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

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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;

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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