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Review Article

Phenolic compounds and biological activities of small-size citrus: Kumquat and calamondin

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ABSTRACT

Kumquat and calamondin are two small-size citrus fruits. Owing to their health benefits, they are traditionally used as folk medicine in Asian countries. However, the research on flavonoids and biological activities of kumquat and calamondin have received less attention. This review summarizes the reported quantitative and qualitative data of phenolic compositions in these two fruits. Effects of maturity, harvest time, various solvent extractions and heat treatment of phenolic compositions, and bioactivities were discussed; distributions of the forms of phenolic compounds existing in kumquat and calamondin were also summarized. Furthermore, biological activities, including antioxidant, anti-tyrosinase, antimicrobial, antitumor, and antimetabolic disorder effects, have also been discussed. Effective phenolic components were proposed for a certain bioactivity. It was found that C-glycoside flavonoids are dominant phenolic compounds in kumquat and calamondin, unlike in other citrus fruits. Up to now, biological activities and chemical characteristics of C-glycoside flavonoids in kumquat and calamondin are largely unknown.

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1. Introduction

Citrus species are rich sources of flavanone glycosides, in particular, hesperidin and naringin, but the presence of dihydrochalcones and C-glycoside flavonoids is not common in fruits of the genus *Citrus*. Kumquat and calamondin bear small-size fruits and contain high amounts of C-glycoside flavonoids and dihydrochalcone compounds [1–5]. Kumquat is an elliptical shaped fruit, closely related to *Citrus*. It has four major cultivated types, including *Fortunella japonica*, *Fortunella*

margarita, *Fortunella crassifolia*, and *Fortunella hindsii* [3]. The whole fruit including peel is used and may be candied or preserved in sugar syrup. Kumquat is also used as traditional folk medicine to manage inflammation of the respiratory tract [6–8]. Health benefits of citrus are well documented; however, few studies report the bioactivity and phenolic composition of kumquat. Moreover, the biological activity of flavonoid C-glycoside in kumquat has not been investigated. Aglycones of flavonoid C-glycosides in kumquat, such as phloretin and acacetin, exhibit a broad spectrum of biological activities such

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as antioxidant activity, antiinflammatory effect, and anticancer effect [9–13].

Calamondin (*Citrus mitis*, *Citrus microcarpa*, or *Citrus manurensis*), a cultivar related to *Citrus*, is a hybrid between *Citrus reticulata* Blanco and *Fortunella* spp. [14]. Hot water extract of immature fruits with green peel has been a popular beverage in Taiwan for many years due in part to its potential health beneficial properties. It may be attributed to the presence of bioactive compounds such as flavonoids in the peel. Calamondin contains a large quantity of 3',5'-di-C- β -glucopyranosylphloretin (DGPP) in its peel, juice sac, and leaves [3]. The flavonoid composition is quite different from that of *Citrus*. However, there are only a few reports on the quantitative data and biological activity of calamondin peel, particularly that of the immature fruits. Therefore, it is desirable to study the flavonoid compositions, especially C-glycoside flavonoids and dihydrochalcone, as well as biological activity of kumquat and calamondin for their potential applications.

2. Kumquat

2.1. Flavonoids and phenolic acids

Fortunellin, a flavone (acacetin-7-O-neohesperidoside), was isolated from kumquat by Matsuno in 1958 [1]. It was known as a major representative flavonoid in kumquat for a long time. In 1985, eight flavonoid glycosides, including 6,8-di-C-glucosylapigenin, 3,6-di-C-glucosylacacetin, 2''-O- α -L-rhamnosyl-4'-O-methylvitexin, 2''-O- α -L-rhamnosyl-4'-O-methylisovitexin, 2''-O- α -L-rhamnosylvitexin, 2''-O- α -L-rhamnosylorientin, 2''-O- α -L-rhamnosyl-4'-O-methylorientin, and poncirin, were isolated and identified in hot water extract of the peel of kumquat (*F. japonica*) [2]. Kawail et al [15] quantified flavonoids in various citrus, including kumquat (*F. japonica*, *F. margarita*, and *F. crassifolia*), extracted by a mixture of methanol and dimethyl sulfoxide. The major quantified flavonoids in the edible part of kumquat were narirutin, rhoifolin, kaempferol, luteolin, poncirin, hesperidin, neoponcirin, eriocitrin, and quercetin. The highest content of narirutin was found in the range of 289–460 μ g/100 mg dried sample. However, as a result of reinvestigation by the same laboratory, “narirutin” was shown to be a dihydrochalcone glucoside, DGPP, which has previously been isolated from kumquat (*F. margarita*) as a major flavonoid [3]. They assumed that the accumulation of DGPP is a generic trait of the genus *Fortunella*, while the genera *Citrus* and *Poncirus* probably lack it. The other three major compounds were identified as 2'-O- α -L-rhamnopyranosylcytisiside, 2'-O- α -L-rhamnopyranosylisocytisiside, and fortunellin. Fortunellin and DGPP in the peel of *F. japonica* had also been identified by Cho et al [16], while acacetin-8-C-neohesperidoside and kaempferide 3-O-rhamnopyranoside were found at the same time. In a study of kumquat peel extracted by ethyl acetate, DGPP and poncirin were also reported [17]. However, they had also found acacetin-6-C-rutinoside, acacetin-8-C-rutinoside, and acacetin-7-O-rutinoside, instead of neohesperidoside, which most former researchers identified.

However, other studies identified naringin, hesperidin, neohesperidin, diosmin, sinensetin, rutin, quercetin, and

kaempferol in the peel of kumquat [18]. Schirra et al [19] demonstrated that only narirutin and rhoifolin were present in kumquat. They found that flavonoids in kumquat are quite different from those reported in other studies. Only five flavonoids, including poncirin, didymin, isorhoifolin, hesperidin, and narirutin, had been quantified in the pulp of kumquat (*F. margarita*) [20]. These indicated a lack of consistent knowledge on the flavonoid compositions of kumquat. It is probably because a few studies in this field focus on kumquat and also because inadequate techniques are used for flavonoid identification.

In the last decade, liquid chromatography tandem mass spectrometry (LC/MS/MS) was widely used to investigate the natural constituents of plants because of its good reliability in quantitative and qualitative analyses. Barreca et al [21] quantified 13 flavonoids in kumquat juice (*F. japonica*) by LC/MS/MS, the main components of which were DGPP, fortunellin, acacetin-6-C-neohesperidoside, acacetin-8-C-neohesperidoside, apigenin-8-C-neohesperidoside, hesperidin, poncirin, didymin, and rhoifolin. In another study, DGPP, poncirin, narirutin, rutin, and apigenin-8-C-rutinoside were observed and quantified in five kumquat extracts using different solvents by LC/MS/MS [22]. In our previous study, 15 flavonoids could be separated from hot water extract of kumquat (*F. margarita*) and characterized by high-performance liquid chromatography (HPLC) (Figure 1) [4]. Seven major flavonoids have been identified and quantified by LC/MS/MS. Their chemical structures and quantitative data are shown in Figure 2 and Table 1 [5]. A similar result about the seven major flavonoids in kumquat has also been reported [23]. However, the authors did not quantify the phenolic compounds. Another study of the leaves of *F. japonica* has identified three flavonoids, including apigenin-7-O- β -D-glucopyranoside, apigenin-7-O- β -D-rhamnoglucoside, and the methoxylated flavonoid cirsimaritin, which are quite different from the flavonoids found in the peel and pulp of kumquat [24].

Seven quantified flavonoids of immature and mature kumquats are listed in Table 1. They were mainly soluble conjugated flavonoids. In immature kumquats, over 90% of total identified flavonoids were C-glycosyl compounds, including DGPP, acacetin-8-C-neohesperidoside, acacetin-6-C-neohesperidoside, and apigenin-8-C-neohesperidoside. The most abundant compound was 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%) [5].

C-glycosides are usually resistant against acidic and enzymatic hydrolysis in contrast to corresponding O-glycosides [25]. DGPP, a phloretin with 6- and 8-C-glycosylation, is a dihydrochalcone glucoside. It is a hydrophilic compound mainly because of its two glucose moieties. Although 98% remained at pH 4.8 after 24 hours of storage, it degraded to 68% at pH 6.8 [26]. Phloretin has been reported to have many biological activities, including antioxidant activity in apple [27], which might partially be because of the stabilization of radical by 2,6-dihydroxyacetophenone via tautomerization [10]. Acacetin-8-C-neohesperidoside and acacetin-6-C-neohesperidoside, also named as margaritene and isomargaritene, respectively, are C-glycosyl flavones

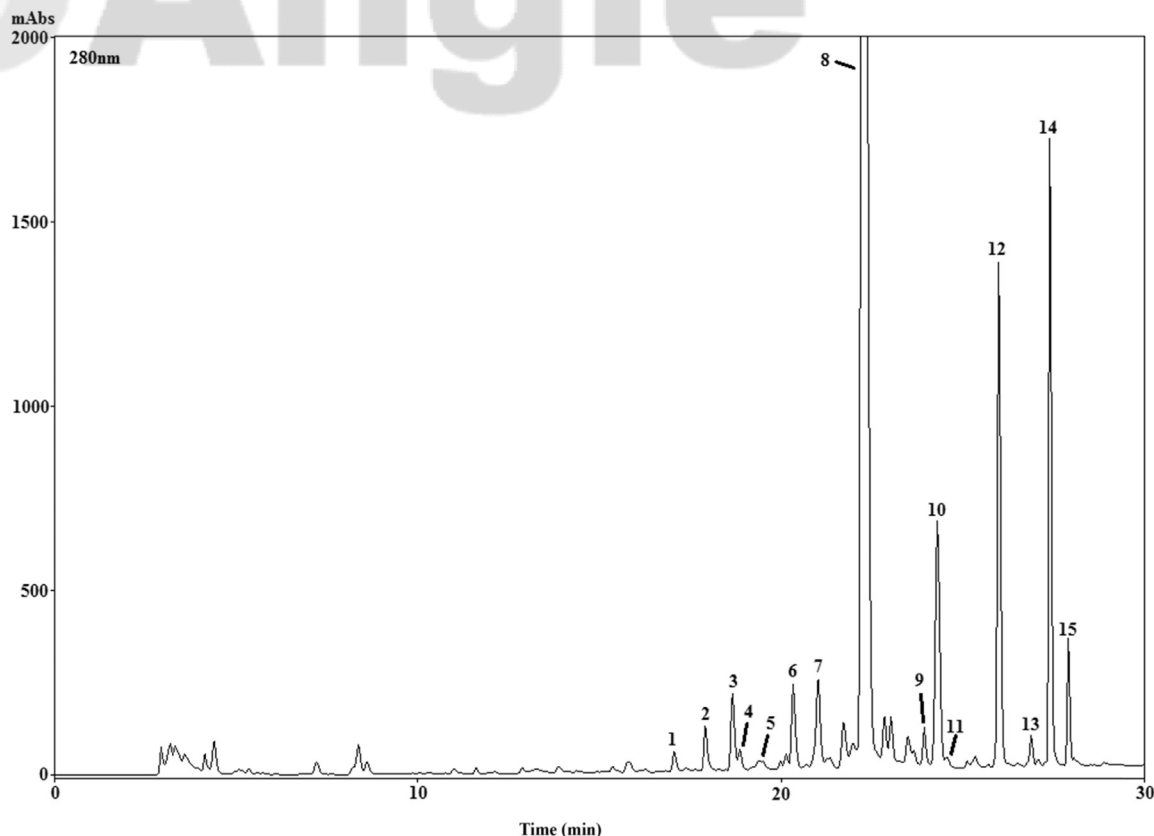


Figure 1 – HPLC profiles of hot water extracts of immature kumquat. Peak numbers represent the flavonoids obtained, which are as follows: 1, apigenin-6,8-di-C-glucoside; 2, flavanone di-C-pentose–hexose; 3, flavanone di-C-pentose–hexose; 4, luteolin-8-C-neohesperidoside; 5, luteolin-6-C-neohesperidoside; 6, flavanone di-C-pentose–hexose; 7, apigenin-8-C-neohesperidoside; 8, 3',5'-di-C- β -glucopyranosylphloretin; 9, apigenin 7-O-neohesperidoside (rhoifolin); 10, acacetin-8-C-neohesperidoside (margaritene); 11, unknown; 12, acacetin-6-C-neohesperidoside (isomargaritene); 13, di-C-hexoxyl derivative; 14, acacetin-7-O-neohesperidoside (fortunellin); and 15, isosakuranetin-O-neohesperidoside (poncirin). HPLC = high-performance liquid chromatography. Note. Adapted from “Drying effect on flavonoid composition and antioxidant activity of immature kumquat,” by S.N. Lou, Y.C. Lai, J.D. Huang, C.T. Ho, L.H. Ferng, and Y.C. Chang, 2015, *Food Chemistry*, 171, p. 356–63.

with a methoxy group at 4' position of the B-ring. Acacetin has been reported to be able to exert antiperoxidative, antiinflammatory, antiplasmodial effects [11,28,29]. It was also suggested that acacetin is a functionally novel agent capable of preventing inflammation-associated tumorigenesis [12]. The amounts of these two C-glycosyl flavones are at the second order after DGPP. Apigenin-8-C-neohesperidoside is also a flavone with 5,7,4'-OH. It contains a 4-oxo group, the C₂–C₃ double bond, and 4'-hydroxy group in the B-ring, which might contribute to its antioxidant activity [30,31].

Fortunellin (acacetin-7-O-neohesperidoside) is another major flavone with the magnitude similar to margaritene and isomargaritene. It is the main O-glycoside flavone in kumquat, the aglycone content of which is the same as that of margaritene and isomargaritene. Poncirin (isosakuranetin-O-neohesperidoside) is the only flavanone in kumquat. A small amount of rhoifolin (apigenin-7-O-neohesperidoside) is also found in kumquat. Thus, it could be concluded that the major flavonoids in kumquat are dihydrochalcone, flavone, and

flavanone, while the main aglycones are phloretin, acacetin, and apigenin.

In mature kumquats, 93.2% of flavonoids were C-glycosyl compounds, while only 6.8% were O-glycosyl compounds. The dihydrochalcone DGPP was still the richest flavonoid in mature kumquats at 76.2%. As expected, the total flavonoid composition of mature kumquats was about 59.1% compared with that of immature kumquats [5].

Flavonoids C-glycosides received less attention than flavonoids O-glycosides in citrus, since most of the flavonoids in citrus are O-glycosides. However, flavonoids C-glycosides also show a broad spectrum of human health benefits [32]. Recently, a review article regarding the current status of the flavonoid C-glycosides and their biological benefits has been published. C-glycosyl flavonoids, in most cases, showed higher antioxidant potential than the corresponding O-glycosyl flavonoids and aglycones [33]. A study on the anti-inflammatory potential of flavone C-glycosides showed that (1) glycosylation in ring A improved the anti-inflammatory activity, (2) methoxylation of hydroxyl groups on flavone ring

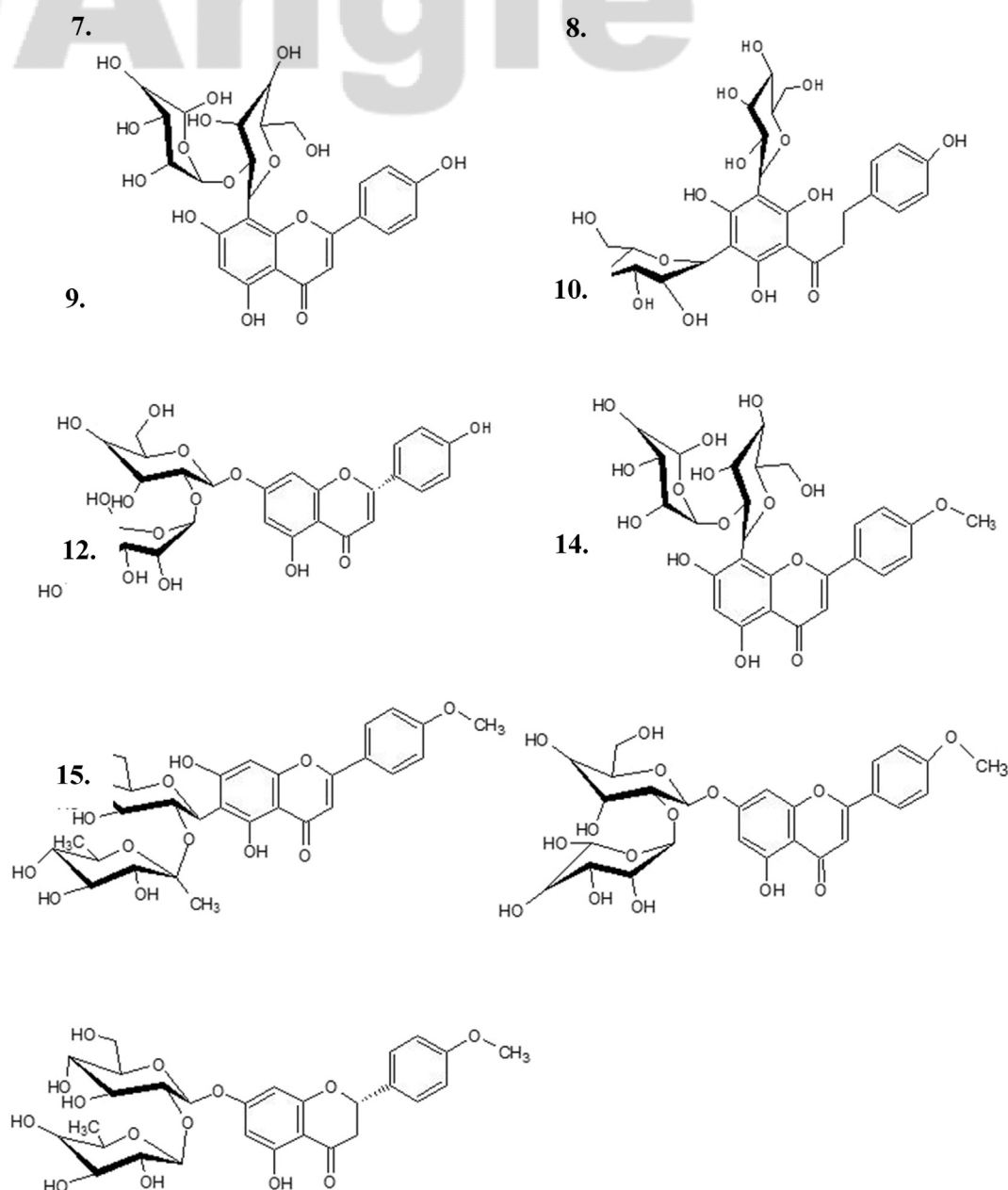


Figure 2 – Chemical structures of the major flavonoids in kumquat: 7, apigenin-8-C-neohesperidoside; 8, 3',5'-di-C-β-glucopyranosylphloretin; 9, apigenin-7-O-neohesperidoside (rhoifolin); 10, acacetin-8-C-neohesperidoside (margaritene); 12, acacetin-6-C-neohesperidoside (isomargaritene); 14, acacetin-7-O-neohesperidoside (fortunellin); and 15, isosakuranetin-7-O-neohesperidoside (poncirin).

reduced the antiinflammatory activity, (3) C-glycosyl flavones showed a stronger antiinflammatory potential than O-glycosyl flavones, and (4) the higher the polarity of the substitute on C''-2, the lower the antiinflammatory potential [33]. However, there are still lack of knowledge regarding the main flavonoid C-glycosides in kumquat, i.e., DGPP, acacetin-8-C-neohesperidoside, and acacetin-6-C-neohesperidoside. For flavonoids O-glycosides, rhoifolin and fortunellin showed inhibitory activities against HIV-1 protease [34] and protection of hepatocytic autophagy [35]. In addition, the antibacterial activity of fortunellin has been reported [36]. Another major O-

glycoside flavonoid is poncirin; its protective effect on gastric disease [37] and antiinflammatory activity [38] have been studied. Poncirin is also demonstrated to act on mesenchymal stem cells by promoting differentiation to osteoblasts while inhibiting adipogenesis [39]. It could be an agent for treatment of osteoporosis and prevention of obesity.

Very few studies reported about phenolic acids in kumquat. It is probably because the phenolic acids are usually covalently bound to insoluble polymers in plant tissue [40,41]. Thus, a small amount of phenolic acids in kumquat can be extracted by various solvents and quantified. In our previous study, we

Table 1 – Flavonoid composition of and immature kumquat peel extracted by hot water.

Flavonoids	Peel of immature kumquat	Peel of mature kumquat
	(mg/100 g dry peel)	
3',5'-Di-C- β -glucopyranosylphloretin	2082 \pm 110 ^a	1348 \pm 43 ^a
Acacetin 8-C-neohesperidoside (margaritene)	372 \pm 10 ^b	179 \pm 6 ^b
Fortunellin	234 \pm 6 ^c	97.8 \pm 3.6 ^c
Acacetin-6-C-neohesperidoside (isomargaritene)	205 \pm 5 ^d	101 \pm 3 ^c
Apigenin-8-C-neohesperidoside	56.5 \pm 3.3 ^e	21.4 \pm 2.5 ^d
Poncirin	33.0 \pm 1.1 ^f	14.8 \pm 0.4 ^f
Rhoifolin	7.4 \pm 0.0 ^g	5.5 \pm 0.2 ^g
Total	2990 \pm 111	1768 \pm 44

Note. Adapted from “Phenolic content, antioxidant activity and effective compounds of kumquat extracted by different solvents” by S.N. Lou, Y.C. Lai, Y.S. Hsu, and C.T. Ho, 2016, *Food Chemistry*, 197, p. 1–6.^{a–g} Values (mean \pm SD, n = 3) in the same column with different superscripts are significantly different ($p < 0.05$). SD = standard deviation.

found that *p*-coumaric acid and ferulic acid existed as ester linkages in kumquat, and no free phenolic acids were found. The major phenolic acid in kumquat is *p*-coumaric acid [42].

2.2. Distribution

The total phenolic and flavonoid contents of kumquat peel extracts were higher than those in pulp extracts. The flavonoid content of immature kumquats was higher than that of mature kumquats [5]. The growth of fruits generally decreases the flavonoid contents per fruit weight in *Citrus* fruits [3]. It is logical to hypothesize that the high level of bitter flavonoids in young fruit tissues may have a role in defense against herbivores, at least until seeds within the fruit are mature [43]. The highest levels of these secondary metabolites were found in young developing fruits [44], although it has been suggested that some polymethoxyflavones might be related to the maturation phase of fruits in some *Citrus* species [45]. However, polymethoxyflavones were not found in mature kumquats, since only hot water extract of kumquat, instead of its hydrophobic extract, was investigated. The distribution of DGPP in kumquat was in the order leaf > peel > juice sac [3]. Traditionally, kumquat, unlike other *Citrus*, is consumed unpeeled. Therefore, much more phenolic compounds can be consumed, leading to good health benefits.

Among the different extraction solvents used, including hot water (80°C, 90°C, and 100°C), ethanol, and methanol, the highest levels of phenolic and flavonoid contents were obtained in hot (90°C) water extracts [5]. A successive extraction procedure with solvents of different polarities has been reported [42]. The results showed that most of the flavonoids in kumquat were in conjugated form, which could be extracted

by hot water. Only a small amount of fortunellin existed in an ester linkage form in kumquat.

2.3. Effects of environmental factors and heat treatments

The total contents of phenolic compounds extracted from immature kumquats were higher than those from mature kumquats [5]. The levels of flavonoids in the juice of unripe kumquats were higher than those from ripe fruits [21]. A progressive decrease in the flavonoid content had been reported in other *Citrus*, e.g., in the juice of *Citrus myrtifolia* [46] and peel of calamondin [47]. Furthermore, the effect of different flowering periods on flavonoid contents of immature kumquats had been studied in Taiwan [26]. It was found that the highest amount of flavonoids in immature kumquats was obtained during the flowering period from June to October. It is proposed that the high flavonoid content is correlated to higher average temperature and total sunshine time during the flowering period [26].

It is well known that dried citrus peel is used as traditional Chinese medicine [7,40]. Studies on the effect of heat treatment on phenolic compounds and the antioxidant activity of citrus peel have been reported [40,41,48,49]. It was suggested that high-temperature drying might be used as an effective method to release bound phenolic compounds from citrus and increase their antioxidant activity [40,41,50]. However, few studies on heat treatment of kumquat have been reported. Heat treatment could enhance the content of total phenolics up to 45% and the antioxidant activities of kumquat peel [8]. In another study, changes in flavonoids of immature kumquats after heat treatment have been investigated [4]. The results indicated that flavonoids of immature kumquats could be better extracted after drying at 110°C for a short time (i.e., 0.5 hours), which might be due to the effect of heat degradation on the cellulose structure of immature kumquats. No significant thermal degradation effect was found on the existing flavonoids during drying at 110°C for 2.0 hours, since most of the flavonoids of immature kumquats were C-glycoside derivatives, which were more heat resistant. However, drying at 130°C for 1.0 hour could enhance the yield of flavonoid extraction, probably due to the destruction of the cell wall structure, i.e., cellulose and lignin. The content of flavonoids extracted at 130°C for 1.0 hour was higher than that at 110°C for 0.5–2.0 hours. Furthermore, flavonoids in immature kumquats could be degraded by high-temperature heating at over 130°C for more than 1.5 hours; especially, the content of DGPP decreased drastically. Therefore, it is suggested that immature kumquats can be dried at 130°C for 1.0 hour before the hot water extraction process to obtain the highest amount of flavonoids with increased antioxidant activity [4].

2.4. Bioactivities

2.4.1. Antioxidant activity

Epidemiological studies have shown the versatile health benefits of flavonoid intake. The possible health beneficial effect might be due to their antioxidant activity [51]. *Kumquat* is a genus close to *Citrus* and contains C-glycoside and O-glycoside flavonoids, the profiles of which are quite different

from those of Citrus. There has been growing interest in the antioxidant activity of kumquat, since few studies on this subject have been reported.

Kumquat showed very good antioxidant activity. Antioxidant activities of the pulp of 21 varieties of citrus fruits extracted by 80% methanol were investigated [20]. The highest value of kumquat assessed by Trolox equivalent antioxidant capacity and ferric reducing antioxidant capacity was observed, while the lowest half maximal inhibitory concentration (IC₅₀) could also be found in kumquat by hypochlorous acid scavenging activity assay.

During the testing of DPPH (2,2-diphenyl-1-picrylhydrazyl free radical scavenging method) and hydroxyl free radical scavenging activities of kumquat extracts, ethyl acetate fraction showed better antioxidant activities than both dichloromethane and butanol fractions [17]. In another study, evaluation of radical scavenging capacities of kumquat extracted by various solvents, including hexane, ethyl acetate, acetone, methanol, and 80% methanol, has been demonstrated. The results indicated that the highest DPPH radical scavenging activity was observed in ethyl acetate extract, while 80% methanol extract exhibited maximum oxygen radical absorbance capacity (ORAC) values and antioxidant capacity by phosphomolybdenum method [22].

Antioxidant activities, including DPPH and ABTS⁺ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) scavenging ability, of kumquat juice has been studied [21]. The juice from ripe kumquats showed significant antioxidant power against DPPH radicals. Scavenging of ABTS⁺ radical cations was more efficient than DPPH quenching. The juice obtained from ripe and unripe kumquats showed similar good ability to quench ABTS⁺ radicals. Furthermore, it is concluded that the flavonoids from unripe fruits are responsible for most of the antioxidant activities against both DPPH (–79%) and ABTS⁺ (–93%). DGPP is responsible for about half of the DPPH scavenging activity and almost all the ABTS⁺ quenching ability.

DPPH radical scavenging potency of kumquats extracted by hot water was higher than that of kumquats extracted by ethanol and methanol [5]. The scavenging potency of immature kumquats was higher than that of mature kumquats. In addition, peel contained higher scavenging potency than pulp. The highest DPPH scavenging potency of immature kumquat peel was observed by hot water extraction at 80°C and 90°C (45.5%/mg/mL and 46.5%/mg/mL, with no significant difference). Six main flavonoids were isolated, and the antioxidant activities were also evaluated by the authors. The major effective antioxidant compounds of kumquat were identified as DGPP and apigenin-8-C-neohesperidoside. The antioxidant activity of DGPP is probably through the action of the A ring [46]. It has also been reported that the antioxidant pharmacophore in the dihydrochalcone phloretin is the 2',6'-dihydroxyacetophenone core [10]. However, DGPP contains a 4-hydroxy group in the B-ring that may also provide good antioxidant ability [47]. In apigenin-8-C-neohesperidoside, the structure of the aglycone apigenin contained a 4-oxo group and the C₂–C₃ double bond that can provide a long chain conjugation system in the B-ring. This might enhance the electron delocalization of the B-ring [30,31]. It also contains a 4'-hydroxy group in the B-ring. The other four flavonoids, acacetin-6-C-neohesperidoside, acacetin-8-C-neohesperidoside, fortunellin, and poncirin,

have a 4-methoxylated group in the B-ring. The blocked 4-hydroxy group in the B-ring might be the reason for the loss of antioxidant activity of the flavonoids.

Effect of drying on the antioxidant activity of immature kumquats has been studied [4]. The results indicated that drying process could enhance the antioxidant activity of hot water extract of immature kumquats. A higher temperature or longer drying duration would lead to higher antioxidant activity. For immature kumquats, higher antioxidant activity of hot water extract was obtained by drying at over 130°C for over 1.5 hours. With drying at a temperature lower than 130°C for 1.5 hours, the antioxidant activity of hot water extract of immature kumquats could be enhanced, which might be due to the increase of phenolic compounds. The antioxidant activity of kumquat could be drastically increased by drying at more than 130°C for 1.5 hours. It is suggested that products of browning reaction might play an important role.

Lin et al [8] investigated the nitric oxide (NO)-suppressing and peroxynitrite-intercepting activities of kumquat after heat treatment by the lipopolysaccharide-activated RAW 264.7 macrophage system. The results showed that heating enhanced the ability of kumquat peel to suppress NO and intercept peroxynitrite as compared with freeze drying. However, the authors did not identify the bioactive components of heated peel.

2.4.2. Antimicrobial activity

The antimicrobial effect of hot water extract of immature kumquat peel has been studied [52]. The extract showed better inhibitory effect against Gram-positive bacteria than against Gram-negative bacteria. The best effect against *Bacillus cereus* was found with a minimum inhibitory concentration (MIC) of 25 mg/mL. Furthermore, the author separated the extract into six fractions by a preparative C₁₈ column and evaluated their antimicrobial activities. Fraction IV showed good antibacterial activity against *B. cereus*, *Listeria monocytogenes*, and *Staphylococcus aureus* with MICs of 25 mg/mL, 12.5 mg/mL, and 25 mg/mL, respectively. The minimum bactericidal concentration of fraction IV against *L. monocytogenes* was 12.5 mg/mL. Flavonoids of fraction IV were also investigated. In 100 g dry fraction, 41.1 g DGPP, 22.3 g acacetin-8-C-neohesperidoside, 1.5 g fortunellin, 1.4 g acacetin-6-C-neohesperidoside, 0.5 g rhoifolin, and 0.2 g poncirin were found. However, Barreca et al [27] isolated phloretin and its glycosylated derivatives from apple and kumquat, and evaluated their antimicrobial activity. It is concluded that dihydrochalcones are able to inhibit the growth of Gram-positive bacteria, in particular *S. aureus*, *L. monocytogenes*, and methicillin-resistant *S. aureus* clinical strain. They also highlighted that the presence of a glycosyl moiety bound to the chalcone structure, i.e., DGPP, dramatically decreases the antimicrobial activity of phloretin. Another study showed antibacterial activity of fortunellin [36].

Thus, it is proposed that acacetin-8-C-neohesperidoside, fortunellin, acacetin-6-C-neohesperidoside, rhoifolin, and poncirin as well as other components might provide the ability to inhibit the growth of bacteria [52]. Some studies have shown that the essential oil of kumquat peel possessed good antimicrobial activity at concentrations of microgram range [53,54].

2.4.3. Tyrosinase inhibitory activity

Tyrosinase inhibitors have a broad range of applications for suppressing unwanted hyperpigmentation in human skin and enzymic browning in fruits. Therefore, searching effective tyrosinase inhibitors from natural products has gained increasing interest. It has been well known that citrus contain a large amount of flavonoids, such as hesperidin, naringin, and nobiletin, which have been studied for their tyrosinase inhibitory activity [55–57]. However, very few studies reported the tyrosinase inhibitory activity of kumquat.

The tyrosinase inhibitory activity of hot water extract of immature kumquat peel has been demonstrated first time that the tyrosinase inhibition effect was 75.5% in concentration of 2.5mM [26]. The effective compound was also isolated and identified as DGPP, which showed 71.7% inhibitory effect at the same concentration. Obviously, DGPP was the major effective component for tyrosinase inhibitory activity in kumquat. The inhibition effect of DGPP is better than that of arbutin (25.7% inhibitory effect at the same concentration).

In a study of tyrosinase inhibitory effect in calamondin, it was confirmed by Lineweaver–Burks plots that DGPP showed a competitive tyrosinase inhibitory effect [47]. The antioxidant pharmacophore in the dihydrochalcone phloretin is the 2,6-dihydroxyacetophenone core [10]. In addition, 3-hydroxyphloretin was demonstrated as the most active constituent from Formosan apple, which exhibited a competitive inhibition type in human epidermal melanocytes [58]. The tyrosinase inhibitory effect of DGPP might also be due to the action of the 2,6-dihydroxyacetophenone core. However, the 4-hydroxy group in the B ring could also contribute to the inhibitory effect of tyrosinase, since the structure is similar to tyrosine, and probably perform competitive inhibition [47].

2.4.4. Anticancer and antitumor activities

The *n*-hexane extract of kumquat (*F. margarita*), at a concentration of 100 mg/mL, exhibited the highest inhibition of human prostate cancer (LNCaP) cells (86.4%) after 96 hours, followed by EtOAc extract (82.8%), MeOH extract (76.7%), and MeOH–water extract (4:1, v/v) (68.2%) [22]. Fragmentation of DNA suggests the ability of extracts to induce apoptosis in LNCaP cells. The authors concluded that EtOAc, MeOH, and MeOH–water (4:1, v/v) extracts showed different levels of cytotoxicity, which may be due to the presence of various levels of rutin, narirutin, poncirin, apigenin-8-C-rutinoside, and DGPP in each extract. However, the maximum cell proliferation inhibition activity of *n*-hexane extract may be due to the presence of any of β -carotene, β -cubebene, and hexadecanoic acid or their cumulative effect [22].

Nagahama et al [59] studied the effect of acetone extracts from kumquat pericarp on natural killer (NK) cell activity *in vitro* and *in vivo*. It has been shown to significantly increase interferon- γ production and NK cytotoxic activity in human KHYG-1 NK cells. Moreover, oral administration of acetone extracts could improve both suppressed plasma interferon- γ levels and NK cytotoxic activity per splenocyte in restraint-stressed mice. It is suggested that the activation effect on NK cells might be caused by carotenoids such as β -cryptoxanthin.

Inhibitory activities of methanol–dimethyl sulfoxide (1:1, v/v) extracts from albedo of kumquat on platelet cyclooxygenase and lipoxygenase have been reported [60]. The IC₅₀ (μ g/mL) of cyclooxygenase was 643–680 and that of lipoxygenase was >1000, respectively. It showed low inhibitory activity compared with other citrus.

2.4.5. Antimetabolic disorder effect

The antimetabolic disorder effects of kumquat fruit extracted by 95% ethanol in high-fat-diet-induced C57BL/6 obese mice was investigated by Tan et al [61]. The authors demonstrated that the ethanol extract of kumquat had ameliorating effects on hyperglycemia, hyperlipidemia, and hepatic lipid accumulation in high-fat-diet-induced obese mice. The expression levels of PRAR α and its target genes involved in glucose and lipid metabolism in the mouse liver were increased by the treatment of ethanol extract of kumquat. Therefore, it is suggested that the ethanol extract of kumquat might be a promising dietary supplement for obesity and obesity-related metabolic disorders. In this study, the flavonoids detected in ethanol extract were only neoeriocitrin and poncirin.

3. Calamondin

3.1. Flavonoids and phenolic acids

Flavonoids of Citrus have widely been studied. It contains a large amount of flavanones, with hesperidin being the most abundant glycoside [20,62,63]. Calamondin, a cultivar related to Citrus, is a hybrid between *C. reticulata* Blanco and *Fortunella* spp. [64], and the flavonoid composition of *Fortunella* species differs from that of the Citrus species [3–5,17]. Therefore, compositions of flavonoids of calamondin might be quite different from those of citrus, and very few studies have been conducted on this subject.

In calamondin, citromitin and 5-O-desmethylecitromitin were isolated by extraction with petroleum ether [65]. However, citromitin and 5-O-desmethylecitromitin were later reidentified as nobiletin and 5-O-desmethylnobiletin [66]. The authors had also identified six other methoxyflavonoids in the peel of calamondin. Kawai et al [15] had reported that narirutin, hesperidin, neohesperidin, rutin, nobiletin, neoeriocitrin, neoponcirin, and 3,3',4',5,6,7,8-heptamethoxyflavone, in order of decreasing concentrations, were identified in calamondin extracted by a solvent mixed with methanol and dimethyl sulfoxide. However, DGPP was identified by Ogawa et al [3] as the major flavonoid in calamondin and kumquat, instead of narirutin as reported by Kawai et al [15]. Therefore, it is suggested that DGPP is present in the genus *Fortunella*. Detection of DGPP, as a chemical marker, by thin-layer chromatography is a rapid and useful method to detect a commercial shiikuwasha juice that is suspected to be adulterated with calamondin juice [67].

In another study, seven flavonoids have been determined in 80% methanol extract of calamondin, which included hesperidin, neohesperidin, poncirin, dydimin, narirutin, diosmin, and isorhoifolin [20]. DGPP and polymethoxyflavones were not identified, although several large unknown peaks existed in the HPLC chromatograms. Shie et al [68] have investigated the

flavonoids in ethyl acetate extract of calamondin. The results indicated that six flavonoids, including hesperidin, diosmin, neohesperidin, nobiletin, tangeretin, and 5-hydroxy-3',4',6,7,8-pentamethoxyflavone, were identified.

HPLC with Photodiode array detector (PAD) and MS/MS detection was used to identify and quantify flavonoids in acidified methanol extract of calamondin [69]. It was confirmed that apigenin-6,8-di-C-glucoside, apigenin-8-C-glucosyl-2''-O-rhamnoside, DGPP, hesperetin-7-O-rutinoside (hesperidin), and hesperetin-7-O-neohesperidoside (neohesperidin) existed in calamondin at levels of 200 µg/g dry fruit, 570 µg/g dry fruit, 675 µg/g dry fruit, 389 µg/g dry fruit, and 296 µg/g dry fruit, respectively. Immature calamondin peel contains a high amount of DGPP (3.69 ± 0.44 g/100 g dry peel), which also shows good tyrosinase inhibitory activity [47]. Furthermore, five major flavonoids, including DGPP, naringin, hesperidin, nobiletin, and tangeretin, were found in hot water extract of immature calamondin peel (ICW) [70]. Four flavonoids, except tangeretin, have also been determined in methanol extract of calamondin [71].

In our previous studies, the flavonoids in hot water extract of immature calamondin have been restudied [44,72]. Seven major flavonoids of immature calamondin were confirmed by LC/MS/MS. The HPLC chromatogram of flavonoids in hot water extract of immature calamondin is shown in Figure 3. The seven flavonoids that were identified are listed in order of decreasing amount as follows: DGPP, apigenin-6,8-di-C-glucoside (vicenin-2), apigenin-8-C-neohesperidoside, hesperetin-7-O-neohesperidoside (neohesperidin), hesperetin-7-O-rutinoside (hesperidin), 3',4',5,6,7,8-hexamethoxyflavone (nobiletin), and 4',5,6,7,8-pentamethoxyflavone (tangeretin); chemical structures of these flavonoids are given in Figure 4. The levels of flavonoids in immature calamondin are listed in Table 2.

The major flavonoids of calamondin are DGPP, apigenin-6,8-di-C-glucoside (vicenin-2), and apigenin-8-C-neohesperidoside, similar to the C-glycosyl flavonoid composition of kumquat, since calamondin is a hybrid between *C. reticulata* Blanco and *Fortunella* spp. [64]. Therefore, calamondin, like *Citrus*, also contains neohesperidin (hesperetin-7-O-neohesperidoside), hesperidin (hesperetin-7-O-rutinoside), nobiletin, and tangeretin, which was not found in kumquat. Collectively, it could be summarized that the major aglycones of flavonoids in calamondin are phloretin, apigenin, and hesperetin, while the main forms of flavonoids are dihydrochalcone, flavone, and flavanone. All these are more hydrophilic compounds. Some polymethoxyflavones, i.e., nobiletin and tangeretin, are present in calamondin, which are in the form of aglycones and show more hydrophobic characters.

Antityrosinase [47] and antioxidant [70] activities of DGPP, also found in kumquat, have been reported. Apigenin-6,8-di-C-glucoside (vicenin-2) could inhibit the angiotensin-converting enzyme *in vitro* [73], while apigenin-8-C-neohesperidoside might provide antioxidant activity [5]. As mentioned previously, a discussion on flavonoid C-glycosides and health benefits was reviewed recently [33]. However, more studies are still needed to evaluate the bioactivities of flavonoid C-glycosides.

For O-glycosides, neohesperidin might be useful for the treatment of and protection against gastritis [37]. It could significantly aggravate gastric damage caused by indomethacin administration [74]. Neohesperidin was also proved to have neuroprotective activity [75] and an antiproliferative effect on human hepatoma cell lines [76], and act as a potential hypoglycemic agent through regulation of glucose metabolism [77]. Hesperidin, also found as a major flavonoid in *Citrus*, has shown many bioactivities, including inhibition of

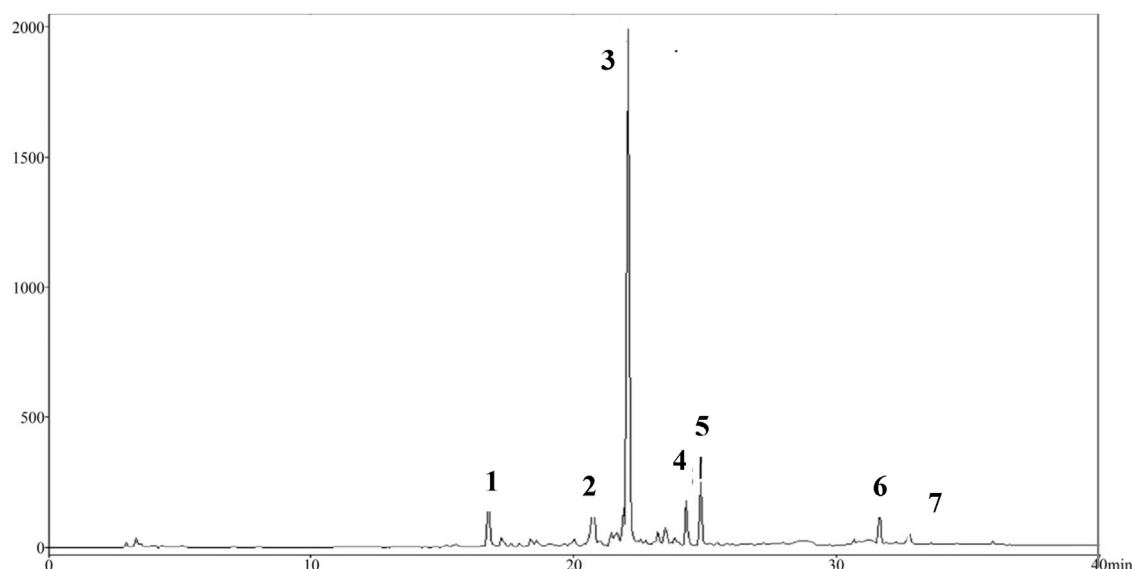


Figure 3 – Flavonoid compositions of hot water extracts of calamondin peel analyzed by HPLC. Peak numbers represent the flavonoids obtained, which are as follows: 1, vicenin-2; 2, apigenin 8-C-neohesperidoside; 3, 3',5'-di-C-β-glucopyranosylphloretin; 4, hesperidin; 5, neohesperidin; 6, nobiletin; and 7, tangeretin. HPLC = high-performance liquid chromatography. Note. Adapted from “Antibacterial activity and effective components in peel of kumquat and calamondin” by J.S. Dai, 2015, Master Thesis, Department of Food Science, National Ilan University, Taiwan.

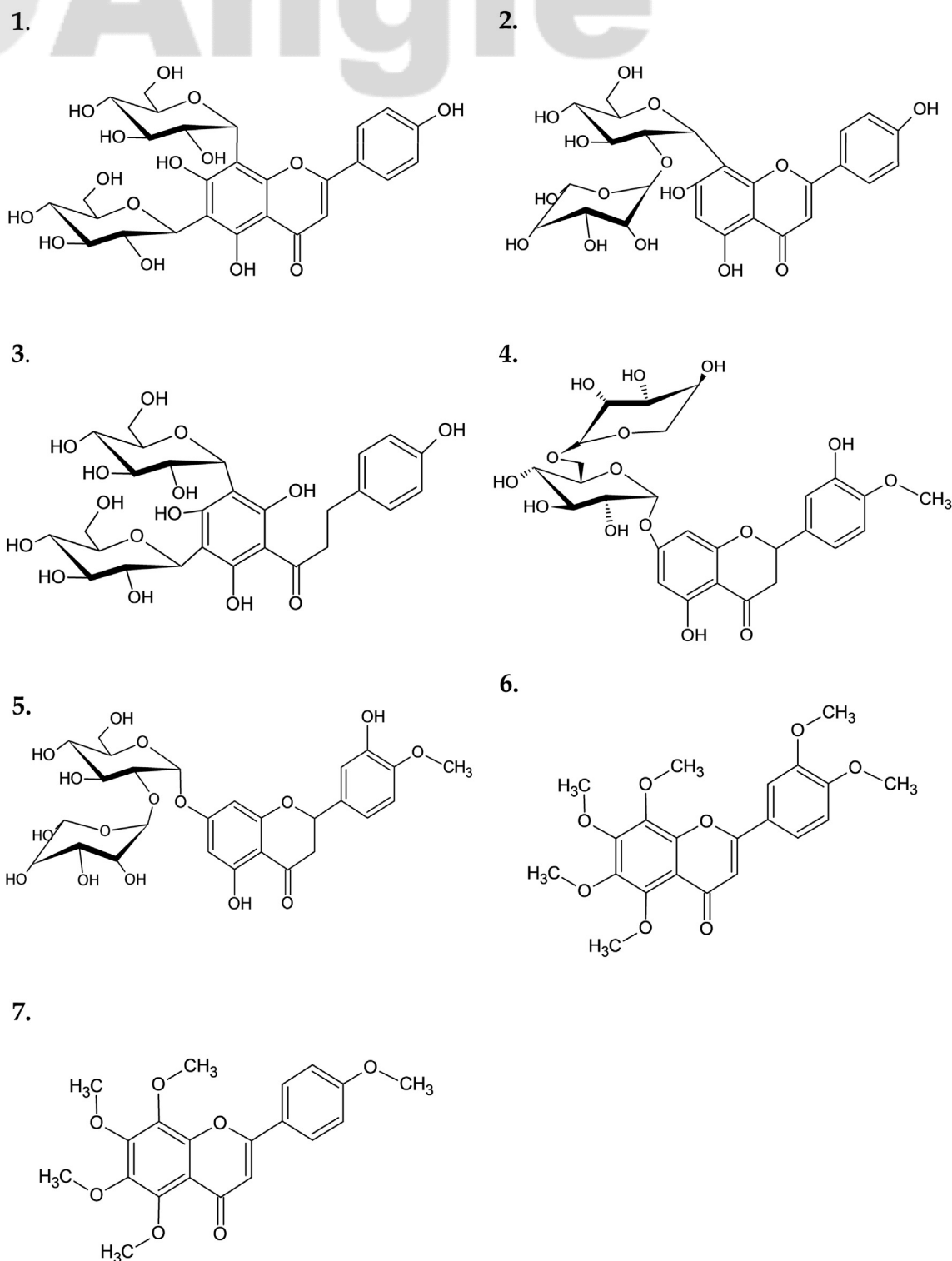


Figure 4 – Chemical structures of major flavonoids in calamondin: 1, vicenin-2; 2, apigenin-8-C-neohesperidoside; 3, 3',5'-di-C-β-glucopyranosylphloretin; 4, hesperidin; 5, neohesperidin; 6, nobiletin; and 7, tangeretin.

bone loss [78], antioxidant activity [79], antiulcer effect [80], and anticancer activity [81]. It has been reported to have a bitter taste. Nobiletin and tangeretin are polymethoxyflavones, which show lower antioxidant activities due to O-methylation [62]. However, polymethoxyflavones have been of particular interest because many of these flavonoids exhibit

a broad spectrum of biological activities, including anti-inflammatory, anticarcinogenic, antiviral, antithrombogenic, and antiatherogenic properties [82].

Very few studies were conducted investigating the phenolic compounds of calamondin. Four phenolic acids, including caffeic acid, *p*-coumaric acid, ferulic acid, and

Table 2 – Content of flavonoid composition of hot water extract from immature calamondin.

Compounds	mg/100 g dry calamondin
Apigenin-6,8-di-C–glucoside (vicenin-2)	637 ± 21
Apigenin-8-C-neohesperidoside	468 ± 22
3',5'-di-C-β-glucopyranosylphloretin	2335 ± 22
Hesperetin-7-O-rutinoside (hesperidin)	125 ± 6
Hesperetin-7-O-neohesperidoside (neohesperidin)	143 ± 6
3',4',5,6,7,8-Hexamethoxyflavone (nobiletin)	43 ± 5
4',5,6,7,8-Pentamethoxyflavone (tangeretin)	10 ± 1

Note. Adapted from “Enzymatic treatments assisted hot water extraction of phenolic components from calamondin and its identification” By S.H. Yang, 2015, Master Thesis, Department of Food Science, National Ilan University, Taiwan.

sinapic acid, were determined in the peel [83] and juice [84] of calamondin. They exist in both free and bound form. The main phenolic acids are ferulic acid and *p*-coumaric acid. However, only caffeic acid was found in hot water extract of the peel of fresh immature calamondin. Moreover, in insoluble compositions, ferulic acid and *p*-coumaric acid were two major bound phenolic acids in ester (205 mg/100 g dry extract and 92 mg/100 g dry extract, respectively) and glycoside linkages (19 mg/100 g dry extract and 15 mg/100 g dry extract, respectively). Furthermore, sinapic acid was observed only in the ester linkage and ellagic acid was found only in the glycoside linkage. These indicated that most of the phenolic acids in immature calamondin peel were in bound form with ester and glycoside linkage [85].

3.2. Distribution

The total phenolic contents of calamondin peel extracts were higher than those of pulp extracts [85,86]; the levels of phenolic acid also showed similar phenomena [83,84]. The content of phenolic compounds in peel was 1054–1894 mg/100 g dry extract, which was higher than that in pulp (189–1053 mg/100 g dry extract). The highest total phenolic content in the peel was observed in its boiling water extract (1894 mg/100 g dry extract). The smallest content of phenolic compounds was obtained in ethyl acetate extract. These indicated that most of the phenolic compounds in calamondin peel were hydrophilic [86].

The major flavonoid in various extracts of calamondin, including hot water, ethanol, and methanol extracts, was DGPP, except in ethyl acetate extract where nobiletin and tangeretin were the only two flavonoids detected. In hot water extracts, the content of DGPP was in the range of 1405–1581 mg/100 g dry extract, which was higher than that in other extracts with different solvents; the highest value was observed in hot (90°C) water extract [86]. Nobiletin and tangeretin are polymethoxyflavones with six and five methoxy groups, respectively. Owing to the hydrophobic nature of methoxy groups, polymethoxyflavones are less polar than hydroxylated flavonoids, such as DGPP and hesperidin [70,82].

Polymethoxyflavones, including nobiletin and tangeretin, existed mainly in the peel [86]. It has been reported that polymethoxyflavones in *Citrus* plays a key role in the defense against microbes [44].

Immature calamondin peel was extracted in successive steps with different solvents (hexane, ethyl acetate, hot water, alkali hydrolysis, and acid hydrolysis) to evaluate the free (soluble) and bound (insoluble) phenolic compounds [85]. For soluble compositions, only two polymethoxyflavones, nobiletin and tangeretin, were found in hexane extract. In hot water extract, a large amount of DGPP was found as the major flavonoid (3621 mg/100 g dry extract), which was similar to the results of a previous study. It is suggested that most of the flavonoids in immature calamondin peel are soluble, while phenolic acids mainly exist in the bound form [85].

3.3. Effects of environmental factors and heat treatments

The ICW contained the largest quantity of flavonoids and showed the highest antioxidant activity among all extracts studied. In hot water extracts, the contents of DGPP, neohesperidin, and hesperidin were much higher in immature calamondin peel than in mature calamondin peel. However, contents of the polymethoxyflavones nobiletin and tangeretin in calamondin peel, extracted by ethyl acetate, increased after ripening [70].

In our previous study, flavonoids and phenolic acid, in hot water extract of immature calamondin after heating at 150°C for 1.5 hours, increased except for DGPP and hesperidin [85]. According to the profiles of free and bound phenolic compounds, it could be found that the bound form of phenolic acids, such as ferulic acid, *p*-coumaric acid, sinapic acid, and ellagic acid, in calamondin peel increased after heating at 150°C for 1.5 hours. Most of them are in the ester linkage form compared with the glycoside linkage form. It is suggested that high-temperature heating (150°C) probably produces two major effects: (1) degradation of flavonoids, such as DGPP and hesperidin, and (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 [85].

The effect of heat treatment (at 110°C and 130°C for 0.5 hours, 1.0 hour, 1.5 hours, and 2.0 hours, and at 150°C for 1.5 hours) on the antioxidant activity and phenolic contents of immature calamondin has been investigated [87]. The major flavonoid DGPP decreased drastically after being heated at ≥130°C for over 1.5 hours. Therefore, the increase of antioxidant activity by heating at 110°C and 130°C for ≤1.0 hour might be due to the increase of phenolic contents, particularly the phenolic acids, i.e., ferulic acid and *p*-coumaric acid.

3.4. Bioactivities

3.4.1. Antioxidant activity

The antioxidant activity of flavedo extract of calamondin was evaluated using three independent methods: the Trolox equivalent antioxidant capacity, ferric reducing antioxidant capacity, and hypochlorous acid assays. The antioxidant activity of calamondin showed lower capability compared with

21 varieties of Citrus [63]. The antioxidant activity of calamondin pulp extract was also investigated [20], and calamondin was grouped as one of the most potent antioxidants in nine Citrus species. Calamondin was reported to have lower antioxidant activity than mango and papaya fruits, while more susceptibility to cold during refrigerated storage was also observed [88].

Yu et al [70] have studied the antioxidant activity of mature and immature calamondin peel extracted by hot water and ethyl acetate. The ICW exhibited the highest ORAC, reducing power, and superoxide scavenging effect. Two compounds, naringin and hesperidin, were determined as the major active components responsible for the antioxidant activity. However, hesperidin and neohesperidin, instead of naringin and hesperidin, were reidentified by the same laboratory [52,72]. DGPP, which revealed a low ORAC value with 7.43 mmol Trolox Equivalent/g fraction, might also contribute to the antioxidant activity of immature calamondin peel due to its greatest quantity.

The DPPH scavenging potency of calamondin peel extracts was significantly higher than that of calamondin pulp extracts ($p < 0.05$) [86]. The highest DPPH scavenging potency was displayed by hot (80°C) water extract of calamondin peel. The correlation coefficient (r) between the total phenolic contents and DPPH scavenging potency of calamondin peel and pulp extracts was 0.6894, which was considered significant ($p < 0.01$). Total flavonoid compositions also showed a significant correlation ($p < 0.05$) to DPPH scavenging potency, with $r = 0.4910$. Collectively, hot water extract of calamondin peel might have great potential for health beneficial food and treatment of hyperpigmentation in cosmetic application.

ORAC assay of each extraction of immature calamondin peel after heating treatment by successive processes with different solvents has been reported [85]. The ORAC value of heated immature calamondin peel was significantly higher than fresh peel in all extractions. After heating, total ORAC values increased from 2815 μmol Trolox/g extract to 3102 μmol Trolox/g extract. In insoluble compositions, contents of the major phenolic acids, including ferulic acid and *p*-coumaric acid, increased after heating treatment, which also probably provided the antioxidant activity.

As mentioned previously, the antioxidant activity of immature calamondin after heat treatment has been studied by the same laboratory [87]. The results indicate that heat treatment can enhance the DPPH scavenging potency and ORAC, while increasing the total phenolic contents significantly. However, the major flavonoid DGPP decreased drastically after being heated at $\geq 130^\circ\text{C}$ for over 1.5 hours. Therefore, the increase of antioxidant activity could not simply be explained by the increase of the total phenolic contents. It is concluded that the products of browning reaction resulted in the increase of the antioxidant activity of immature calamondin heated at $\geq 130^\circ\text{C}$ for over 1.5 hours, while the increase of antioxidant activity by heating at 110°C and 130°C for ≤ 1.0 hour was due to increased phenolic content.

3.4.2. Antimicrobial activity

The ICW showed better antibacterial effect against Gram positive than against Gram negative, while the best effect on *L. monocytogenes* was observed with an MIC of 10 mg/mL [52]. The

MIC of hot water extract of *B. cereus*, *Enterococcus faecalis*, and *Streptococcus sanguis* was 20 mg/mL. Furthermore, the authors separated the extracts into five fractions by a preparative C18 column. The highest antibacterial effect has been found in fraction V, which contained mainly nobiletin (3.4 g/100 g dry fraction) and tangeretin (1 g/100 g dry fraction). Therefore, they suggested that nobiletin might be the major antibacterial compound in immature calamondin peel. Nobiletin has also been purified from the leaves of calamondin and its antibacterial activity has been evaluated [89]. It showed moderate activity against the fungus *Candida albicans*, and low activity against the bacteria *P. aeruginosa* and *Bacillus subtilis* and the fungi *Trichophyton mentagrophytes* and *Aspergillus niger*.

3.4.3. Tyrosinase inhibitory activity

Owing to the color interference of the extract, the tyrosinase inhibitory effect of the ICW was unable to be determined. Therefore, the ICW was first separated and collected as fractions I–IX on a semipreparative HPLC C₁₈ column. Each fraction was then subjected to enzymatic assay of tyrosinase inhibitory activity [47]. Only two fractions, fractions V (ICW-V) and VI (ICW-VI), showed tyrosinase inhibitory activity with 89.90% and 19.11% of inhibition, respectively. The IC₅₀ of ICW-V was 0.87 mg/mL, which was about twofold that of the IC₅₀ of arbutin (positive control). Fraction ICW-VI showed less potency and its IC₅₀ value was 3.3 mg/mL. DGPP was identified in fraction ICW-V, which might contribute to strong inhibitory activity against tyrosinase. In fraction ICW-VI, hesperidin and neohesperidin were determined, which also expressed tyrosinase inhibitory property.

3.4.4. Antihepatitis B virus activity

Antihepatitis B virus activity of the extracts (dichloromethane, ethyl acetate, *n*-butanol, acetone, and methanol) of calamondin fruits (peel and pulp) have been studied [68]. Ethyl acetate and acetone extracts of the peel reduced the hepatitis B surface antigen expression of hepatitis B virus by 41.6% and 71.4%, respectively, at a dose of 50 $\mu\text{g/mL}$. Ethyl acetate extract exhibited a high inhibitory effect. In this extract, three polymethoxyflavones, namely, nobiletin, tangeretin, and 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone, were found to significantly inhibit the hepatitis B virus surface antigen. The IC₅₀ value of 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone was 5.12 μM and it had the lowest inhibiting concentration, followed by tangeretin (20.7 μM) and nobiletin (33.9 μM).

So far, very little research has been conducted on the bioactivity of calamondin. Some flavonoids in calamondin have been identified, which DGPP, hesperidin and neohesperidin are the major phenolic compounds. Thus, it is reasonable for certain bioactivity of calamondin. In particular, in Asian countries, hot water extract of calamondin is a popular hot drink as folk medicine due to its health benefits. Therefore, more research on bioactivity of calamondin is needed to elucidate the potential application of calamondin fruits.

Conflicts of interest

There are no conflicts of interest.

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