:3, v/v) and the flow rate was 1 mL/min. The change in peak area of DPPH was determined after the reaction. Radical scavenging activity was expressed as percent inhibition and was calculated using the following formula: % DPPH radical scavenging activity = (1 − peak area in sample/peak area in blank) × 100.

2.10. Oxygen radical absorbance capacity (ORAC) assay

The reaction was carried out in 75 mM phosphate buffer (pH 7.4) in cuvettes (Ou, Hampsch-Woodill, & Prior, 2001). Fifty microliters of plant extract solution and 50 μL of disodium fluorescein (70 nM final concentration) were mixed in the cuvette and preincubated for 15 min at 37 °C. Twenty-five microliters of AAPH solution (221 mM final concentration) was then added, and fluorescence was recorded for 70 min at excitation and emission wavelengths of 485 and 520 nm every 5 min. A blank sample containing phosphate buffer in the reaction mix was measured. Five calibration solutions of Trolox (10, 20, 30, 40, and 50 μM final concentration) were also tested to establish a standard curve. All samples were analysed in triplicate. The area under the curve (AUC) was calculated for each sample by integrating the relative fluorescence curve. The net AUC of the sample was calculated by subtracting the AUC of the blank. The regression equation between net AUC and Trolox concentration was determined, and ORAC values were expressed as mmol Trolox equivalents/g plant extract or fractions using the standard curve established previously.

2.11. Statistical analysis

The data were subjected to analysis of variance (ANOVA) and the significance of the difference between means was determined by Duncan's multiple range test ($p < 0.05$), using SAS (SAS Inst., Cary, NC, USA).

3. Results and discussion

3.1. Total phenolic and flavonoid contents of immature calamondin in different extracts

Total phenolic contents of hot water, ethanol, methanol, and ethyl acetate extracts of immature calamondin are shown in Table 1. The total phenolic content in peel was ca. two or five times higher than that in pulp, regardless of what solvent was used for extraction. Citrus peel contains rich phenolic compounds, such as flavonoids and phenolic acids (Choi et al., 2011; Li, Lo, & Ho, 2006; Manthey & Grohmann, 2001; Tripoli, La Guardia, Giammanco, Di Majo, & Giammanco, 2007). In peel, the total phenolic content of hot water extract was 4034–5264 GAE mg/100 g dry extract, which is almost two times larger than that of ethanol and methanol extracts (2268–2763 GAE mg/100 g dry extract). However, the lowest total phenolic contents was found in ethyl acetate extract (522 GAE mg/100 g dry extract). The total phenolic contents of immature calamondin peel is about three fold higher than that of mature calamondin peel, which contains only 1690–1894 GAE mg/100 g dry extract in hot water extract (Lou et al., 2014). Flavanones are usually present in diglycoside form in citrus (Tripoli et al., 2007). In mature calamondin peel, some water soluble *C*- and *O*-glycosyl flavanones and a dihydrochalcone were found (Lou et al., 2014). In the pulp of immature calamondin, the highest phenolic content was obtained in ethanol extract within 50–80%, which is slightly higher than in hot water extract. The lowest total phenolic content (163 GAE mg/100 g dry extract) was found in ethyl acetate extract. There is no obvious different between the total phenolic contents in pulp of mature and immature calamondin.

Table 1 also shows total flavonoid contents of peel and pulp in immature calamondin extracted by different solvents. The highest content was obtained in peel by hot water extract at 80 and 90 °C (932 and 960 QE mg/100 g dry extract). The extract of ethyl acetate in peel showed a very high value of total flavonoids, which might be due to artefact from the interference of some pigments having similar detection absorbance for flavonoids (Yu et al., 2013). Therefore, the data of ethyl acetate in peel and pulp were not taken into consideration. Similar to total phenolic contents, the total contents of flavonoids in hot water extract of immature calamondin peel were much higher than that in mature calamondin peel. The contents in peel were higher than in pulp of all extractions.

3.2. DPPH scavenging activity of different extracts from immature calamondin

In order to investigate the antioxidant activity of different extracts, various extracts obtained were subjected to DPPH free radical scavenging assay. The results indicate that the DPPH scavenging potency of extracts from immature calamondin peel was significantly higher than the calamondin pulp ($p < 0.05$) (Fig. 1). The highest DPPH scavenging potency was observed in the 80 and 90 °C water extract. The DPPH scavenging potency of hot water extracts from peel was higher than that extracted by ethanol, meth-

Table 1
Total phenolic and flavonoids contents of pulp and peel in immature calamondin extracted by different solvents.

Solvents	Total phenolic contents (GAE mg/100 g dry extract)		Total flavonoids contents (QE mg/100 g dry extract)	
	Pulp	Peel	Pulp	Peel
Hot water				
80 °C	1040 ± 29[b]	5264 ± 21[a]	363 ± 9[b]	932 ± 16[a]
90 °C	1048 ± 29[b]	4989 ± 123[b]	455 ± 5[a]	960 ± 13[a]
100 °C	995 ± 55[b]	4034 ± 242[c]	477 ± 32[a]	765 ± 62[b]
Ethanol				
50%	1150 ± 8[a]	2582 ± 40[d,e]	118 ± 11[c]	384 ± 5[d]
60%	1133 ± 19[a]	2643 ± 49[d,e]	54 ± 5[d]	384 ± 22[d]
70%	1152 ± 42[a]	2763 ± 76[e]	62 ± 1[d]	489 ± 23[c,d]
80%	1168 ± 27[a]	2737 ± 14[d,e]	68 ± 5[d]	499 ± 13[c,d]
95%	1014 ± 3[b]	2268 ± 11[f]	78 ± 5[d]	643 ± 33[b,c]
Methanol	1049 ± 32[b]	2568 ± 59[e]	58 ± 1[d]	699 ± 9[b]
Ethyl acetate	163 ± 3[c]	522 ± 52[f]	147 ± 4	5205 ± 286

[a–f] Values (mean ± S.D., $n = 3$) in the same column with different superscripts are significantly different ($p < 0.05$).

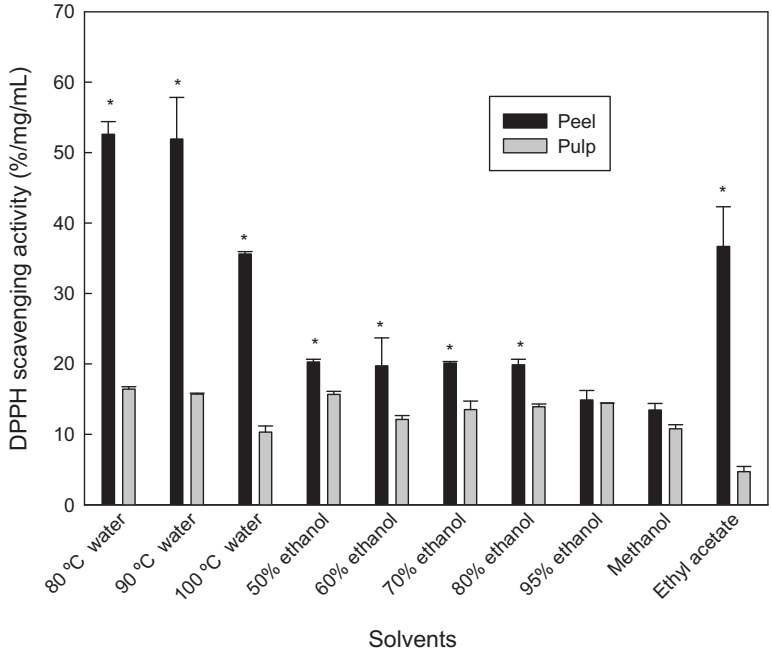

Fig. 1. DPPH scavenging potency of immature calamondin peel and pulp extracted by different solvents. (*Means significantly different ($p < 0.05$) between peel and pulp of same extraction.)

anol or ethyl acetate (Fig. 1). With the ethyl acetate extract, a higher DPPH scavenging potency (36%/mg/mL) was found in the calamondin peel, while a relatively low scavenging potency was observed in the calamondin pulp. The correlation coefficient (r) between total phenolic content and DPPH scavenging potency in extracts of immature calamondin peel and pulp was 0.7911, which is considered significant ($p < 0.01$). Total flavonoid compositions also showed significant correlation ($p < 0.01$) to DPPH scavenging potency with $r = 0.7582$ (data not shown).

Collectively, 80–90 °C water extracts of immature calamondin peel show the highest level of phenolic compounds, such as flavonoids, which also reveal the best antioxidant activity in all extractions. These lead to a good understanding of the health effect of the traditional hot drink of immature calamondin.

3.3. Effect of heat treatments on phenolic compounds of immature calamondin peel

Immature calamondin peel was dried at 70 °C for 11 h, 85 °C for 9 h, 100 °C for 4 h, and 150 °C for 1.5 h, then extracted by 90 °C hot water to gain high level phenolic content. The total phenolic content and total flavonoids content of hot water extract from immature calamondin peel are shown in Table 2. Total phenolic content increased significantly after heating at 150 °C for 1.5 h from 4989 to 5946 GAE mg/100 g dry extract. No obvious difference was observed after heating at 70 °C for 11 h, 85 °C for 9 h, or 100 °C for 4 h. In the water extract from citrus peel (*Citrus unshiu*) heat-treated at 150 °C for 1.0 h, the total phenolic contents increased from 84.4 to 204.9 μM (Jeong et al., 2004). Jeong et al. concluded that phenolic compounds can be released by simple heat treatment, since several low molecular weight phenolic compounds were newly formed at 150 °C for 0.5 h.

Total flavonoid content of hot water extract from immature calamondin peel increased by heat treatment at 150 °C from 960 to 1160 QE mg/100 g dry extract. However, they decreased by heating below 100 °C. It has been studied that total phenolic and flavonoid contents of dried treated orange peels (*Citrus sinensis*) were decreased by lower temperature (<60 °C) and increased by higher temperature (70–100 °C) heating (Chen et al., 2011). Xu et al. (2007) mentioned that total flavanone glycoside content of

Table 2
Total phenolic and flavonoids contents of hot water extract from peel of immature calamondin treated with different heating process.

Temperature (°C)	Time (h)	Total phenolic content (GAE mg/100 g dry extract)	Total flavonoids content (QE mg/100 g dry extract)
Raw materials		4989 ± 123[b]	960 ± 13[b]
70	11	4690 ± 146[b]	756 ± 26[c]
85	9	4245 ± 149[c]	623 ± 9[d]
100	4	4887 ± 126[b]	776 ± 3[c]
150	1.5	5946 ± 16 [a]	1160 ± 38[a]

[a–d] Values (mean ± S.D., $n = 3$) in the same column with different superscripts are significantly different ($p < 0.05$).

150 °C heated Huyou peel was lower than that of the 120 °C peel. They indicated that higher temperature (>100 °C) treatment might destroy the flavonoid compounds of citrus peel. Jeong et al. (2004) indicated that phenolic compounds of plants should be present in different bound status depending on species. Thus, the effective processing step for liberating phenolic compounds from various plants may be different.

The changes in phenolic composition of hot water extract from immature calamondin after heating at 150 °C for 1.5 h is shown in Table 3. Five flavonoids and one phenolic acid were found in immature calamondin, including 3′,5′-di-C-β-glucopyranosylphloretin (DGPP), hesperidin, diosmin, naringin, nobiletin, and caffeic acid. The major flavonoid was DGPP with a value of 4322 mg/100 g dry extract, which is a dihydrochalcone derivative and has been isolated and identified from hot water extract of immature calamondin in remarkably large amounts (Lou et al., 2012; Ogawa et al., 2001). In our previous study we reported that DGPP contained good tyrosinase inhibitory activity and contributed to antioxidant activity due to its greatest quantity (Lou et al., 2012; Yu et al., 2013). Hesperidin was the second major flavonoid (430 mg/100 g dry extract), while diosmin, naringin and nobiletin were in the range from 54 to 87 mg/100 g dry extract. The flavanones, naringin and hesperidin, existed commonly in citrus (Kawail, Tomono, Katase, Ogawa, & Yano, 1999; Kawail et al., 2000; Yoo, Lee, Park, Lee, & Hwang, 2004) and in calamondin (Ramful et al., 2011; Yu et al., 2013). The antioxidant activities of naringin and hesperidin have previously been reported (Wilmsen, Spada, & Salvador, 2005; Yu et al., 2005, 2013). Nobiletin is a polymethoxyflavone with six methoxy groups and showed a hydrophobic nature that is also found in hot water extract. However, tangeretin, the other common polymethoxyflavone in citrus, was not detected. For the phenolic acids, only caffeic acid was observed in a level of 22 mg/100 g dry extract.

The content of DGPP, hesperidin and caffeic acid decreased after heating at 150 °C for 1.5 h. DGPP decreased tremendously from 4322 to 90 mg/100 g dry extract. The level of hesperidin and caffeic acid also decreased from 430 to 290 mg/100 g dry extract and from 22 to undetectable, respectively. Higher temperature (>100 °C) treatment might destroy the flavonoid compounds of citrus peel (Chen et al., 2011; Xu et al., 2007). However, the heating also enhances the release of some phenolic compounds from the immature calamondin peel, i.e., naringin, tangeretin, ferulic acid, *p*-coumaric acid, and gallic acid. Tangeretin and three phenolic acids, including ferulic acid, *p*-coumaric acid, and gallic acid, increased from undetectable to 20–212 mg/100 g dry extract. Total flavonoid composition decreased about 4253 mg/100 g dry extract, while the total phenolic acids increased about 243 mg/100 g dry extract. This indicates that the release of phenolic acids from immature calamondin by hot water extract could be enhanced by heating at 150 °C for 1.5 h. This might be due to the degradation of cell wall structure or that some bound status of phenolic acids was broken down during heating treatment, since phenolic acids are usually covalently bound to insoluble polymers (Choi et al., 2011; Xu et al., 2007). However, DGPP, a chalcone derivative, and hesperidin are probably degraded drastically due to high temperature effect.

Total phenolic and flavonoid contents increased after heating at 150 °C for 1.5 h (Table 2), while the identifiable phenolic compositions decreased enormously (Table 3). This hints that some other phenolic compounds, that were not identified, might exist in hot water extract of immature calamondin peel after heating. Interestingly, the content of phenolic compositions (4993 mg/100 g dry extract) (Table 3), including total flavonoid composition and phenolic acid composition, were almost equal to the content of total phenolic content (4989 GAE mg/100 g dry extract) (Table 1), but much higher than the content of total flavonoid content (960 QE mg/100 g dry extract) (Table 1). This is most likely due to the variation in method of determination. Total flavonoid content was determined by photometric method with a calibration curve of quercetin, of which the molecular weight is 302 Dalton. However, the content of flavonoid compositions was determined by the HPLC method with the relative flavonoids standard, such as DGPP and hesperidin, that have molecular weights of 598.0 and 610.6 Dalton, respectively. This might lead to the difference in quantitation. The method for the determination of total flavonoid content with aluminium chloride is probably unsuitable for use with immature calamondin peel.

Table 3
Phenolic compositions of hot water extract from peel of immature calamondin after heating at 150 °C for 1.5 h.

Phenolic compounds	Fresh	After heating
Contents (mg/100 g dry extract)		
3′,5′-Di-C-β-glucopyranosylphloretin	4322 ± 94[a]	90 ± 14[b]
Hesperidin	430 ± 78[a]	290 ± 14[b]
Diosmin	78 ± 47[a]	86 ± 9[a]
Naringin	54 ± 20[b]	130 ± 11[a]
Nobiletin	87 ± 25[a]	78 ± 9[a]
Tangeretin	n.d.	45 ± 5[a]
Total flavonoids	4971 ± 264[a]	718 ± 63[b]
Caffeic acid	22 ± 11[a]	n.d.
Ferulic acid	n.d.	33 ± 8[a]
p-Coumaric acid	n.d.	20 ± 2[a]
Gallic acid	n.d.	212 ± 14[a]
Total phenolic acids	22 ± 11[b]	265 ± 24[a]

n.d.: Not detectable.
[a,b] Values (mean ± S.D., $n = 3$) in the same raw with different superscripts are significantly different ($p < 0.05$).

3.4. Profiles of soluble and insoluble phenolic compounds in immature calamondin peel after heating

In order to investigate the heating effect on phenolic compounds, immature calamondin peel was heated at 150 °C for 1.5 h. Then, the immature calamondin peel was extracted by successive steps with different solvents in the following order: hexane, ethyl acetate, hot water, alkali hydrolysis, and acid hydrolysis to evaluate the soluble (free) and insoluble (bound) phenolic compounds (Tables 4 and 5). In the soluble compositions, only nobiletin and tangeretin, two polymethoxyflavones, were found in hexane extract (Table 4). Hesperidin (1106 mg/100 g dry extract) was observed in ethyl acetate extract accompanied by nobiletin and tangeretin. In hot water extract, a large magnitude of DGPP was found as the major flavonoid (3621 mg/100 g dry extract), that is similar to our previous study. The other identified flavonoids in hot water extract listed in decreasing level are as follows: hesperidin, diosmin, naringin, nobiletin, and tangeretin, of which the contents are in the range of 3–270 mg/100 g dry extract.

Table 4
Profiles of phenolic compounds and ORAC of immature calamondin peel extracted by successive processes with various solvents.

Phenolic compounds	Soluble			Insoluble		Total flavonoids contents
	Hexane	Ethyl acetate	Hot water	Ester linkage	Glycoside linkage	
Flavonoids	*Contents (mg/100 g dry extract)*					
3′,5′-Di-C-β-glucopyranosylphloretin	n.d.	n.d.	3621 ± 157	n.d.	n.d.	3621 ± 157^{a}
Hesperidin	n.d.	1106 ± 71	270 ± 22	n.d.	n.d.	1376 ± 74^{c}
Diosmin	n.d.	n.d.	80 ± 7	n.d.	n.d.	80 ± 7^{d}
Naringin	n.d.	n.d.	50 ± 2	n.d.	n.d.	50 ± 2^{e}
Nobiletin	1151 ± 132	1039 ± 97	51 ± 3	n.d.	n.d.	2241 ± 164^{b}
Tangeretin	1172 ± 34	276 ± 18	3 ± 1	5 ± 5	n.d.	1456 ± 38^{c}
Total	2323 ± 136^{y}	2421 ± 122^{y}	4075 ± 159^{x}	5 ± 5^{z}	n.d.	8824 ± 242
Phenolic acids						
Caffeic acid	n.d.	n.d.	19 ± 2	n.d.	n.d.	19 ± 2^{d}
Ferulic acid	n.d.	n.d.	n.d.	205 ± 10	19 ± 1	224 ± 10^{a}
p-Coumaric acid	n.d.	n.d.	n.d.	92 ± 11	15 ± 1	107 ± 11^{b}
Sinapic acid	n.d.	n.d.	n.d.	32 ± 7	n. d.	32 ± 7^{c}
Ellagic acid	n.d.	n.d.	n.d.	n.d.	15 ± 7	15 ± 7^{d}
Gallic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	n.d.	n.d.	19 ± 2^{y}	329 ± 16^{x}	49 ± 7^{y}	397 ± 17
ORAC (μmole Trolox/g extract)	493 ± 17	656 ± 13	1031 ± 45	331 ± 12	304 ± 5	2815 ± 51 (ORAC)

n.d.: Not detectable.
Values (means ± S.D., $n = 3$) with different superscripts (a–e) within the same column and with different superscripts (x–z) within the same raw in separated group are significantly different ($p < 0.05$).

Table 5
Profiles of phenolic compounds and ORAC of immature calamondin peel after heating at 150 °C for 1.5 h extracted by successive processes with various solvents.

Phenolic compounds	Soluble			Insoluble		Total phenolic acids contents
	Hexane	Ethyl acetate	Hot water	Ester linkage	Glycoside linkage	
Flavonoids	*Contents (mg/100 g dry extract)*					
3′,5′-Di-C-β-glucopyranosylphloretin	n.d.	n.d.	65 ± 2	n.d.	n.d.	65 ± 2^{e}
Hesperidin	n.d.	165 ± 12	255 ± 11	n.d.	n.d.	420 ± 16^{c}
Diosmin	n.d.	n.d.	56 ± 3	n.d.	n.d.	56 ± 3^{f}
Naringin	n.d.	n.d.	79 ± 1	n.d.	n.d.	79 ± 1^{d}
Nobiletin	2549 ± 62	1949 ± 111	16 ± 1	37 ± 24	n.d.	4551 ± 129^{a}
Tangeretin	1662 ± 55	460 ± 33	16 ± 3	19 ± 8	n.d.	2157 ± 84^{b}
Total	4211 ± 83^{w}	2574 ± 116^{x}	487 ± 12^{y}	56 ± 25^{z}	n.d.	7328 ± 145
Phenolic acids						
Caffeic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ferulic acid	n.d.	94 ± 3	22 ± 1	277 ± 24	88 ± 4	481 ± 24^{a}
p-Coumaric acid	n.d.	73 ± 3	13 ± 1	194 ± 15	75 ± 17	355 ± 23^{b}
Sinapic acid	n.d.	n.d.	n.d.	30 ± 5	17 ± 9	47 ± 10^{c}
Ellagic acid	n.d.	n.d.	n.d.	n.d.	14 ± 9	14 ± 9^{d}
Gallic acid	n.d.	339 ± 36	102 ± 8	n.d.	n.d.	441 ± 37^{a}
Total	n.d.	506 ± 36^{w}	137 ± 8^{x}	501 ± 29^{w}	194 ± 22^{x}	1338 ± 52
ORAC (μmole Trolox/g extract)	487 ± 15	708 ± 12	1154 ± 20	369 ± 20	384 ± 9	3102 ± 35 (ORAC)

n.d.: Not detectable.
Values (means ± S.D., $n = 3$) with different superscripts (a–f) within the same column and with different superscripts (w–z) within the same raw in separated group are significantly different ($p < 0.05$).

In the insoluble composition (i.e. bound compounds), such as ester and glycoside that are linkage phenolic compounds, a small amount of tangeretin (5 mg/100 g dry extract) was the only compound found in flavonoids, and which was extracted after alkali hydrolysis. Since tangeretin does not have a hydroxyl group, it could not be an ester linkage in calamondin peel.

In the soluble compositions, there were also only nobiletin and tangeretin observed in hexane extract from immature calamondin after heat treatment (Table 5). The content of nobiletin and tangeretin were higher than that in fresh sample. A similar phenomenon existed in ethyl acetate extract. This indicates that heat treatment at 150 °C leading to a better extraction rate might be due either to the destruction of the cell wall structure or degradation of hydrophobic interaction between polymethoxyflavones and matrix of calamondin peel. However, the content of insoluble polymethoxyflavones increased after heat treatment. This hints that the better release of polymethoxyflavones is mainly due to cell wall structure degradation, not degradation of hydrophobic interaction. In addition, the content of hesperidin in ethyl acetate extract and DGPP in hot water extract were decreased drastically after heat treatment. This might indicate that DGPP and hesperidin were heat sensitive and degraded during high temperature heating (150 °C).

Tables 4 and 5 show the changes in soluble and insoluble phenolic acids of immature calamondin peel after heat treatment. No soluble phenolic acid was found in extracts of immature calamondin peel, except for caffeic acid in hot water extract with 19 mg/100 g dry extract. In the insoluble compositions, ferulic acid and *p*-coumaric acid were the two major bound phenolic acid in ester (205 and 92 mg/100 g dry extract, respectively) and glycoside link-

ages (19 and 15 mg/100 g dry extract, respectively). Furthermore, sinapic acid was observed only in ester linkage and ellagic acid was found only in glycoside linkage. This indicates that most of the phenolic acids in immature calamondin peel were in bound form with ester and glycoside linkages.

After heating, gallic acid, ferulic acid and *p*-coumaric acid increased in ethyl acetate extract from undetectable to 339, 94, and 73 mg/100 g dry extract, respectively (Tables 4 and 5). In hot water extract, these three phenolic acids existed in lower levels, while caffeic acid disappeared after heating. For the insoluble compositions, the content of ferulic acid and *p*-coumaric acid increased similarly after heating. It seems that both soluble and insoluble phenolic acids can be better extracted from immature calamondin after heat treatment at 150 °C, except for caffeic acid in hot water extract.

Collectively, it is proposed that heating might mainly lead to better extracting and releasing of phenolic compounds due to a thermal destruction of cell wall in immature calamondin peel. The major increasing phenolic compounds extracted from immature calamondin peel after heat treatment were phenolic acids, such as gallic acid, ferulic acid and *p*-coumaric acid, and flavonoids, such as nobiletin and tangeretin. However, the effect of thermal degradation of phenolic compounds was also observed for DGPP, hesperidin and caffeic acid.

3.5. Antioxidant activity of soluble and insoluble phenolic compounds in immature calamondin peel after heating

The ORAC assay of each extraction from immature calamondin peel after heat treatment by successive processes with different solvents is shown in Tables 4 and 5. The ORAC value of heated immature calamondin peel was significantly higher than fresh peel in all extracts, with the exception of hexane extract. After heating, total ORAC increased from 2815 to 3102 μmol Trolox/g extract. The change in DPPH scavenging activity was similar (data not shown). In ethyl acetate and hot water extracts, gallic acid might contribute to the antioxidant activity, since the content increased after heat treatment. In insoluble compositions, the major phenolic acids, including ferulic acid and *p*-coumaric acid, increased after heat treatment, which might also provide the antioxidant activity. Collectively, the enhanced antioxidant activity of immature calamondin peel after heat treatment at 150 °C could be a result of better release of phenolic acids, i.e., gallic acid, ferulic acid, and *p*-coumaric acid.

4. Conclusion

Hot water extract from immature calamondin peel could provide a higher level of phenolic compounds and good antioxidant activity than other methods. The total phenolic contents and DPPH scavenging activity of various extracts from immature calamondin peel showed a significantly positive relationship ($p < 0.01$). Most of the flavonoids in immature calamondin peel are soluble, while phenolic acids mainly exist in bound form. High temperature heating (150 °C) can enhance the extraction of phenolic acids in both soluble and insoluble form, and flavonoids in soluble form. However, some phenolic compounds, such as DGPP, hesperidin and caffeic acid, could be degraded during heating. After heat treatment, the increase in phenolic acids, both in soluble and insoluble forms, might contribute to the increase in antioxidant activity.

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