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Tyrosinase inhibitory components of immature calamondin peel

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ABSTRACT

There is broad range of applications in the use of tyrosinase inhibitors for suppressing unwanted hyperpigmentation in human skin and enzymic browning in fruits. In searching effective tyrosinase inhibitors from natural products, the components in unripe calamondin (*Citrus mitis* Blanco) peel were investigated by performing bioassay-directed fractionation and chromatographic separation coupled with tyrosinase inhibition assay. Herein it is reported for the first time that (1) there is a rich content of 3′,5′-di-C-β-glucopyranosylphloretin in unripe calamondin peel, 3.69 ± 0.44 g/100 g dry basis, (2) this C-glycosylated flavonoid showed the strongest inhibitory activity against tyrosinase among the components in this fruit, with an IC_{50} of 0.87 mg/ml, and (3) that unripe calamondin peel is also a rich source of naringin and hesperidin, 1.25% and 0.73% by dry weight, respectively, which also expressed strong tyrosinase inhibitory property.

1. Introduction

Tyrosinase is a polyphenol oxidase (PPO) with a dinuclear copper active site. It has dual functionalities as both a monophenolase and an *o*-diphenolase (Seo, Sharma, & Sharma, 2003; Kim & Uyama, 2005) and catalyses the hydroxylation of monophenols to *o*-diphenols and the consequent oxidation of *o*-diphenols to *o*-quinones. It is responsible for the formation of mammalian melanin pigments as well as enzymatic browning of fruits and vegetables. Thus, tyrosinase has received great attention, since it is known as a key enzyme in melanin biosynthesis, which plays a crucial protective role against skin photocarcinogenesis (Kubo, Nitoda, & Nihei, 2007; Xue et al., 2011). However, overproduction and accumulation of melanin in the skin can result in pigmentation disorders.

It has been known that citrus contains large amounts of flavonoids, such as hesperidin, naringin and nobiletin, which have been studied for their tyrosinase inhibition activity. For instance, it is reported that the IC_{50} value of naringin and hesperidin were 1.9 mM (Itoh et al., 2009) and 16.08 mM (Zhang et al., 2007), respectively. The tyrosinase inhibition potency of nobiletin has also been reported (Sasaki & Yoshizaki, 2002). Calamondin (*Citrus mitis* Blanco), a cultivar related to citrus, is a hybrid between *Citrus reticulate* Blanco and *Fortunella* spp. (Moshonas & Shaw, 1996). However, the flavonoids composition of *Fortunella* species differs from those of the *Citrus* species (Sadek, Makris, & Kefalas, 2009).

Calamondin bears small size citrus fruits. The hot water extract of immature fruit with green peel has been a popular beverage in Taiwan for many years due in part to its potential health beneficial properties. The levels of total polyphenols and flavonoids of immature calamondin peel were found to be higher than those of mature calamondin peel (Hsu, 2009). Antioxidant activity and the DPPH free radical scavenging effect of immature calamondin peel (56.9–64.3%) was also found to be higher than that of mature calamondin peel (35.0–48.9%). Calamondin contains a large quantity of 3′,5′-di-C-β-glucopyranosylphloretin in its peel, juice sac and leaf (Ogawa, Kawasaki, Omura, & Yoshida, 2001). However, there is no reported quantitative data on unripe calamondin peel and also no systematic research being done on the effect of tyrosinase inhibition on calamondin components.

The present study evaluated the inhibitory effect of the water extract of immature calamondin peel on the monophenolase activated form of tyrosinase *in vitro*. The active fractions were collected by a semi-preparative HPLC equipped with a reversed phase column and then confirmed by a tyrosinase enzymatic assay. The isolated active compounds were identified and their mode of inhibition studied.

2. Materials and methods

2.1. Materials

Immature calamondin (*Citrus mitis* Blanco) with whole green appearance was collected during November–December 2009. The average weight of a whole calamondin was 14.53 ± 0.53 g. The immature calamondin was manually peeled and the peels were

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collected and lyophilised for 48 h. Prior to extraction, the peels were pulverised with a blender and passed through a 40 mesh sieve. The obtained peel powders were stored in a suitable brown bottle with screw cap at −18 °C.

2.2. Chemicals

Kojic acid, Arbutin, tyrosine, L-DOPA (3′,4′-dihydroxyphenylalanine), mushroom tyrosinase and flavonoid and phenolic acid standards including neoeriocitrin, rutin, naringin, hesperidin, neohesperidin, naringenin, hesperetin, limettin, nobiletin, tangeretin, and protocatechuic acid, gentisic acid, β-resorcylic acid, caffeic acid, syringic acid, kuromanin acid, *p*-coumaric acid, isoferulic acid, ferulic acid, *o*-coumaric acid, and cinnamic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The other chemicals including acetonitrile, acetic acid, sodium dihydrogen phosphate monohydrate, di-sodium hydrogen phosphate dihydrate, and methanol-D4 were of analytical grade from Merck Chemical Co. (Darmstadt, Germany).

2.3. Extraction procedure

Three grams of dried and powdered immature calamondin peel were mixed with 60 ml of deionised water at 90 °C. The resulted suspension was shaking for 1 h in a water bath at 90 °C with a shaking rate of 100 rpm. Then, the extract was filtered and the filtrate collected. The solid residue was extracted two more times with the same extraction procedure. The three collected filtrates were combined and concentrated on a rotary vacuum evaporator at 50 °C. To thus formed residue, deionised water was added to the flask. The resulted solution was defined as immature calamondin peel water extract (ICW) and poured into a brown bottle with screw cap and stored at −18 °C until further use.

2.4. Isolation of tyrosinase inhibitory compounds

The tyrosinase inhibitory compounds were isolated from immature calamondin peel water extract (ICW) on a semi-preparative column. The chromatographic system consisted of a Shimadzu binary pump and Shimadzu SPD-M10 photodiode array detector. A semi-preparative C18 column (Hibar Pre-Packed Column, Lichrospher 100 RP-18 endcapped 5 μm, 250 × 10 mm, Merck, Darmstadt, Germany) was used for separation. Mobile phase A consisted of 2% acetic acid and 98% water, and mobile phase B consisted of 1:1 (v:v) mixture of 0.5% aqueous acetic acid and acetonitrile. The flow rate was 1 ml/min and photodiode array detection (PDA) was performed between 220 and 350 nm. The gradient separation was programmed as the followings: mobile phase B was started with 5% and ramped up to 10% in 10 min, to 55% in next 45 min, to 80% in next 5 min and to 100% in another 5 min. The 100% mobile phase B was kept for 75 min till the separation programme ended. Nine fractions, i.e. ICW-I to ICW-IX, were collected and concentrated on a vacuum evaporation to complete dryness. The extracts were then re-dissolved with deionised water to a proper concentration for the enzymatic assay of tyrosinase and HPLC analyses.

2.5. Enzymatic assay of tyrosinase

Enzymatic assay was performed according to the procedure of Masuda et al. (Masuda, Yamashita, Takeda, & Yonemori, 2005). Mushroom tyrosinase was used for this assay. A mixture of 60 μl of 1/15 M phosphate buffer solution (pH 6.8) and 100 μl of 2.5 mM tyrosine was designated as solution A. Solution B was 160 μl of 1/15 M phosphate buffer solution (pH 6.8). The solution C consisted of 20 μl of 1/15 M phosphate buffer solution (pH 6.8), 40 μl sample, and 100 μl of 2.5 mM tyrosine. 120 μl of 1/15 M phosphate buffer solution (pH 6.8) mixed with 40 μl sample was designated as solution D. Solutions A, B, C and D were added to 40 μl tyrosinase (50 U/ml) for 40 min reaction time at 23 °C, individually. Absorbance of the resulting solutions was then determined by ELISA reader at 475 nm. The tyrosinase inhibition was calculated as the following formula: percentage (%) = $\{[(A_A - A_B) - (A_C - A_D)]/(A_A - A_B)\} \times 100$, while A_A: absorbance at 475 nm with tyrosine; A_B: absorbance at 475 nm without tyrosine; A_C: absorbance at 475 nm with test substance and tyrosine; A_D: absorbance at 475 nm with test substance. The following mixtures for this assay were carried out under the same conditions, including mixture 1: tyrosine, tyrosinase, and the fraction-V of immature calamondin peel extract (ICW-V); mixture 2: tyrosine and ICW-V; mixture 3: tyrosinase and the ICW-V; mixture 4: tyrosine and tyrosinase.

2.6. HPLC analysis

The fraction of ICW-V and ICW-VI were analysed on a RP-18 column (250 mm × 4.6 mm, 5 μm, Merck, Darmstadt, Germany) using gradient mode with 2% acetic acid in water as solvent A and 0.5% acetic acid in water/acetonitrile (1:1, v/v) as solvent B (Schieber, Keller, & Carle, 2001). The gradient was carried out as 0–10 min, 5–10% B; 10–55 min, 10–55% B; 55–60 min, 55–80% B; 60–65 min, 80–100% B; 65–70 min, 100% B; 70–75 min, 100–5% B. The flow rate was 1 ml/min. The PDA detector was performed at 280 and 340 nm. Ten flavonoids and eleven phenolic acids were used as standards including neoeriocitrin, rutin, naringin, hesperidin, neohesperidin, naringenin, hesperetin, limettin, nobiletin, tangeretin, and protocatechuic acid, gentisic acid, β-resorcylic acid, caffeic acid, syringic acid, kuromanin acid, *p*-coumaric acid, isoferulic acid, ferulic acid, *o*-coumaric acid, cinnamic acid.

2.7. Determination of the inhibition type

The work was performed according to Ley and Bertram's procedure (2001). Taking various concentrations of tyrosine (0.5, 1.0, 1.5, and 2.0 mM) as substrate, the tyrosinase inhibitory activity was measured according to the method described above. Inhibitory kinetics of the sample of tyrosinase was analysed by Lineweaver–Burk plots.

2.8. Identification of effective compounds

Pure compounds were isolated and characterised based on their UV spectrum, MS and MS–MS data, and NMR spectra. A LCQ-FLEET ion trap mass spectrometer (Thermo Fisher Scientific, USA) equipped with electrospray ionisation source (ESI) was used. The operating parameters of the mass spectrometer were capillary temperature 350 °C; and the spray needle voltage set at 5.0 kV for negative polarity. The ^{1}H NMR and ^{13}C NMR spectra were obtained on a Bruker Avance 500 MHz FT-NMR spectrometer. Compounds in fraction ICW-VI were identified by retention time of HPLC and UV spectrum using a PDA detector in comparison with standard compounds.

2.9. Statistical analysis

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

Table 1
Inhibitory effect on mushroom tyrosinase of different fractions from ICW obtained by a semi-preparative reversed-phase HPLC separation.

Fraction	Concentration (mg/ml)	Tyrosinase inhibition (%)	Inhibition potency[h] (%/mg/ml)	IC_{50} (mg/ml)
I	0.08	−92.59 ± 6.85[g]	–[i]	–
II	1.00	−92.29 ± 1.13[g]	–	–
III	0.50	−3.65 ± 3.45[c]	–	–
IV	1.42	−43.30 ± 4.97[de]	–	–
V	1.75	89.90 ± 4.22[a]	51.37 ± 2.41[a]	0.87 ± 0.01[b]
VI	0.50	19.11 ± 4.07[b]	38.22 ± 8.15[b]	3.30 ± 0.09[a]
VII	0.33	−50.00 ± 7.25[e]	–	–
VIII	0.50	−74.15 ± 4.14[f]	–	–
IX	0.25	−35.19 ± 0.00[d]	–	–
Arbutin	–	–	–	0.46
Kojic acid	–	–	–	0.01

[a–g] Values (Mean ± S.D., $n = 3$) in the same column with different superscripts are significantly different ($p < 0.05$).
[h] Inhibition potency = Inhibition (%)/concentration in the reaction.
[i] No inhibitory effect.

3. Results and discussion

3.1. Tyrosinase inhibitory activity of various fractions separated from Calamondin peel

Due to the colour interference of the extract, the tyrosinase inhibitory effect of hot water extract from immature calamondin peel was unable to be determined. Therefore, the hot water extract of immature calamondin peel (ICW) was first separated and collected as fractions I–IX on a semi-preparative HPLC C18 column. Each fraction was then subjected to enzymatic assay of tyrosinase inhibitory activity. As illustrated in Table 1, only two fractions, fractions V (ICW-V) and VI (ICW-VI), had shown tyrosinase inhibitory activity with 89.90% and 19.11% of inhibition, respectively. The inhibition potency, defined as inhibition percentage divided by the concentration, was 51.4%/mg/ml for ICW-V and 38.2%/mg/ml for ICW-VI. The IC_{50} of ICW-V was 0.87 mg/ml, which was about two folds than the IC_{50} of arbutin (positive control). Fraction ICW-VI had shown less potency and its IC_{50} value was 3.3 mg/ml.

3.2. Analysis and identification of phenolic compounds in extract of Calamondin peel

The composition of phenolic compounds in fraction ICW-V was analysed by HPLC with an UV photodiode array detector. There was a major peak with a retention time of 43.5 min (ICW-V-a) in the HPLC chromatogram. A relatively minor peak was eluted at 44.9 min.

Compound ICW-V-a was further purified on semi-preparative HPLC and pure compound obtained as a yellow powders. The UV spectrum showed the maxima absorption (UV max) at 284, 237, and 223 nm. The structural information of ICW-V-a was obtained using ESI-MS and NMR. Compound ICW-V-a had a molecular ion at m/z 597 $[M-H]^-$. It also showed an ion at m/z 477 $[M-120-H]^-$ that indicates a characteristic fragment ion of C-glycosyl flavonoids (Liang, Jin, Feng, & Ke, 2011). It has ^1H NMR and ^{13}C NMR spectral data as the followings: ^1H NMR (CD_3OD, 500 MHz): phloretin (aglycone) moiety δ7.03 (2H, d, $J = 8.4$ Hz, H2 and H6), 6.67 (2H, d, $J = 8.4$ Hz, H3 and H5), 3.34 (2H, m, Hα), 2.85 (2H, m, Hβ); glucose moiety δ4.93 (2H, d, $J = 9.8$ Hz, H1″, H1‴), 3.84 (2H, dd, $J = 12.2$ Hz and 2.1 Hz, H6a″, H6a‴), 3.80 (2H, dd, $J = 12.2$ Hz and 4.2 Hz, H6b″, H6b‴), 3.61 (2H, dd, $J = 9$ Hz and 9.5 Hz, H2″, H2‴), 3.54 (2H, dd, $J = 9$ Hz and 9 Hz, H3″, H3‴), 3.50 (2H, dd, $J = 8.8$ Hz and 8.8 Hz, H4″, H4‴), 3.42 (2H, m, H5″, H5‴). ^{13}C NMR (CD_3OD, 125 MHz): phloretin (aglycone) moiety δ207.1 (carbonyl), 163.3 (C4′), 162.5 (C2′, C6′), 156.5 (C4), 133.9 (C1), 130.4 (C2, C6), 116.1 (C3, C5), 106.1 (C1′), 104.4 (C3′, C5′), 47.8 (Cα), 31.1 (Cβ); glucose moiety δ 82.7 (C5″, C5‴), 79.1 (C3″, C3‴), 76.8 (C1″, C1‴), 74.1 (C2″, C2‴), 71.1 (C4″, C4‴), 61.8 (C6″, C6‴). All spectral data were consistent with the data of 3′,5′-di-C-β-glucopyranosylphloretin (Fig. 1) (Sato, Akiya, Nishizawa, & Suzuki, 2006; Ogawa et al., 2001).

The compound, 3′,5′-di-C-β-glucopyranosylphloretin, a dihydrochalcone derivative, has been reported in the kumquat (*Fortunella margarita* and *japonica*) (Jayaprakasha, Murthy, Etlinger, Mantur, & Patil, 2012; Sadek et al., 2009; Cho et al., 2005) and in Kumquat juice (Barreca, Bellocco, Caristi, Leuzzi, & Gattuso, 2010). A large quantity of 3′,5′-di-C-β-glucopyranosylphloretin has also been found in genus *Fortunella* as well as calamondin (Ogawa et al., 2001), although calamondin is believed to be a hybrid between *Citrus reticulata* Blanco and *Fortunella* spp. (Moshonas & Shaw, 1996). In this study, we isolated an average of 3.69 ± 0.44 g 3′,5′-di-C-β-glucopyranosylphloretin/100 g dry basis from immature calamondin peel.

In the fraction of ICW-VI, two major peaks were identified as naringin and hesperidin by retention times on HPLC and UV spectra as compared with standards. Immature calamondin peel has been reported to contain naringin, hesperidin, diosmin, and nobiletin in hot water extract (Hsu, 2009). The tyrosinase inhibitory activities of naringin and hesperidin have previously been reported (Itoh et al., 2009; Zhang et al., 2007). It is therefore suggested that naringin and hesperidin probably contributed to the major tyrosinase inhibitory activity of the ICW-VI fraction. The levels of naringin and hesperidin were 1.25 ± 0.04 g/100 g dry basis and 0.73 ± 0.03 g/ 100 g dry basis, respectively, in immature calamondin peel.

3.3. Changes of related components during tyrosinase enzymatic assay

To confirm the effective component of ICW-V, we analysed the change of compounds in solution of tyrosinase enzymatic assay by HPLC. The change of component profile in the mixture of ICW-V, tyrosine, and tyrosinase during 60 min reaction time was investigated (Fig. 1). The results indicated that the content of tyrosine remained steady during the reaction, while the compound 3′,5′-di-C-β-glucopyranosylphloretin decreased, and L-DOPA (peak 2) and three unknown peaks (peaks 3, 4, 5) increased. The percentage of 3′,5′-di-C-β-glucopyranosylphloretin decreased 2.71–97.29% after 20 min reaction, 9.07% to 90.93% after 40 min reaction, and 20.03–79.97% after 60 min reaction (Table 2). Meanwhile, three new unknown peaks, 3, 4, and 5, were detected after 20 min reaction and increased with prolonged reaction time until 60 min reaction.

In a mixture containing only tyrosine and ICW-V, there was no change of tyrosine and 3′,5′-di-C-β-glucopyranosylphloretin being observed (data not shown). This suggests that there was no reaction between 3′,5′-di-C-β-glucopyranosylphloretin and tyrosine. However, in another mixture, containing only tyrosinase and 3′,5′-di-C-β-glucopyranosylphloretin, the percentage of 3′,5′-di-C-β -glucopyranosylphloretin decreased to 76.9% during 60 min reaction time (Fig. 2), while three unknown peaks 3, 4, and 5 also appeared and increased during the reaction time. No level of L-DOPA was observed in this reaction. The decreasing rate of 3′,5′-di-C-β-glucopyranosylphloretin in this mixture was faster than that in the mixture of tyrosine, tyrosinase, and fraction ICW-V. It showed that a direct reaction between tyrosinase and 3′,5′-di-C-β-glucopyranosylphloretin occurred and during the reaction, three products were formed indicated by the three unknown peaks 3, 4, and 5 in the HPLC traces. This suggests that, indeed, tyrosine can be protected by 3′,5′-di-C-β-glucopyranosylphloretin against the catalytic reaction of tyrosinase. At the same time, the 3′,5′-di-C-β-

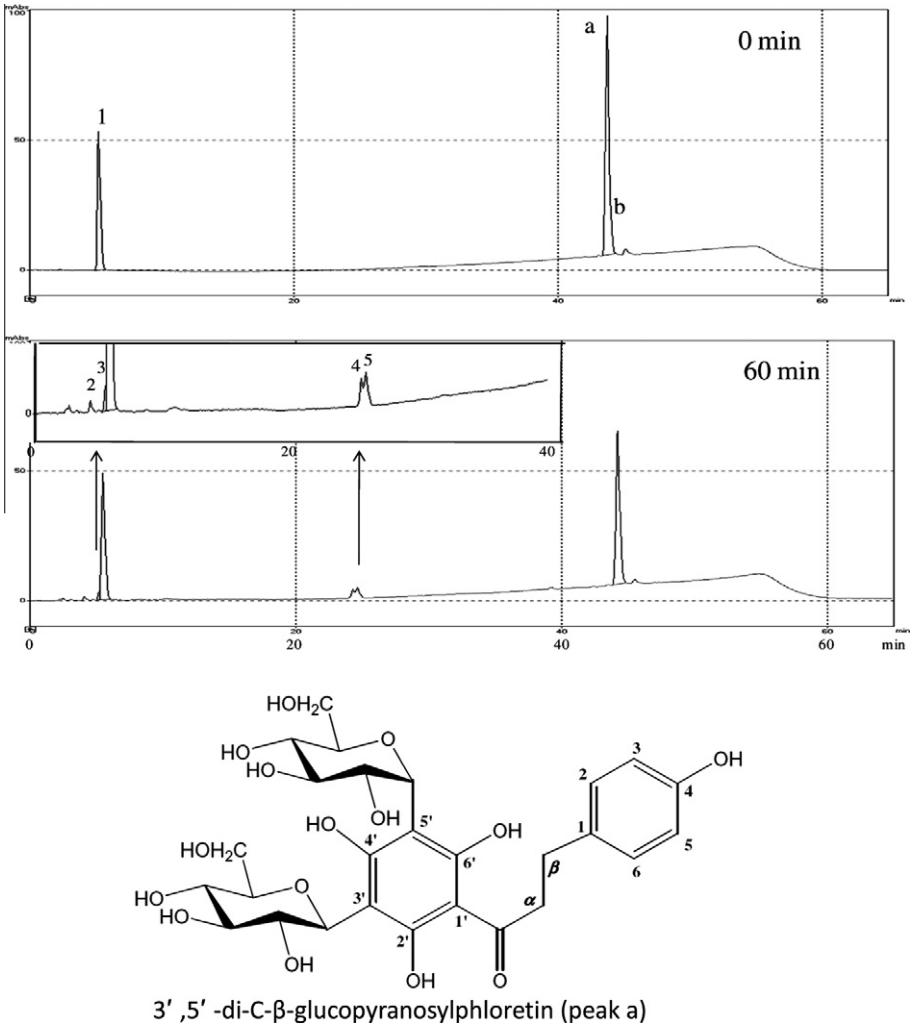

Fig. 1. HPLC analysis of the reaction solution after addition of ICW-V, tyrosine and tyrosinase for different duration. (a: 3′,5′-di-C-β-glucopyranosylphloretin, b: unknown, 1: tyrosine, 2: DOPA, 3, 4, and 5: unknown).

Table 2
Change of components in the reaction solution of ICW-V, tyrosine and tyrosinase for different duration.

Reaction time (min)	Tyrosine (μM)	L-DOPA (μM)	Decrease of 3′,5′-di-C-β-glucopyranosylphloretin (%)	Peak 3 (area)	Peak 4	Peak 5
0	621 ± 14[a]	–[h]	–	–	–	–
10	630 ± 26[a]	–	–	–	–	–
20	636 ± 6[a]	–	2.71 ± 0.48[c]	–	9511 ± 2677	12,073 ± 326
40	654 ± 21[a]	1.95 ± 0.94[a]	9.07 ± 2.56[b]	17,190 ± 4777	26,886 ± 4995	36,954 ± 2104
60	638 ± 42[a]	3.67 ± 1.87[a]	20.03 ± 3.16[a]	30,945 ± 8899	53,638 ± 4480	76,411 ± 4515

[a–g] Values (Mean ± S.D., $n = 3$) in the same column with different superscripts are significantly different ($p < 0.05$).
[h] Not detected.

glucopyranosylphloretin was catalysed by tyrosinase during a 20 min reaction and to produce new molecules (unknown peaks 3, 4, and 5), simultaneously.

Compound 3,4-dihydroxyphenylalanine (L-DOPA) was found in the mixture of fraction ICW-V, tyrosine, and tyrosinase after 40 min reaction with a concentration level of 1.95 μM and after 60 min reaction reached to 3.67 μM (Table 2). The magnitude of produced L-DOPA only reached to 0.30% and 0.58% of tyrosine at 40 and 60 min reaction. However, in the mixture of only tyrosinase and ICW-V, no L-DOPA was detected during the reaction. In another mixture as control system, only tyrosine and tyrosinase, a concentration of 5.33 μM of L-DOPA at 20 min during the reaction was found (Fig. 3). After that, the content of L-DOPA increased gradually to 18.48 μM at 40 min and 23.61 μM at 60 min, while the level of tyrosine decreased. This indicates that tyrosine was catalytically oxidised by tyrosinase after 20 min in the mixture and produced L-DOPA, since L-DOPA is the hydroxylation product of tyrosine by monophenolase action (Seo et al., 2003; Kim & Uyama, 2005). The compound 3′,5′-di-C-β-glucopyranosylphloretin might inhibit the activity of monophenolase. The inhibition effect was 84.5–89.4% after 40–60 min reaction.

3.4. Inhibition type of 3′,5′-di-C-β-glucopyranosylphloretin

Due to the decreasing rate of 3′,5′-di-C-β-glucopyranosylphloretin in the mixture of tyrosinase and ICW-V was higher than that in

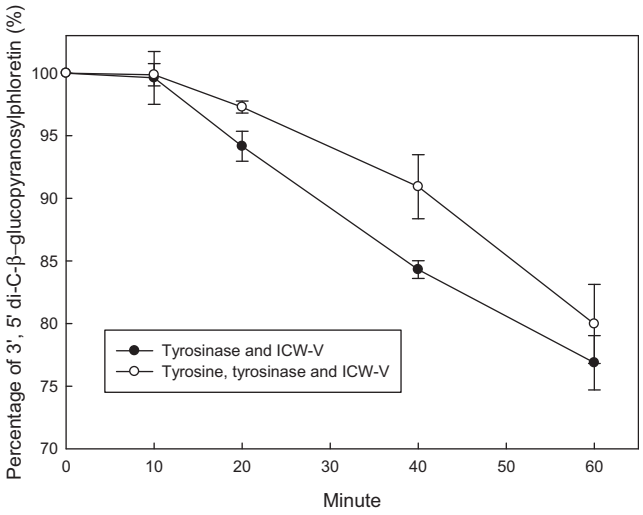

Fig. 2. Changes in percentage of 3′,5′-di-C-β-glucopyranosylphloretin during various tyrosinase enzymatic assay.

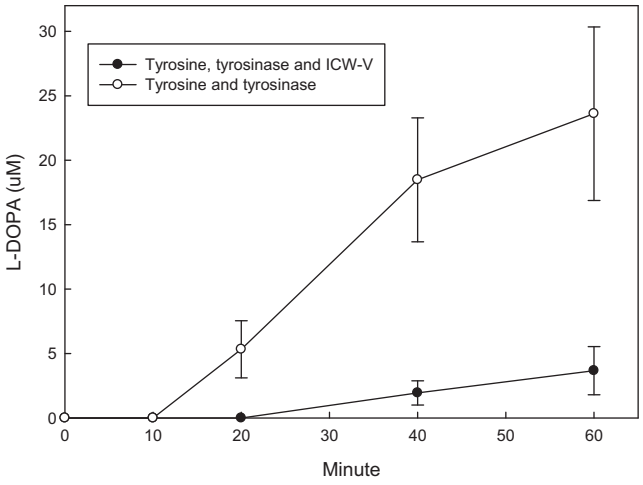

Fig. 3. Changes in L-DOPA content during various tyrosinase enzymatic assay.

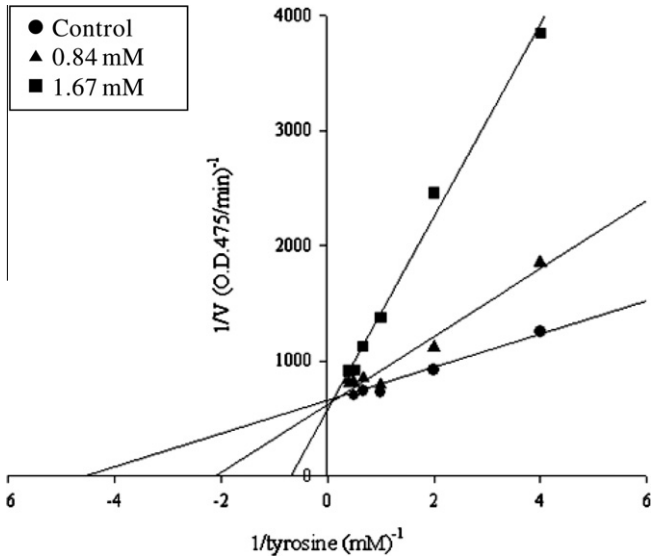

Fig. 4. Kinetic assay of tyrosinase inhibitory activity by 3′,5′-di-C-β-glucopyranosylphloretin. A Lineweaver–Burk plot was constructed for the inhibition of tyrosine oxidase by 3′,5′-di-C-β-glucopyranosylphloretin.

the mixture of tyrosine, tyrosinase and ICW-V, the inhibition type of 3′,5′-di-C-β-glucopyranosylphloretin most probably belongs to the competitive mode. The isolated 3′,5′-di-C-β-glucopyranosylphloretin was a yellow powder and the kinetic assay of tyrosinase inhibitory activity by the 3′,5′-di-C-β-glucopyranosylphloretin is shown in Fig. 4. The Lineweaver–Burks plots led us to support the view that tyrosinase inhibition of 3′,5′-di-C-β-glucopyranosylphloretin was indeed in a competitive mode. 3′,5′-Di-C-β-glucopyranosylphloretin has been reported as an antioxidant, which performs its antioxidant activity most probably through the action of the A ring (Barreca et al., 2010). The antioxidant pharmacophore in the dihydrochalcone phloretin is the 2,6-dihydroxyacetophenone core (Rezk et al., 2002). In addition, 3-hydroxyphloretin was demonstrated as the most active constituent from the Formosan apple, which exhibited a competitive inhibition type in human epidermal melanocytes (Lin, Hsu, Chen, Chern, & Lee, 2007). The tyrosinase inhibition effect of 3′,5′-di-C-β-glucopyranosylphloretin might also be due to the action of 2,6-dihydroxyacetophenone core. However, the 4-hydroxy group in B ring could also contribute to the inhibitory effect of tyrosinase, since the structure is similar to tyrosine, and probably performed as competitive inhibition.

4. Conclusions

In summary, based on our study, it was concluded that the hot water extract of immature calamondin peel contained a large quantity of 3′,5′-di-C-β-glucopyranosylphloretin, which demonstrated good tyrosinase inhibitory activity in the competitive mode. A certain amount of naringin and hesperidin was also observed in the extract that could also contribute to the tyrosinase inhibitory activity. Thus, the hot water extract of immature calamondin peel has a good potential for use in the cosmetic industry.

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