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Phenolic content, antioxidant activity and effective compounds of kumquat extracted by different solvents

Shyi-Neng Lou [a,*], Yi-Chun Lai [a], Ya-Siou Hsu [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

The total phenolic and flavonoid content of extracts from peel of kumquat were higher than those from pulp, and those extracted from immature kumquat were higher than those from mature kumquat. The highest levels of phenolic and flavonoid content were obtained in hot water extracts. The flavonoids of kumquat extracted from hot water were mainly soluble conjugated compounds, including *C*-glycosides, such as 3′,5′-di-*C*-β-glucopyranosylphloretin (DGPP), acacetin 8-*C*-neohesperidoside (margaritene), acacetin 6-*C*-neohesperidoside (isomargaritene), apigenin 8-*C*-neohesperidoside, and *O*-glycosides, such as acacetin 7-*O*-neohesperidoside (fortunellin), isosakuranetin 7-*O*-neohesperidoside (poncirin) and apigenin 7-*O*-neohesperidoside (rhoifolin). A positive relationship existed between total phenolic content and DPPH scavenging potency ($p < 0.001$). Total flavonoid content showed a similar correlation ($p < 0.001$) to DPPH scavenging potency. The effective flavonoids contributing to antioxidant activity were DGPP and apigenin 8-*C*-neohesperidoside, which could be extracted in high amounts, by hot water at 90 °C, from immature kumquat peel.

1. Introduction

Kumquat (*Citrus japonica* var. margarita) is a small, elliptical shaped fruit, closely related to *Citrus*. The main growing area of kumquats in Taiwan is in Ilan county with over 90% of kumquats grown in Taiwan in last decade. They are used as traditional folk medicine to manage inflammation of the respiratory tract (Chiu & Chang, 1998; Lin, Hung, & Ho, 2008; Zang, 2005). The health benefits of citrus are well-documented. Their bioactivity is attributed to the presence of flavonoid compounds (Roowi & Crozier, 2011). The major flavonoids in citrus are flavanone glycosides and polymethoxyflavones (Kawail, Tomono, Katase, Ogawa, & Yano, 1999; Li et al., 2009; Ogawa, Kawasaki, Omura, & Yoshida, 2001). However, the flavonoid compositions of kumquats are very different from those of other *Citrus* species (Barreca, Bellocco, Caristi, Leuzzi, & Gattuso, 2011; Jayaprakasa, Murthy, Etlinger, Mantur, & Patil, 2012; Kumamoto, Matsubara, Iizuka, Okamoto, & Yokoi, 1985; Lou et al., 2015; Ogawa et al., 2001; Ramful, Tarnus, Aruoma, Bourdon, & Bahorun, 2011; Sadek, Makris, & Kefalas, 2009).

Few studies report the antioxidant activity and flavonoid composition of kumquat as affected by various extraction solvents. Eight flavonoids of kumquat namely, eriocitrin, narirutin, hesperidin, neohesperidin, luteolin, neoponcirin, poncirin, and kaempferol extracted with methanol/DMSO have been reported and quantified (Kawail et al., 1999). Only poncirin, didymin, isorhoifolin, hesperidin, and narirutin (in 80% methanol extract) have been both quantified and the antioxidant activity evaluated (Ramful et al., 2011). In another study, 3′,5′-di-*C*-β-glucopyranosylphloretin (DGPP), poncirin, narirutin, rutin, and apigenin 8-C-rutinoside are observed and quantified in five kumquat extracts using different solvents (Jayaprakasa et al., 2012). In our previous study seven flavonoids are identified and quantified from hot water extract of immature kumquats (Lou et al., 2015). Barreca et al. (2011) quantified thirteen flavonoids of kumquat juice (*Fortunella japonica*). Eight flavonoids were found and studied during qualitative analysis of kumquats (*F. japonica*) in hot water extract (Kumamoto et al., 1985) and ten were found using methanol as the extracting solvent (Cho et al., 2005). Ogawa et al. (2001) demonstrated that DGPP, acacetin 8-*C*-neohesperidoside (margaritene), acacetin 6-*C*-neohesperidoside (isomargaritene) and fortunellin (acacetin 7-*O*-neohesperidoside) exist in ethanol extract of kumquats (*F. japonica*). However, DGPP and rutinoside derivatives of acacetin, instead of neohesperidoside derivatives of acacetin, are found using ethyl acetate, dichloromethane, and butanol extracts

* Corresponding author.
E-mail address: snlou@niu.edu.tw (S.-N. Lou).

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of kumquat (*Fortunella margarita*) but no quantitative data are available (Sadek et al., 2009). Thus, the reported flavonoid compositions in kumquat extracts using various extraction solvents are quite different. Very little quantitative data and antioxidant activity of different extracts from kumquats have been reported.

To improve their application we studied the flavonoid compositions and antioxidant activity of kumquat extracts prepared by different solvents. Mature and immature, as well as peel and pulp, of kumquat were investigated separately. The relationship between the content of phenolic compounds and antioxidant activity was evaluated. The effective antioxidant flavonoids were also isolated and identified.

2. Materials and methods

2.1. Materials

Kumquats (*C. japonica* var. margarita) were collected from a kumquat estate in the Jao-Si region, Ilan, Taiwan in November 2009. Green kumquats were sorted in the laboratory as immature kumquats, and kumquats with whole yellow appearance were defined as mature kumquats. After washing and manual peeling, the separated peels and pulps of mature and immature kumquats 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 kumquat peel and pulp were stored in suitable brown bottles with screw caps at −18 °C.

2.2. Chemicals

Methanol, ethanol, and acetonitrile were LC grade from Merck Chemical Co. (Darmstadt, Germany). Acetic acid, Na_2CO_3, 2, 2′-diphenyl-1-picrylhydrazyl (DPPH), disodium fluorescein (FL), 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) were analytical grade. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, 97% (Trolox), and standards of flavonoids, including fortunellin, poncirin, and rhoifolin, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3′,5′-Di-*C*-β-glucopyranosylphloretin (DGPP), acacetin 8-*C*-neohesperidoside (margaritene), acacetin 6-*C*-neohesperidoside (isomargaritene), apigenin 8-*C*-neohesperidoside were separated from hot water extract of immature kumquat in our laboratory. The extract was separated by a preparative HPLC, VP Nucleodur 100-5 C_{18} column (250 mm × 21.0 mm id, 5 μm) (Macherey–Nagel, Düren, Germany) and the compounds were collected. They were then subjected to LC/MS^n identification by comparison with data in literature. The purity for DGPP, margaritene, isomargaritene and apigenin 8-*C*-neohesperidoside were determined as 97.8%, 97.9%, 90.4%, and 90.1%, respectively, based on area of HPLC chromatogram by UV 280 nm measurement. After lyophilization, the residues were redissolved in suitable solvents and stored at −18 °C for further use.

2.3. Preparation of extracts by different solvents

Three grams of dried and powdered kumquat peels and pulps, including mature and immature, were extracted with (1) 50 mL deionized hot water (80, 90, and 100 °C) for 1 h in a shaking water bath at the same temperature, or extracted with (2) 30 mL ethanol (50%, 60%, 70%, 80%, and 95%), or (3) 30 mL methanol in a shaker at room temperature for 1 h. The shaking rate was 100 rpm (rpm). The extract was filtered with Whatman No. 1 filter paper. The obtained residue was extracted by the same procedure two additional times. Three resulting filtrates were transferred into a 250 mL flask and dried using a rotary vacuum evaporator at 40 °C. A suitable volume of deionized water, ethanol, and methanol was added to each flask to dissolve the filtrate. The obtained solutions were poured into brown bottles with screw caps and stored at −18 °C until further use. Triplicate determinations (n = 3) were carried out during the study.

2.4. Determination of total phenolic content

A hundred microliters of extract from kumquat or standard solution, were mixed with 100 μL of Folin–Ciocalteu's phenol reagent for 3 min (Taga, Miller, & Pratt, 1984). The mixture was added to 1 mL of 20% Na_2CO_3 solution and incubated in the dark for 30 min at room temperature. After incubation, absorbance was measured at 750 nm against the blank. 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 a standard curve. All samples were analyzed in triplicate.

2.5. Determination of total flavonoid content

Five hundred microliters of kumquat 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 analyzed in triplicate. The total flavonoid content was expressed as mg quercetin equivalents (QE) per 100 g dry extract using the standard curve established previously.

2.6. HPLC analysis of flavonoid composition and identified by HPLC–UV-ESI/MS

Hot water extracts of kumquat were subjected to HPLC analysis (Shimadzu LC-10AT) with a Discovery RP-C18 column (250 mm × 4.6 mm, 5 μm, Supelco, Bellefonte, PA, USA) using a gradient with deionized water as solvent A and acetonitrile as solvent B (Barreca et al., 2011). The gradient was carried out at 0 min, 5% B; 15 min, 20% B; 35 min, 100% B; 40 min, 5% B; 50 min, 5% B for equilibrium. The flow rate was 1 mL/min. Photodiode array (PDA) detection was performed between 220 and 350 nm, with a resolution of 2 nm. Seven flavonoids used as standards were available in our laboratory as follows: DGPP, margaritene, isomargaritene, apigenin 8-*C*-neohesperidoside, fortunellin, poncirin, and rhoifolin. The flavonoid compounds were quantified from their peak area at 280 nm by an external standard method, using calibration curves. Their concentrations were expressed as milligram per 100 g dry peel.

For the identification of flavonoids, a Thermo Fisher Spectra System P4000 HPLC coupled with a UV detector (Shimadzu SPD 10 Avp, Tokyo, Japan) and a Thermo Fisher Scientific LCQ-Fleet mass spectrometer (Waltham, MA, USA) were used. The separation was performed as described previously. The ion trap mass spectrometer was equipped with an electrospray ionization (ESI) source. Mass spectra were obtained at positive and negative ion modes. The source parameters were as follows: ESI source voltage of 5 kV, capillary temperature 300 °C, Sheath gas 40 arbitrary. The tube lens voltage was 0 for positive ion mode and −25 V for negative ion mode. Full scan MS was measured from m/z 160 to 1000. Collision-induced fragmentation experiments were performed using helium as the collision gas. The instrument operated under the Xcalibur version 2.5 delivered by Thermo Fisher.

2.7. DPPH radical scavenging activity

The DPPH radical scavenging activity of kumquat extracts was measured according to a slightly modified method of Yamaguchi, Takamura, Matoba, and Terao (1998). After 0.5 mL of kumquat 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:

$$\%\ \text{DPPH radical scavenging activity} = (1 - \text{Peak area in sample}/\text{Peak area in blank}) \times 100$$

2.8. 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 kumquat extract solution and 50 μL of disodium fluorescein (70 nM final concentration) were mixed in a cuvette and preincubated for 15 min at 37 °C. Twenty-five microliters of APPH 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 concentrations) were also tested to establish a standard curve. All samples were analyzed in triplicate. The area under the curve (AUC) was calculated for each sample by integrating the relative fluorescence curve. The net AUC of each 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 dry extract using the standard curve established previously.

2.9. 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 content of different solvent extracts of immature and mature kumquats

Total phenolic contents of immature and mature kumquat extracted by hot water, ethanol, and methanol are shown in Table 1. For immature kumquats, the total phenolic content in peel extracted by hot water was in a range of 2346–3000 mg GAE/100 g dry extract, while the highest content was obtained by 80 and 90 °C extraction. The extracts of methanol and ethanol (50–95%) showed 2082 mg GAE/100 g dry extract and 1537–1848 mg GAE/100 g dry extract for peel, respectively. In pulp, the phenolic content was significantly lower than in peel. The total phenolic content of immature kumquats was over two times higher than those in mature kumquats. The highest phenolic content for mature kumquats was also obtained by hot water extract. These indicated that phenolic compounds in kumquats are mainly hydrophilic. of the calamondin. Total flavonoid content of kumquats showed a similar tendency of extraction of total phenolic

Table 1
Total phenolic contents of mature and immature kumquat extracted by different solvents.

Solvents	GAE mg/100 g dry extract			
	Immature		Mature	
	Peel	Pulp	Peel	Pulp
Hot water				
80 °C	3000 ± 58^{a}	1540 ± 31^{b}	1362 ± 19^{a}	799 ± 8^{b}
90 °C	2984 ± 60^{a}	1930 ± 33^{a}	1042 ± 6^{bc}	768 ± 18^{bc}
100 °C	2346 ± 20^{b}	1477 ± 20^{b}	1014 ± 59^{bcd}	861 ± 36^{a}
Ethanol				
50%	1848 ± 77^{d}	1129 ± 5de	919 ± 38^{d}	746 ± 24^{c}
60%	1823 ± 41^{d}	1093 ± 9^{e}	956 ± 22^{cd}	720 ± 9^{c}
70%	1836 ± 16^{d}	1139 ± 56de	937 ± 7^{d}	733 ± 10^{c}
80%	1832 ± 67^{d}	1206 ± 59^{d}	984 ± 13^{cd}	724 ± 26^{c}
95%	1537 ± 155^{e}	1204 ± 19^{d}	551 ± 65^{f}	571 ± 20^{d}
Methanol	2082 ± 177^{c}	1375 ± 68^{c}	1096 ± 40^{b}	848 ± 23^{a}

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

Table 2
Total flavonoid contents of mature and immature kumquat extracted by different solvents.

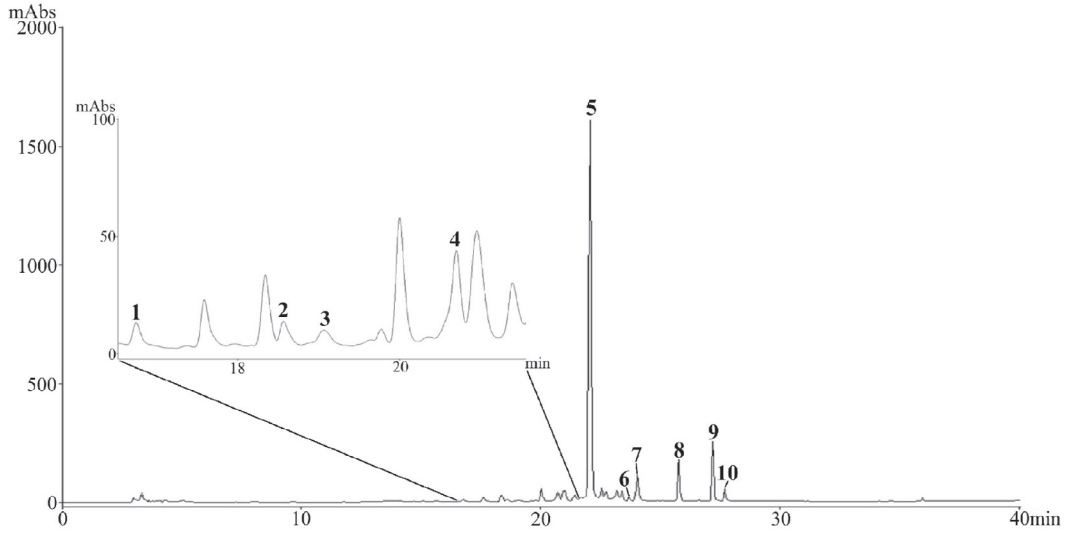
Solvents	QE mg/100 g dry extract			
	Immature		Mature	
	Peel	Pulp	Peel	Pulp
Hot water				
80 °C	288 ± 7^{b}	156 ± 11^{b}	173 ± 14^{a}	98 ± 7^{a}
90 °C	326 ± 12^{a}	207 ± 1^{a}	153 ± 14^{a}	95 ± 5^{a}
100 °C	265 ± 5^{bc}	159 ± 1^{b}	160 ± 18^{a}	103 ± 6^{a}
Ethanol				
50%	132 ± 6^{f}	53 ± 14^{f}	54 ± 2^{d}	28 ± 9^{e}
60%	147 ± 6^{f}	72 ± 2de	68 ± 1^{d}	25 ± 1^{e}
70%	179 ± 2^{e}	80 ± 6^{cd}	80 ± 2^{c}	44 ± 1^{bc}
80%	215 ± 12^{d}	55 ± 8^{ef}	93 ± 1^{c}	36 ± 4^{bc}
95%	241 ± 27^{cd}	90 ± 4^{c}	122 ± 1^{b}	46 ± 2^{bc}
Methanol	218 ± 22^{d}	57 ± 3^{ef}	128 ± 5^{b}	56 ± 3^{b}

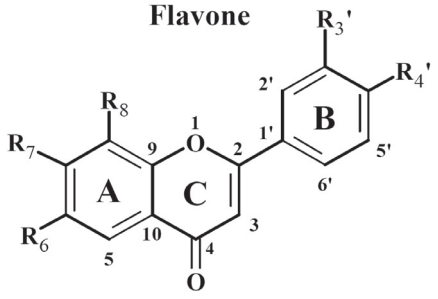
$^{a–f}$ Values (mean ± S.D., $n = 3$) in the same column with different superscripts are significantly different ($p < 0.05$).

Table 3
DPPH radical scavenging potency of mature and immature kumquat extracted by different solvents.

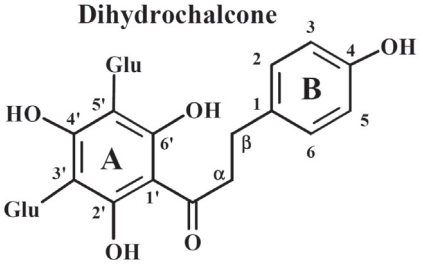
Solvents	Scavenging potency (%/mg/mL)^{A}			
	Immature		Mature	
	Peel	Pulp	Peel	Pulp
Hot water				
80 °C	45.5 ± 2.4^{a}	24.3 ± 2.8^{a}	23.3 ± 1.1^{a}	15.7 ± 1.1^{a}
90 °C	46.5 ± 5.3^{a}	27.4 ± 3.9^{a}	18.2 ± 0.6^{b}	14.2 ± 1.0^{b}
100 °C	32.3 ± 1.0^{b}	19.7 ± 2.9^{b}	15.5 ± 2.5^{c}	11.9 ± 0.2^{c}
Ethanol				
50%	8.3 ± 0.6^{c}	7.0 ± 0.3^{cd}	5.6 ± 0.2^{de}	5.1 ± 0.3^{d}
60%	7.1 ± 0.3^{cd}	9.0 ± 0.2^{c}	5.2 ± 0.2^{de}	4.9 ± 0.2^{d}
70%	7.1 ± 0.9^{cd}	7.8 ± 1.0^{c}	4.9 ± 0.2^{de}	3.8 ± 0.2^{e}
80%	6.0 ± 0.3^{d}	8.6 ± 0.9^{c}	4.5 ± 0.4^{e}	3.6 ± 0.1^{e}
95%	4.2 ± 0.8^{e}	6.0 ± 0.1^{cd}	2.1 ± 0.3^{f}	2.4 ± 0.1^{f}
Methanol	6.6 ± 1.4^{cd}	7.1 ± 0.5^{cd}	6.9 ± 0.8^{d}	4.7 ± 0.5^{d}

^{A} Scavenging potency = Scavenging effect (%)/Solid concentration in the reaction.
$^{a–f}$ Values (mean ± S.D., $n = 3$) in the same column with different superscripts are significantly different ($p < 0.05$).

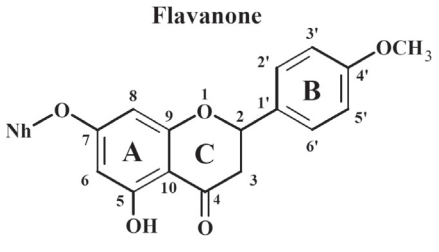




Peak	Compounds	$R_{3'}$	$R_{4'}$	R_6	R_7	R_8
1	apigenin 6,8–di–*C*–glucoside (vicenin–2)	H	OH	Glu	OH	Glu
2	luteolin 8–*C*–neohesperidoside	OH	OH	H	OH	Nh
3	luteolin 6–*C*–neohesperidoside	OH	OH	Nh	OH	H
4	apigenin 8–*C*–neohesperidoside	H	OH	H	OH	Nh
6	apigenin 7–*O*–neohesperidoside (rhoifolin)	H	OH	H	*O*–Nh	H
7	acacetin 8–*C*–neohesperidoside (margaritene)	H	OCH_3	H	OH	Nh
8	acacetin 6–*C*–neohesperidoside (isomargaritene)	H	OCH_3	Nh	OH	H
9	acacetin 7–*O*–neohesperidoside (fortunellin)	H	OCH_3	H	*O*–Nh	H



5: 3 , 5 –di–*C*– –glucopyranosylphloretin (DGPP)



10: isosakuranetin 7–*O*–neohesperidoside (poncirin)

Fig. 1. Flavonoid compositions of 90 °C hot water extract from peel of immature kumquat analyzed by HPLC. (Peak number: (1) apigenin 6,8-di-*C*-glucoside (vicenin-2), (2) luteolin 8-*C*-neohesperidoside, (3) luteolin 6-*C*-neohesperidoside, (4) apigenin 8-*C*-neohesperidoside, (5) 3′,5′-Di-*C*-β-glucopyranosylphloretin (DGPP), (6) apigenin 7-*O*-neohesperidoside (rhoifolin), (7) acacetin 8-*C*-neohesperidoside (margaritene), (8) acacetin 6-*C*-neohesperidoside (isomargaritene), (9) acacetin 7-*O*-neohesperidoside (fortunellin), (10) isosakuranetin 7-*O*-neohesperidoside (poncirin).

Table 4
Flavonoid composition of peel from mature and immature kumquat extracted by hot water at 90 °C.

Flavonoids	Peel of immature kumquat	Peel of mature kumquat
	(mg/100 g dry peel)	
3′,5′-Di-*C*-β-glucopyranosylphloretin	2082 ± 110[a]	1348 ± 43[a]
Acacetin 8-*C*-neohesperidoside	372 ± 10[b]	179 ± 6[b]
Fortunellin	234 ± 6[c]	97.8 ± 3.6[c]
Acacetin 6-*C*-neohesperidoside	205 ± 5[d]	101 ± 3[c]
Apigenin 8-*C*-neohesperidoside	56.5 ± 3.3[e]	21.4 ± 2.5[d]
Poncirin	33.0 ± 1.0[f]	14.8 ± 0.4[f]
Rhoifolin	7.4 ± 0.0[g]	5.5 ± 0.2[g]
Total	2990 ± 111	1768 ± 44

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

contents by various solvents (Table 2). The highest content was obtained in peel by hot water extract at 90 °C (326 mg QE/100 g dry extract). The flavonoid content of immature kumquats was higher than those in mature kumquat, and in peel higher than in pulp.

3.2. Antioxidant activity of different extracts and maturities from kumquat

DPPH radical scavenging potency of kumquats extracted by hot water was higher than those extracted by ethanol, and methanol (Table 3). The highest scavenging potency in peel of immature kumquats was found by hot water extraction at 80 and 90 °C (45.5 and 46.5%/mg/mL, with no significant difference). This coincides with the traditional use of kumquats, the hot drink of the small fruit is popular locally as a healthy drink for the respiratory tract (Lin et al., 2008; Lou et al., 2015; Zang, 2005). However, extraction by boiling water showed lower radical scavenging potency than those extracted by 80 and 90 °C. This phenomenon was observed regardless of the ripening stage of peel or pulp. It is most likely the result of the decrease in antioxidant activity due to the thermal effect.

Similar to the changes of total phenolic content, the scavenging potency of immature kumquats was higher than those in mature kumquats. In addition, peel contained higher scavenging potency than in pulp. The correlation coefficient (r) between total phenolic content and DPPH scavenging potency in extracts of kumquats peel and pulp was 0.7376, which is considered significant ($p < 0.001$) (data not shown). Total flavonoids content also showed significant correlation ($p < 0.001$) to DPPH scavenging potency with $r = 0.7352$.

3.3. Effective antioxidant phenolic compounds of immature kumquat peel

Chromatograms of flavonoids from 90 °C hot water extract of immature kumquat peel by HPLC coupled with PDA detector are shown in Fig. 1. Ten compounds, listed in Fig. 1, were numbered and identified based on retention time, UV spectra, MS data, and compared with the data of literature. Seven quantified flavonoids of immature and mature kumquats are listed in Table 4. They were mainly soluble conjugated flavonoids. In immature kumquats, over 90% of total identified flavonoids were *C*-glycosyl compounds, including DGPP, margaritene, isomargaritene, and apigenin 8-*C*-neohesperidoside. The most abundant compound is DGPP in 70% of total flavonoids. The level of *O*-glycosyl compounds are about 10%, including fortunellin, poncirin, and rhoifolin. The major *O*-glycosyl flavonoid was fortunellin (7%). In mature kumquats, *C*-glycosyl compounds were 93.2%, while *O*-glycosyl compounds were 6.8%. DGPP was the richest flavonoid in mature kumquats at 76.2%. As expected, total flavonoid composition of mature kumquats was about 59.1% to immature kumquats.

The profiles of flavonoids in kumquats were quite different to citrus in which the most abundant flavonoids are naringin, hesperidin, and polymethoxyflavones, such as nobiletin and tangeretin. No nobiletin and tangeretin were found in kumquat. A large quantity of DGPP has already been reported in *Fortunella* spp. (Barreca et al., 2011; Jayaprakasa et al., 2012; Kumamoto et al., 1985; Lou et al., 2015; Ogawa et al., 2001; Sadek et al., 2009). DGPP showed good tyrosinase inhibitory activity (Lou, Yu, & Ho, 2012). The aglycones of DGPP and margaritene, such as phloretin and acacetin, exhibit a broad spectrum of biological activities, including antioxidant, anti-inflammatory and anticancer effects (Hsu, Kuo, & Lin, 2004; Liao, Houghton, & Hoult, 1999; Pan, Lai, Wang, & Ho, 2006; Rezk, Haenen, Van der Vijgh, & Bast, 2002; Shao et al., 2008). The antibacterial activity of fortunellin (Rizvi et al., 2009) and the protective effect of poncirin on gastric disease have been reported (Lee, Lee, Kim, & Jeong, 2009).

In order to elucidate the effective antioxidant flavonoids of kumquat, the hot water extract at 90 °C of immature kumquat peel was subjected to a preparative HPLC. Six main flavonoids were isolated, and the antioxidant activities were evaluated. The results indicate that DGPP showed the highest DPPH scavenging potency and ORAC in 153.6%/mg/mL and 481.7 mmol Trolox/g dry extract, respectively (Table 5). Apigenin 8-*C*-neohesperidoside had lower antioxidant activity than DGPP, while the other four flavonoids showed very low or no antioxidant activity. The antioxidant activity of DGPP is probably through the action of the A ring (Barreca, Bellocco, Caristi, Leuzzi, & Gattuso, 2010). It has also been reported that the antioxidant pharmacophore in the dihydrochalcone phloretin is the 2′,6′-dihydroxyacetophenone core (Rezk et al., 2002). However, DGPP contains a 4-hydroxy group in the B-ring that may also provide good antioxidant ability (Lou et al., 2012). In apigenin 8-*C*-neohesperidoside, the structure of the aglycone,

Table 5
The scavenging effect on DPPH radical of isolated compounds from 90 °C hot water extract of immature kumquat peel.

Compounds	Scavenging potency (%/mg/mL)	ORAC (mmole Trolox/g dry extract)
Apigenin 8-*C*-neohesperidoside	80.9 ± 3.2[b]	133.1 ± 5.9[b]
3′,5′-Di-*C*-β-glucopyranosylphloretin	153.6 ± 2.7[a]	481.7 ± 12.6[a]
Acacetin 8-*C*-neohesperidoside	4.6 ± 1.9[d]	12.3 ± 1.5[c]
Acacetin 6-*C*-neohesperidoside	13.9 ± 0.6[c]	4.0 ± 1.7[d]
Fortunellin	–[e]	10.2 ± 2.1[c]
Poncirin	–	6.1 ± 1.4[d]

[a–d] Values (mean ± S.D., $n = 3$) in the same column with different superscripts are significantly different ($p < 0.05$).
[e] No inhibition effect.

apigenin, contained a 4-oxo group and the C_2–C_3 double bond that can provide a long chain conjugation system in the B-ring. This might enhance the electron delocalization of the B-ring (Amaral, Mira, Nogueita, da Silva, & Florencio, 2009; Bors & Saran, 1987). It also contains a 4-hydroxy group in the B-ring. The other four flavonoids, margaritene, isomargaritene, fortunellin, and poncirin, have a 4-methoxylated group in the B-ring. The blocked 4-hydroxy group in the B-ring might be the result of the loss antioxidant activity of the flavonoids.

4. Conclusion

The highest content of phenolic and flavonoid compounds were obtained by hot water extraction. The major extractable phenolic compounds in kumquats were soluble conjugated flavonoids, including about 90% *C*-glycoside and 10% *O*-glycoside flavonoids. The phenolic and flavonoid content of immature kumquat were higher than that in mature kumquat, while that in peel was higher than in pulp. A significantly positive relationship ($p < 0.001$) existed between DPPH scavenging potency and total phenolic content, as well as total flavonoid content. The major effective antioxidant compounds of kumquat were identified as DGPP and apigenin 8-*C*-neohesperidoside.

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References

Amaral, S., Mira, L., Nogueita, J. M. F., da Silva, A. P., & Florencio, M. H. (2009). Plant extracts with anti-inflammatory properties – A new approach for characterization of their bioactive compounds and establishment of structure–antioxidant activity relationship. *Bioorganic & Medicinal Chemistry, 17*, 1876–1883.

Barreca, D., Bellocco, E., Caristi, C., Leuzzi, U., & Gattuso, G. (2010). Flavonoid composition and antioxidant activity of juices from Chinotto (*Citrus* × *myrtifolia* Raf.) fruits at different ripening stages. *Journal of Agricultural and Food Chemistry, 58*, 3031–3036.

Barreca, D., Bellocco, E., Caristi, C., Leuzzi, U., & Gattuso, G. (2011). Kumquat (*Fortunella japonica* Swingle) juice: Flavonoid distribution and antioxidant properties. *Food Research International, 44*, 2190–2197.

Bors, W., & Saran, M. (1987). Radical scavenging flavonoid antioxidants. *Free Radical Research Communication, 2*, 289–294.

Chiu, N. C., & Chang, K. S. (1998). *The illustrated medicinal plants of Taiwan*. Taiwan: SMC publishing Ltd.

Cho, J. Y., Kawazoe, K., Moon, J. H., Park, K. H., Murakami, K., & Takaishi, Y. (2005). Chemical constituents from the fruit peels of *Fortunella japonica*. *Food Science and Biotechnology, 14*, 599–603.

Christel, Q. D., Gressier, B., Vasseur, J., Dine, T., Brunet, C., Luyckx, M., ... Trotin, F. (2000). Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum* Moench) hulls and flour. *Journal of Ethnopharmacology, 72*, 35–42.

Hsu, Y. L., Kuo, P. L., & Lin, C. C. (2004). Acacetin inhibits the proliferation of Hep G2 by blocking cell cycle progression and inducing apoptosis. *Biochemical Pharmacology, 67*, 823–829.

Jayaprakasa, K. G., Murthy, K. N. C., Etlinger, M., Mantur, S. M., & Patil, B. S. (2012). Radical scavenging capacities and inhibition of human prostate (LNCaP) cell proliferation by *Fortunella margarita*. *Food Chemistry, 131*, 184–191.

Kawail, S., Tomono, Y., Katase, E., Ogawa, K., & Yano, M. (1999). Quantitation of flavonoid constituents in citrus fruits. *Journal of Agricultural and Food Chemistry, 47*, 3565–3571.

Kumamoto, H., Matsubara, Y., Iizuka, Y., Okamoto, K., & Yokoi, K. (1985). Structure and hypotensive effect of flavonoids glycosides in Kinkan (*Fortunella japonica*) peelings. *Agricultural and Biological Chemistry, 49*, 2613–2618.

Lee, J. H., Lee, S. H., Kim, Y. S., & Jeong, C. S. (2009). Protective effects of neohesperidin and poncirin isolated from the fruits of *Poncirus trifoliata* on potential gastric disease. *Phytotherapy Research, 23*, 1748–1753.

Li, S., Pan, M. H., Lo, C. Y., Tan, D., Wang, Y., Shahidi, F., & Ho, C. T. (2009). Chemistry and health effects of polymethoxyflavones and hydroxylated polymethoxyflavones. *Journal of Functional Foods, 1*, 2–12.

Liao, Y. H., Houghton, P. J., & Hoult, J. R. (1999). Novel and known constituents from Buddleja species and their activity against leukocyte eicosanoid generation. *Journal of Natural Products, 62*, 1241–1245.

Lin, C. C., Hung, P. F., & Ho, S. C. (2008). Heat treatment enhances the NO-suppressing and peroxynitrite-intercepting activities of kumquat (*Fortunella margarita* Swingle) peel. *Food Chemistry, 109*, 95–103.

Lou, S. N., Lai, Y. C., Huang, J. D., Ho, C. T., Ferng, L. H. A., & Chang, Y. C. (2015). Drying effect on flavonoid composition and antioxidant activity of immature kumquat. *Food Chemistry, 171*, 356–363.

Lou, S. N., Yu, M. W., & Ho, C. T. (2012). Tyrosinase inhibitory components of immature calamondin peel. *Food Chemistry, 135*, 1091–1096.

Ogawa, K., Kawasaki, A., Omura, M., & Yoshida, T. (2001). 3′,5′-Di-C-β-glucopyranosylphloretin, a flavonoid characteristic of the genus *Fortunella*. *Phytochemistry, 57*, 737–742.

Ou, B., Hampsch-Woodill, M., & Prior, R. L. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry, 49*, 4619–4626.

Pan, M. H., Lai, C. S., Wang, Y. J., & Ho, C. T. (2006). Acacetin suppressed LPS-induced up-expression of iNOS and COX-2 in murine macrophages and TPA-induced tumor promotion in mice. *Biochemical Pharmacology, 72*, 1293–1303.

Ramful, D., Tarnus, E., Aruoma, O. I., Bourdon, E., & Bahorun, T. (2011). Polyphenol composition, vitamin C content and antioxidant capacity of Mauritian citrus fruit pulps. *Food Research International, 44*, 2088–2099.

Rezk, B. M., Haenen, G. R. M. M., Van der Vijgh, W. J. F., & Bast, A. (2002). The antioxidant activity of phloretin: The disclosure of a new antioxidant pharmacophore in flavonoids. *Biochemical and Biophysical Research Communications, 295*, 9–13.

Rizvi, W., Rizvi, M., Kumar, R., Kumar, A., Shukla, I., & Parveen, M. (2009). Antibacterial activity of *Ficus lyrata* – An *in vitro* study. *The Internet Journal of Pharmacology, 8*(2).

Roowi, S., & Crozier, A. (2011). Flavonoids in tropical citrus species. *Journal of Agricultural and Food Chemistry, 59*, 12217–12225.

Sadek, E. S., Makris, D. P., & Kefalas, P. (2009). Polyphenolic composition and antioxidant characteristics of kumquat (*Fortunella margarita*) peel fractions. *Plant Foods for Human Nutrition, 64*, 297–302.

Shao, X., Bai, N., He, K., Ho, C. T., Yang, C. S., & Sang, S. (2008). Apple polyphenols, phloretin and phloridzin: New trapping agents of reactive dicarbonyl species. *Chemical Research in Toxicology, 21*, 2042–2050.

Taga, M. S., Miller, E. E., & Pratt, D. E. (1984). Chia seeds as a source of natural lipid antioxidants. *Journal American Oil Chemists' Society, 61*, 928–931.

Yamaguchi, T., Takamura, H., Matoba, T., & Terao, J. (1998). HPLC method for evaluation of the free radical scavenging activity of foods by using 1,1-diphenyl-2-picrylhydrazyl. *Bioscience Biotechnology Biochemistry, 62*, 1201–1204.

Zang, K. T. (2005). *Small encyclopedia of Chinese herbal medicine*. Heliopolis Culture Group, Taiwan: Neptune Culture Publishing Ltd..