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Tyrosinase inhibitory effects and inhibition mechanisms of nobiletin and hesperidin from citrus peel crude extracts

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Abstract

The inhibitory effects of nobiletin and hesperidin from citrus peel crude extracts on tyrosinase diphenolase activity are evaluated. IC_{50} of nobiletin and hesperidin is 1.49 mM and 16.08 mM, respectively and their inhibition mechanism is competitive type with $K_i = 2.82$ mM and noncompetitive with $K_i = 9.16$ mM, respectively. Crude extracts from citrus peel (*C. unshiu* Marc.) were extracted with 95% ethanol and fractionated by petroleum ether (PCPE). The ethanol phase (ECPE) was further desorbed from macroporous adsorption resin (FGRE). Their IC_{50} values were 8.09 mg/mL, 7.53 mg/mL and 4.80 mg/mL, respectively. Their inhibition on melanogenesis in B16 mouse melanoma cells was also evaluated. FGRE showed a significant inhibition (42.5% at 31.25 μg/mL, $p < 0.01$) while hesperidin showed almost no inhibition. Nobiletin and PCPE give efficacious antiproliferation effects on B16 mouse melanoma cell with IC_{50} values 88.6 μM and 62.96 μg/mL, respectively, by the MTT test. Hesperidin and other crude extracts showed very low cytotoxity to the B16 cell.

Keywords: *Citrus unshiu, Marc, nobiletin, hesperidin, B16 mouse melanoma, crude extract, tyrosinase inhibitor, melanin, traditional Chinese medicine, SAR*

Introduction

Owing to severe exposure to the ultraviolet radiation of sunlight, rapidly growing numbers of melanoma cases in the west, *esp.* Caucasians, like USA and Australia [1,2], and the continuous needs for skin whitening agents from the east, is increasing research interests focus on suppressing accumulation of the pigment melanin. Melanin biogenesis, stimulated by ultraviolet radiation, occurs in insects, animals, and plants by an oxidation process starting with L-tyrosine. The major step is the oxidation of tyrosine to 3,4-dihydroxyphenyl-L-alanine (L-DOPA) (monophenolase or cresolase activity) followed by the oxidation of L-DOPA to dopaquinone (diphenolase or catecholase activity) [3,4]. During melanogenesis, the key enzyme is tyrosinase (EC 1.14.18.1), which contains a binuclear copper cluster in the common mushroom (*Agaricus bisporus*) and in human malignant melanoma [5,6]. Therefore, most whitening agents act specifically to reduce the function of this enzyme by means of several mechanisms: (i) interference with its transcription and/or glycosylation, (ii) inhibition by different modalities, (iii) reduction of by-products and (iv) post-transcriptional control [7].

Plant sources are rich in bioactive chemicals, mostly without harmful side effects, and are attracting increasing efforts in screening for tyrosinase inhibitors. Kojic acid and arbutin, for examples, are currently used as natural tyrosinase inhibitors in the cosmetic industry. These natural sources can be categorized into two subgroups [4]: aldehydes like cinnamic acid, anisic acid and cuminaldehyde [8], etc. and polyphenols like kaempferol [9], quercetin [10],

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etc. The polyphenol group is widely distributed in natural plants from the bark, root to the leaves and peels, which provides a wide choice for the source of tyrosinase inhibitors such as green tea [11], black rice bran [12] and grape seed [13].

Citrus peel as a by-product of the citrus juice industry contains a large amount of pectin and flavonoids. Citrus flavonoids like hesperidin and naringin, the flavanone glycosides, have been well studied for their toxicities and activities like improving vascular integrity, decreasing capillary permeability [14] and anti-oral carcinogenesis [15]. In China, citrus peel is even used as a common food and traditional Chinese medicinal ingredients [16–18]. However, there are few reports on its inhibitory effect on tyrosinase. Citrus polymethoxylated flavones, another group like nobiletin and tangeretin, have attracted recent interest for their potent activities against tumors and other targets [19–27] due to their unique polymethoxyl radicals (Figure 1). Though one report has previously demonstrated the inhibitory potency of nobiletin towards mushroom tyrosinase [28], further work needs to be carried out on their type of inhibition mechanism and intracellular performance in cell models due to the wide availability and commercial advantage of these citrus flavonoids. The present study examined the inhibitory effect of hesperidin, nobiletin and industrial crude extracts of citrus peel on mushroom tyrosinase, and furthermore, elucidated their inhibition mechanism. Their inhibition of melanogenesis in B16 mouse melanoma cells was also evaluated as intracellular evidence.

Materials and methods

Chemicals

Tyrosinase from mushroom (EC 1.14.18.1, T3824, tyrosinase activity 3900 unit/mg solid) was purchased from Sigma-Aldrich (St. Louis, US). RPMI Medium 1640 was purchased from GIBCO BRL, Grand Island (New York, US). Synthetic melanin was purchased from Sigma-Aldrich Chemie GmbH (Germany). L-3,4-dihydroxyphenylalanine (L-DOPA) from Qiude biotech Ltd. (Shanghai, China), Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Co., Ltd. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was purchased from Bioseen Technology, Inc. (Shanghai, China). Standard compounds of hesperidin (CAS No. 520-26-3, 98%), nobiletin (CAS No. 10236-47-2, 98%) were gifts from Tianjin Jianfeng Natural Products Ltd. (Tianjin, China).

Preparation of citrus peel crude extracts

Citrus peel (*C. unshiu* Marc.) was purchased from a local factory in Jinhua, Zhejiang (China) in November 2004 and the cultivar was authenticated by the Citrus Research Institute, Zhejiang Academy of Science, Huangyan, Zhejiang. The peel was milled, extracted with 95% ethanol, and liquid-liquid extracted by petroleum ether to obtain the petroleum ether phase (citrus peel extract from petroleum ether, PCPE) and ethanol phase (citrus peel extract from ethanol, ECPE) of citrus peel extract (Figure 2). The flavanone glycosides-rich extract (FGRE) was further obtained

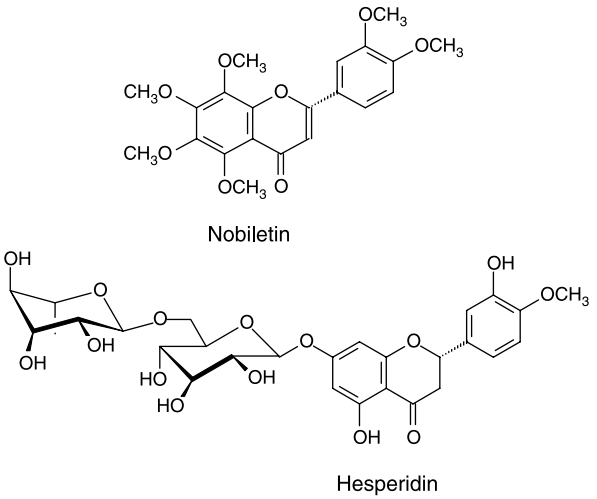

Figure 1. Structure of nobiletin and hesperidin.

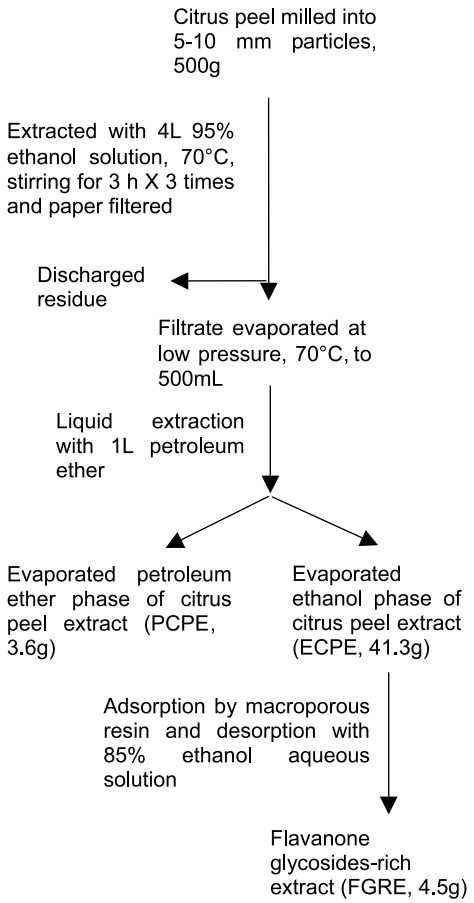

Figure 2. Process flow diagram of citrus peel extracts.

from ECPE by the following process: ECPE was suspended in 30% (v/v) ethanol aqueous solution followed by ultrasonication, centrifugation and paper filtration. The clear filtrate was adsorbed on macroporous resin (AB-8, partial polar cross-linked polystyrene resin, Nankai University, Tianjin, China), washed with de-ionized water to remove water soluble impurities and desorbed with 85% (v/v) ethanol aqueous solution to finally obtain FGRE.

HPLC-DAD assay

Quantitative analysis of flavonoids was performed on an Agilent 1100 series HPLC system. Samples were separated on an Agilent Eclipse XDB-C18 reverse phase column (θ4.6 × 250 mm, 5 μm) protected by a guard column (XDB-C18, θ4.6 × 12.5 mm), at 35°C, flow rate 1 mL/min. The UV profiles at 284 and 332 nm were recorded. The gradient elution program was as follows: an initial 10-min run of 35% methanol-water solution (v/v) was followed by a 10-min linear gradient to 100% methanol and held for 5 min, linear gradient to 35% methanol-water in 5 min. Comparison with the standard compounds, peak purity check and compound identification were carried out by a 190 ~ 400 nm UV scan through a diode array detector (DAD) on software Agilent ChemStation Plus rev. A.10.02.

Tyrosinase assay

The assay employed is based on Mason and Peterson's method [29] adapted to a 96-well plate: 70 μL of 0.1M phosphate buffer pH 6.8 (PBS), 30 μL of mushroom tyrosinase diluted in phosphate buffer (167 units/mL) and various concentration of different test samples dissolved in 20 μL dimethyl sulfoxide (DMSO) were inserted into 96-well plates for 5 min pre-incubation at 30°C. 100 μL L-DOPA was added to start the enzymatic reaction. Optical density (OD) at 492 nm was measured on a Sunrise absorbance microplate reader (Tecan Trading AG, Switzerland) to observe dopachrome formation for 10 min. The percentage of inhibition is calculated from the following equation,

$$\% \text{ inhibition} = [(A - B) - (C - D)]/(A - B) \times 100,$$

where (A) = OD at 492 nm with tyrosinase but without test substance; (B) = OD at 492 nm without test substance and tyrosinase; (C) = OD at 492 nm with test substance and tyrosinase; (D) = OD at 492 nm with test substance but without tyrosinase. Kojic acid and arbutin were tested as two positive control samples. 50% of tyrosinase inhibition (IC_{50}) and the 95% confidence range were estimated with the Trimmed Spearman-Karber method [30] calculated by program SPEARMAN (Montana State University, 1999).

Cell cultures

The B16 mouse melanoma cells were purchased from Chinese Type Culture Collection (Shanghai Institute of Cell Biology, Chinese Academy of Science, Shanghai, China). The cells were cultured in RPMI Medium 1640 supplemented with 10% dialyzed heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 U/ml) in a humidified atmosphere with 5% CO_2 at 37°C.

Cell viability assay

Subcultures of B16 cells were seeded in 96-well plates at a density of 3×10^3 cells/mL and cultured for 24 h. The medium was then replaced with 200 μL fresh 10% FBS-RPMI1640 medium containing 0.5% DMSO (v/v) to dissolve various test substances, which were all filtered through a 0.22 μm membrane. After 3-day culturing, the medium was replaced with 90 μL fresh medium and 10 μL 5.0 mg/mL MTT-PBS solution. After culturing for 4 h, the medium and non-metabolized MTT were carefully removed and 100 μL of DMSO were added to each well to dissolve the MTT formazan produced by the living cultured cells. After shaking for 30 min at room temperature, the plates were read with an automated Bio-Rad 550 microtiter plate reader (Bio-Rad Laboratories, CA) using 570 nm for the reading and 690 nm for the reference wavelength. 10% FBS-RPMI1640 medium containing 0.5% DMSO was used as a control blank.

Melanin determination assay

The assay followed Kubo's method [31] with slight modification. Briefly, subcultures of B16 cells were seeded in 24-well plates at a density of 2×10^5 cells/mL and cultured for 24 h. The medium was then replaced with 1.0 mL fresh 10% FBS-RPMI1640 medium containing 0.5% DMSO (v/v) to dissolve various test substances, which were all filtered through 0.22 μm membrane. After 3-day culturing, the cells were harvested and suspended in 0.5 mL 1N NaOH-10% DMSO solution (v/v), kept at 80°C for 2 h and incubated at 37°C overnight. 200 μL test solution was transferred into 96-well plate and measured on a Sunrise absorbance microplate reader (Tecan Trading AG, Switzerland) at 405 nm. 10% FBS-RPMI1640 medium containing 0.5% DMSO was used as control. The melanin content was determined by calculation from a synthetic melanin standard curve.

Statistical analysis

All the experiments were run at least in triplicate. SPSS 11.5 (SPSS Inc. Chichago) was used for all the statistical analysis: a descriptive statistical analysis was made by calculating the mean and standard deviation

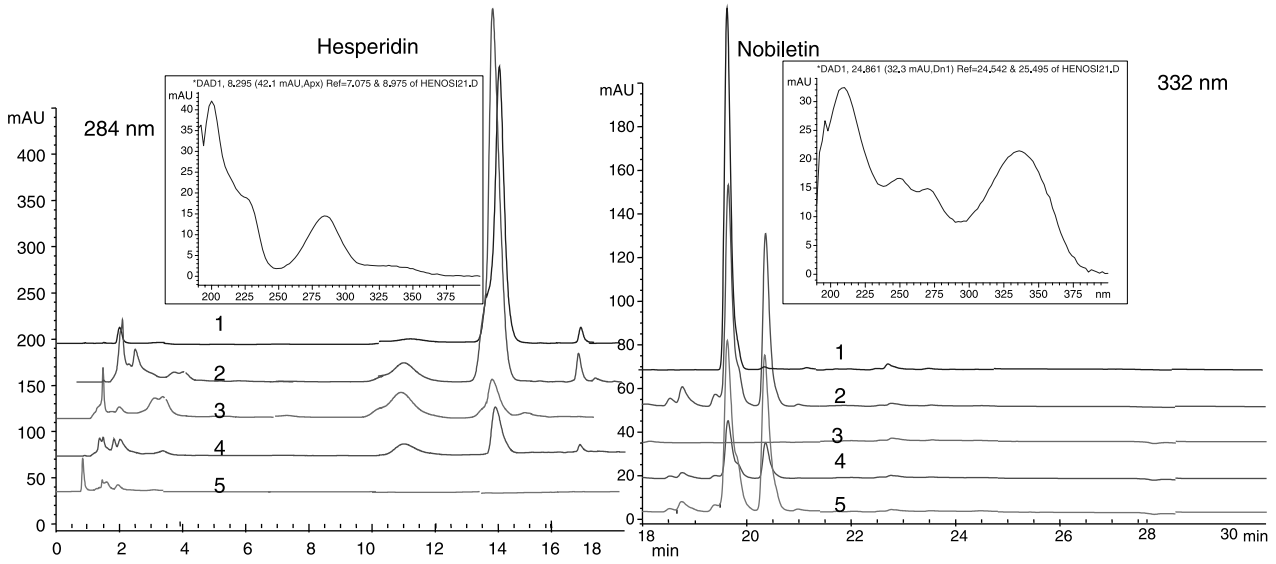

Figure 3. HPLC profiles of citrus extracts at dual wavelength: 284 nm and 332 nm. 332 nm profiles followed 284 nm profiles from 18.0 min. 1: hesperidin and nobiletin mixed standard solution, 2: dried citrus peel, 3: FGRE, 4: ECPE, 5: PCPE; The 2 insets are DAD UV scan of hesperidin and nobiletin peak (190 ~ 400 nm).

and comparison between groups was complemented by a comparison between means (pairwise t-test). Values of $p < 0.05$ were considered to be significant.

Results and discussion

Quantitative analysis

The HPLC profiles and the compounds were identified by comparison with standard compounds and the DAD scan of the flavonoids peaks (Figure 3). The flavonoids contents, mainly hesperidin, were 2.81% ± 0.09%, 6.49% ± 0.23%, 21.24% ± 0.85% in dried citrus peel, ECPE and FGRE, respectively, and negative in PCPE. The nobiletin contents, the majority of polymethoxylated flavones in the citrus cultivar, were 0.27% ± 0.03%, 0.77% ± 0.09%, 3.76% ± 0.11% in dried citrus peel, ECPE and PCPE, respectively, and negative in FGRE, (n = 3).

Inhibition of the diphenolase activity of tyrosinase by citrus extract

Though the sample of mushroom tyrosinase differs from those from different sources, it was still employed in the current experiments due to its commercial availability. The reaction courses are presented in Figure 4. All the experiments were run under saturated-air conditions, though this is a bi-substrates (oxygen and L-DOPA) enzymatic reaction. Among these reaction curves (Figure 4), dopachrome formation reaches a plateau during 190 ~ 300 seconds as the available oxygen in the reaction system was consumed. However, kojic acid is different from those curves above, which can be evidence of its slow-binding inhibition mechanism [32]. The changes in percentage inhibition with reaction time are shown in Figure 5. Almost all the test samples appeared to have a turnover of inhibition as the time increased which normally occured at the same time as the plateau appeared in Figure 4. It could result from a secondary reaction in the later oxygen-consumed system. On the other hand, it cannot be ruled out that the test substances could be fast-binding inhibitor in classical manner to tyrosinase activity [33].

The potency of the tyrosinase inhibitors in citrus extracts, presented as 50% of inhibition (IC_{50}) of the DMSO control by comparison with two positive

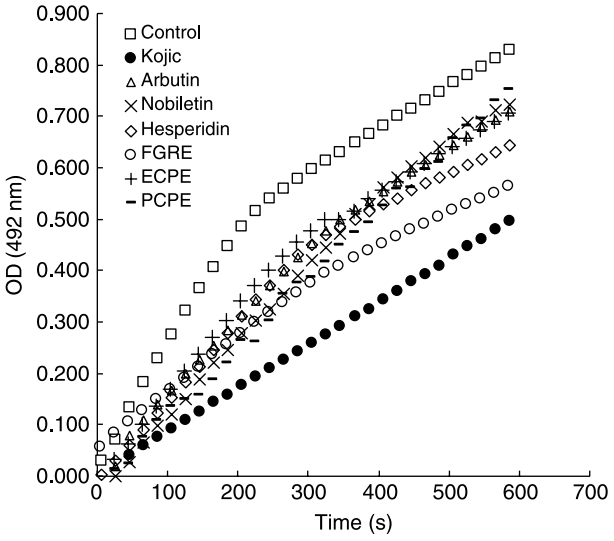

Figure 4. Effect of diphenolase activity of tyrosinase, L-DOPA (10 mM) as substrate and inhibitors: kojic acid, 0.128 mM; arbutin, 14.15 mM; nobiletin, 2.58 mM; hesperidin, 2.41 mM; FGRE, 3.89 mg/mL; ECPE, 2.61 mg/mL; PCPE, 5.98 mg/mL; DMSO used as control.

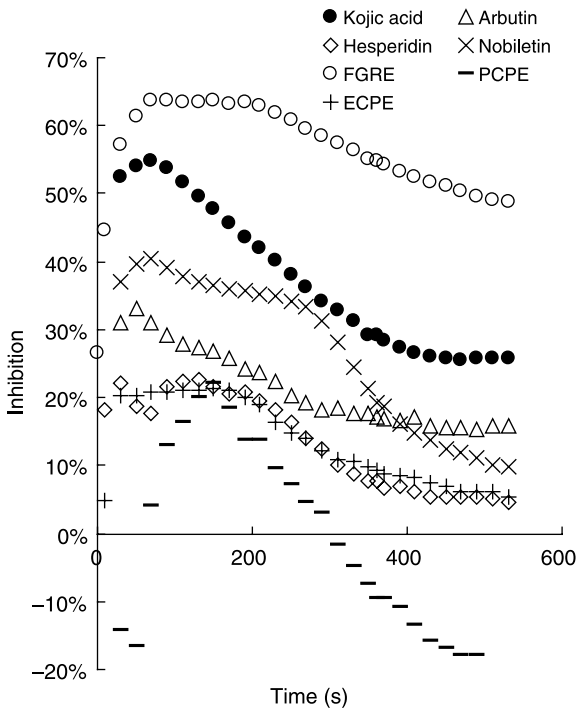

Figure 5. Effect of reaction time on tyrosinase inhibition, L-DOPA (10 mM) as substrate and inhibitors: kojic acid, 0.133 mM; arbutin, 28.29 mM; nobiletin, 1.29 mM; hesperidin, 4.81 mM; FGRE, 7.78 mg/mL; ECPE, 2.61 mg/mL; PCPE, 2.99 mg/mL.

controls, kojic acid and arbutin, is shown in Table I. As previously reported, the IC_{50} values of the positive control samples, kojic acid and arbutin, are basically in the range 0.04 ~ 0.774 mM [28,33–39] and 8 ~ 24 mM [4,34,35,38–41], respectively. However, the IC_{50} of nobiletin is much higher than a previous reported value, 0.0462 mM [28], mainly due to the different tyrosinase assay used including the influence of DMSO [42], whereas it is still the strongest inhibitory component from citrus extracts and is much more potent than arbutin (IC_{50} 27.31 mM). Meanwhile, hesperidin, the major flavonoid in this cultivar, shows only a mild inhibitory effect, 16.08 mM, on tyrosinase, the weakest in the citrus extracts.

The inhibition type of hesperidin and nobiletin on the diphenolase activity of mushroom tyrosinase

The initial velocities were determined in the early stage of the tyrosinase reaction (no longer than one minute), that is to say, the reaction for the mechanism study was under air-saturated condition, and the Michaelis constant (Km) and the maximum velocity (Vm) were apparent. Lineweaver-Burk plots of hesperidin inhibition of the diphenolase activity of tyrosinase were obtained through a series reaction with various concentrations of substrate, L-DOPA and inhibitor, hesperidin (Figure 6). The set of straight lines obtained by linear regression intersected each other on the x-axis indicating that the inhibition mechanism for hesperidin is non-competitive type [43]. The inset secondary plots obtained from different intercepts and different slopes versus inhibitor concentration were used to estimated the inhibition constant for free enzyme (K_i) and for the complex of enzyme-substrate (K_{is}), respectively, (inset of Figure 6). The estimated values shown in Table I indicate that the affinity of the inhibitor, hesperidin, to free enzyme and the enzyme-substrate complex is the same ($K_i = K_{is} = 9.16$ mM).

The same model was employed to obtain the Lineweaver-Burk plots of nobiletin inhibition of the diphenolase activity of tyrosinase. The family of straight lines intersected each other on the y-axis indicating a competitive type of inhibition mechanism for nobiletin (Figure 7). The plots of slope (apparent Km) versus concentration of inhibitor, nobiletin, are shown in the inset of Figure 7. The K_i value of nobiletin is estimated as 2.82 mM (inset of Figure 7 and Table I).

This suggests that the polymethoxylated flavone, nobiletin, effectively binds the active site reversibly (chelating copper in the enzyme) and its active radical is an exception to the previous understanding of tyrosinase inhibition by flavonoids: not a free 3-hydroxy group [4], not an α-keto group [44] and not a flavon-3-ol skeleton with a galloyl moiety at the 3-position [11], but a methoxyl group on the flavone skeleton.

Cell viability test

An MTT assay was employed for evaluating the antiproliferation activity of the test substances on B16 mouse melanoma cells. After 72 hours culture, most inhibitors showed no significant change of cell viability within the concentration range 15.63 ~ 250 μg/mL while nobiletin and PCPE showed significant antiproliferation activity (Figure 8). The estimated IC_{50}

Table I. IC_{50} values, estimated confidence ranges and inhibition constants (Ki) of citrus extracts and related components.

	IC_{50}	95% confidence range	K_i (mM)
Kojic acid	0.0372 mM	0.0348 ~ 0.0398 mM	
Arbutin	27.31 mM	23.31 ~ 33.58 mM	
Nobiletin	1.49 mM	1.47 ~ 1.51 mM	2.82
Hesperidin	16.08 mM	15.74 ~ 16.42 mM	$K_i = K_{is} = 9.16$
PCPE	8.09 mg/mL	8.02 ~ 8.16 mg/mL	
ECPE	7.53 mg/mL	7.37 ~ 7.69 mg/mL	
FGRE	4.80 mg/mL	4.68 ~ 4.94 mg/mL	

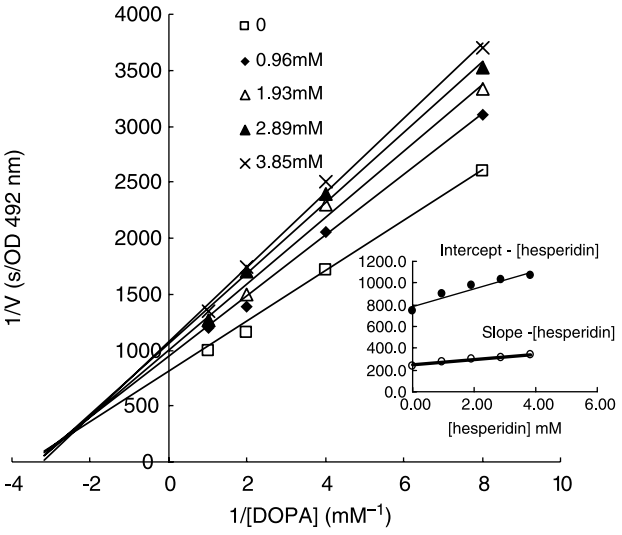

Figure 6. Lineweaver-Burk plots of hesperidin inhibition on diphenolase activity of tyrosinase with substrate, L-DOPA (0.125 mM, 0.25 mM, 0.5 mM, 1 mM). Five curves of five concentration of hesperidin are: 0, 0.96 mM, 1.93 mM, 2.89 mM and 3.85 mM, respectively. The inset is the secondary plots of the intercept versus concentration of inhibitor (hesperidin) and slope versus concentration of hesperidin.

values for nobiletin and PCPE are 35.65 μg/mL (88.6 μM) and 62.96 μg/mL, respectively. A similar MTT test has been carried out by another group on tangeretin, a 5-methoxylated flavone in citrus, with an IC_{50} value of 11.2 μM [20].

Although this cell viability test was aimed at finding a relative safe dosage for further intracellular

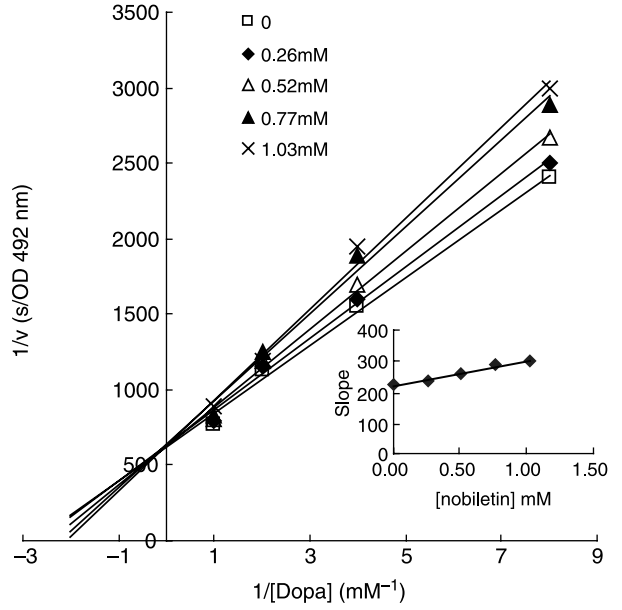

Figure 7. Lineweaver-Burk plots of nobiletin inhibition on diphenolase activity of tyrosinase with substrate, L-DOPA (0.125 mM, 0.25 mM, 0.5 mM, 1 mM). Five curves of five concentration of hesperidin are: 0, 0.26 mM, 0.52 mM, 0.77 mM and 1.03 mM, respectively. The inset is the secondary plot of the intercept versus concentration of inhibitor (nobiletin).

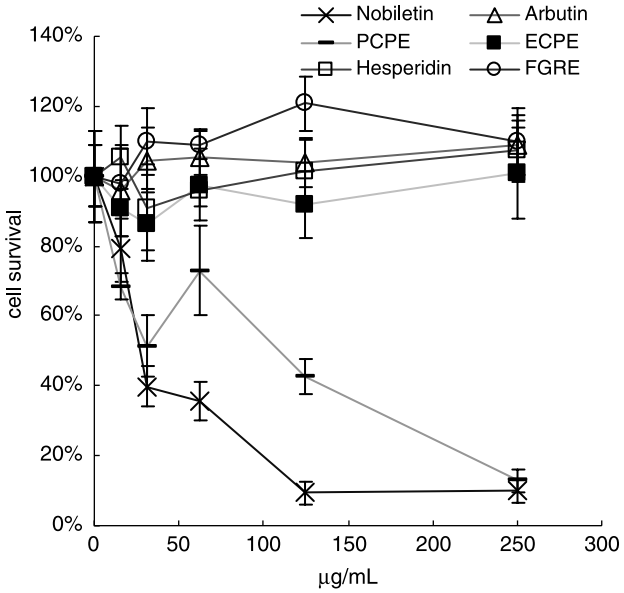

Figure 8. Cell viability determined by MTT assay after 72 h exposure to various concentrations of inhibitors: arbutin, nobiletin, hesperidin, FGRE, ECPE, PCPE, n = 4.

determination of melanin formation in B16 melanoma cells, nobiletin and the crude extract PCPE provide preliminary evidence that polymethoxylated flavones possess significant antiproliferation activity against melanoma cell. Previous reports have focused more on tangeretin for its potent antiproliferative effect on B16 melanoma cell lines [19,20] and correlated to the structure-activity relationship (SAR) that the presence of three or more methoxylated radicals in a flavone-type flavonoid skeleton with double C2-C3 bond imparts antiproliferative capacity [45,46]. Positive MTT test results on tangeretin and nobiletin in a human melanoma cell line were also reported by Manthey and Guthrie [27]. All the above results agree with our cell viability test results on the efficacy of nobiletin, which has one more methoxylated radical than tangeretin, on B16 mouse melanoma cell.

Measurement of melanin content

Based on the results from the MTT test, concentrations of 31.25 μg/mL and 7.8 μg/mL of various test substances were chosen for determination of the melanin content (Figure 9). The inhibitory potency of arbutin (12.31% at 31.25 μg/mL, 0.12 mM) is consistent with a previous report (0.1 mM for ~18% inhibition) [31] i.e., %inhibition = [(melanin of control cells − melanin of arbutin inhibited cells)/melanin of control cells) × 100]. Regardless of the influence of nobiletin and PCPE on cell growth (only at 7.8 μg/mL), their intracellular tyrosinase inhibitory effects are mild (both are 5.2%) and much weaker than those on extracellular mushroom tyrosinase. Hesperidin at 7.8 μg/mL does not inhibit melanin

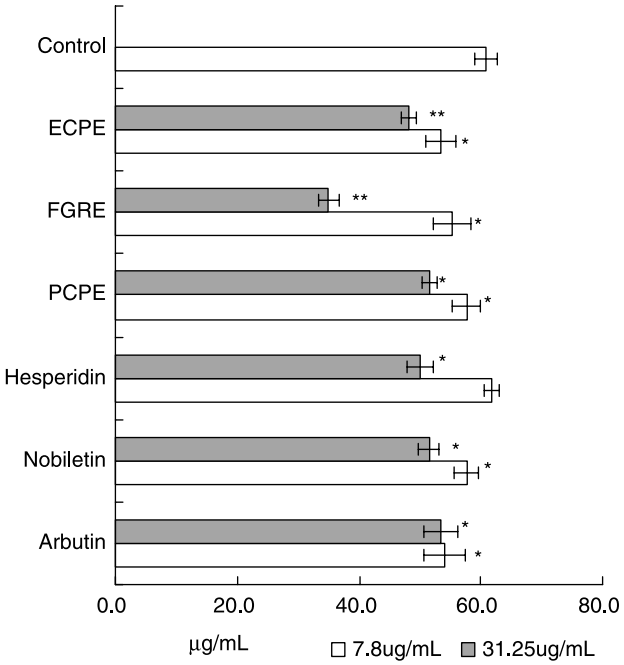

Figure 9. Melanin content (μg/mL 1N NaOH +10% DMSO), Concentration of test substances: 7.8 μg/mL, 31.25 μg/mL. n = 4, ⋆p < 0.05, ⋆⋆ p < 0.01.

formation in B16 cells. ECPE also show a significant inhibitory effect (20.65% at 32.25 μg/mL, $p < 0.01$). Interestingly, FGRE shows significant strong inhibition of B16 melanin formation ($p < 0.01$), especially at the level of 31.25 μg/mL (42.48%). These results lead to the conclusion that the presence of hesperidin in ECPE and FGRE does not play a key role on tyrosinase inhibition. Although further work is needed, a hypothesis could be given that, other constituents like polysaccharides might influence tyrosinase inhibition with respect to the activity of FGRE.

Conclusion

In conclusion, citrus peel, a widely available by-product was extracted by a novel industrialized process in the present work. The extracted products such as ECPE, PCPE and FGRE and its main citrus flavonoids, hesperidin and nobiletin were evaluated extracellular and intracellular for their tyrosinase inhibitory effect. The crude extracts exhibited promising inhibition on mushroom tyrosinase, even higher than arbutin. Although effective inhibition by citrus crude extracts needs a relative high dosage, it is still practical due to their low cytotoxicity. Furthermore, the fact that the crude extract FGRE shows significant inhibition of both mushroom tyrosinase and melanin formation of B16 cells suggests that the active compound may not be the flavonoid, hesperidin. The inhibition mechanism of hesperidin and nobiletin on mushroom tyrosinase diphenolase activity were of noncompetitive and competitive types, respectively. Although nobiletin showed potent inhibition of mushroom tyrosinase, its intracellular activity is more antiproliferative against B16 murine melanoma cells than inhibitory on melanin formation. Moreover, nobiletin, which is the major polymethoxylated flavone in this citrus cultivar, gives an interesting exception [4], to the accepted flavonoid structure-activity relationship that polymethoxylated flavones including tangeretin, sinensetin may suppress tyrosinase activity by their electron donating groups, the methoxyl radicals [4]. Therefore, it is necessary to carry out further research on more citrus flavonoids and correlate their structure-activity relationship to tyrosinase inhibition.

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