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

CHONGWEI ZHANG†, YANHUA LU†, LIN TAO, XINYI TAO, XIAOCHUN SU, & DONGZHI WEI

State Key Laboratory of Bioreactor Engineering, New World Institute of Biotechnology, East China University of Science and Technology, Shanghai, China

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Abstract

The inhibitory effects of nobiletin and hesperidin from citrus peel crude extracts on tyrosinase diphenolase activity have been evaluated. IC_{50} of nobiletin and hesperidin were 1.49 mM and 16.08 mM, respectively and their inhibition mechanisms are competitive type with inhibition constant (K_i) 2.82 mM and noncompetitive type 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 of melanogenesis in B16 mouse melanoma cells was also evaluated. FGRE showed a significant inhibition (42.48% at 31.25 μg/mL, $p < 0.01$) while hesperidin showed almost no inhibition. Nobiletin and PCPE gave efficacious antiproliferation effects on the B16 mouse melanoma cell with IC_{50} values 88.6 μM and 62.96 μg/mL, respectively, through 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 under the ultraviolet radiation of sunlight producing rapidly growing numbers of melanoma cases in the west, *esp.* Caucasians, like USA and Australia [1,2], and the continuous need for skin whitening agents from the East, increasing research interest has focused 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 the 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 rich in bioactive chemicals, mostly without harmful side effects, are attracting increasing efforts on screening for tyrosinase inhibitors. Kojic acid and arbutin, for examples, are currently used as natural tyrosinase inhibitors in the cosmetic industry.

Correspondence: Prof. Dr. Y. Lu, P. O. #311, East China University of Science and Technology, No. 130, Meilong RD, Shanghai 200237, China. Tel: 86 21 64252981. Fax: 86 21 64250068. E-mail: luyanhua@ecust.edu.cn
†Co-author, who gave the same contribution to this research work.

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These natural sources can be categorized into two subgroups [4]: aldehydes like cinnamic acid, anisic acid and cuminaldehyde [8], etc.; polyphenols like kaempferol [9], quercetin [10], etc. The polyphenol group is widely distributed in natural plants from the bark and root to 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 their 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 a Traditional Chinese Medicinal ingredient [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 on their potent activities against tumors and others [19–27] due to their unique polymethoxyl radicals (Figure 1). Although one report has already demonstrated the inhibitory potency of nobiletin on mushroom tyrosinase [28], further work needs to be carried out on their types, 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 the B16 mouse melanoma cells was also evaluated as intracellular evidence.

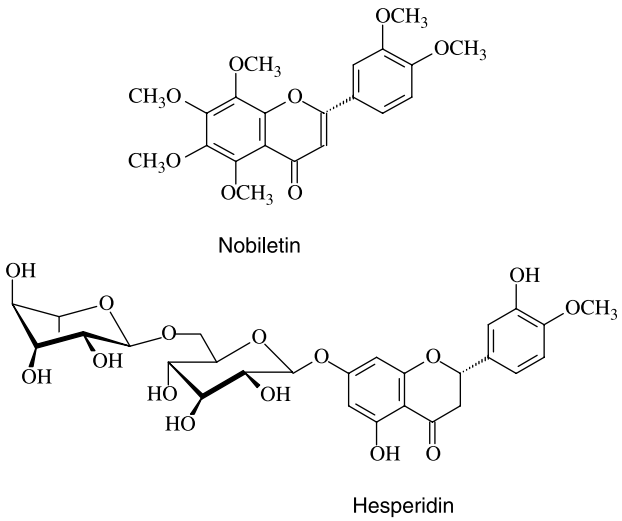

Figure 1. Structure of nobiletin and hesperidin.

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 from GIBCO BRL, Grand Island (New York, US), synthetic melanin from Sigma-Aldrich Chemie GmbH (Germany), L-3,4-dihydroxyphenylalanine (L-DOPA) from Qiude biotech Ltd. (Shanghai, China), Fetal bovine serum (FBS) from Hangzhou Sijiqing Co., Ltd. and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) 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).

Citrus peel crude extracts preparation

Citrus peel (*C. unshiu* Marc.) was purchased from a local factory in Jinhua, Zhejiang (China) in November 2004 and the cultivar was authenticated by 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 a 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). Flavanone glycosides-rich extract (FGRE) was further obtained from ECPE by the following process: ECPE was suspended in 30% (v/v) ethanol aqueous solution which was there ultrasonicated, centrifuged and paper filtered. The clear filtrate was adsorbed on a 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 (4.6 × 250 mm, 5 μm) protected by a guard column (XDB-C18, 4.6 × 12.5 mm), at 35°C, flow rate 1 mL/min. The profiles at λ284 and 332 nm were recorded. The gradient elution program was as follows: initial 10-min run with 35% methanol-water solution (v/v), followed by a 10-min linear gradient to 100% methanol which was held for 5 min and then linear gradient to 35% methanol-water in 5 min. Comparing with the standard compounds, peak purity check and compound identification were carried out at 190 ~ 400 nm UV scan through a diode array

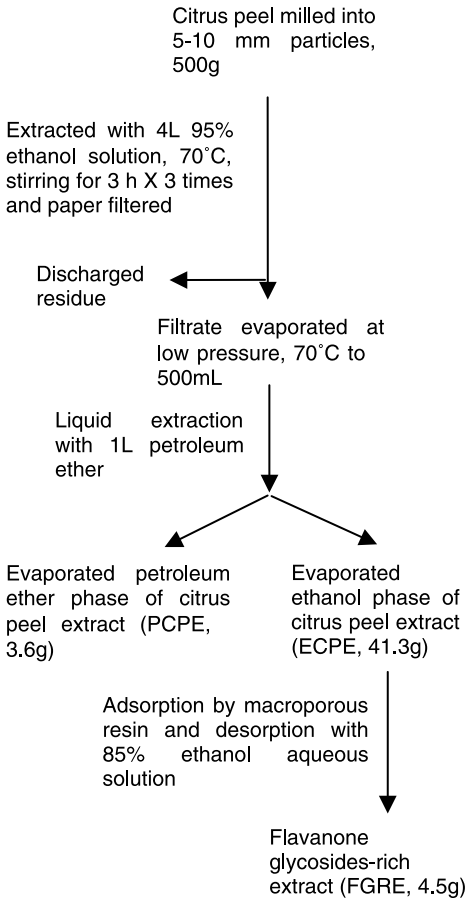

Figure 2. Process flow diagram for citrus peel extracts.

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 the 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 then 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 was calculated as follows:

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

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 controls. 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 nonmetabolized MTT were carefully removed and 100 μL of DMSO was 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 blank control.

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 a 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.

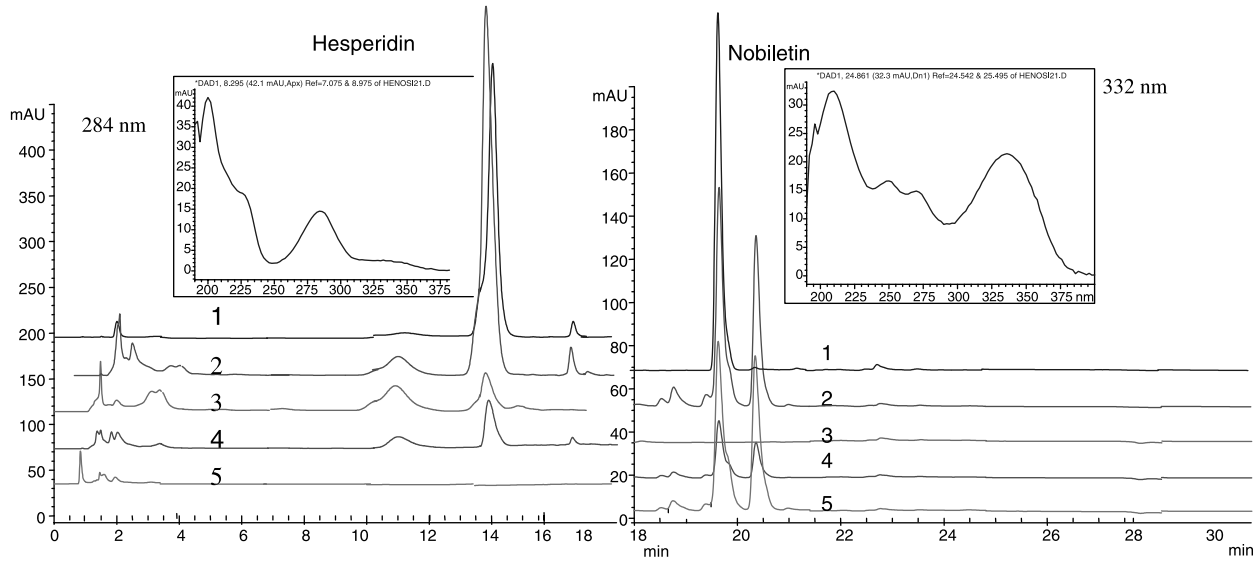

Figure 3. HPLC profiles of citrus extracts at dual wavelength: 284 nm and 332 nm. 332 nm profiles followed 284 nm profiles continuing from 18.0 min. 1: hesperidin and nobiletin mixed standard solution, 2: dried citrus peel, 3: FGRE, 4: ECPE, 5: PCPE; The 2 insets are DAD UV scan of hesperidin and nobiletin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

The melanin content was determined and calculated with 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 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 are 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, FGRE respectively and negative in PCPE. The nobiletin content, 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, PCPE respectively and negative in FGRE, (n = 3).

Inhibition of diphenolase activity of tyrosinase by citrus extract

Though the sample of mushroom tyrosinase differs from those from other 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 the saturated-air condition, though this is a bi-substrate (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 is consumed. However, the kojic acid curve is different from those curves above, which can be evidence of its slow-binding inhibition mechanism [32]. The changes in percentage of inhibition with reaction time were observed in Figure 5. Almost all the test samples

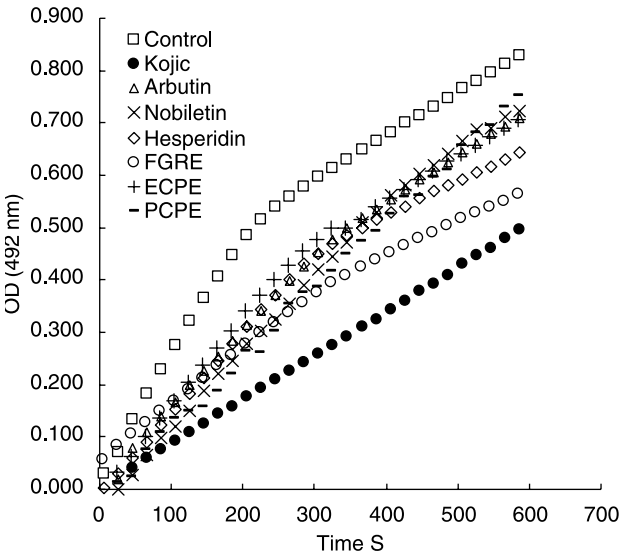

Figure 4. Effect on diphenolase activity of tyrosinase, L-DOPA (10 mM) as substrate and inhibitors: kojic acid, 0.128 mM; arbutin, 14.15 mM; nobiletin, 2.58 mM; hesperidin, 2.41 mM; FGRE, 3.89 mg/mL; ECPE, 2.61 mg/mL; PCPE, 5.98 mg/mL; DMSO used as control.

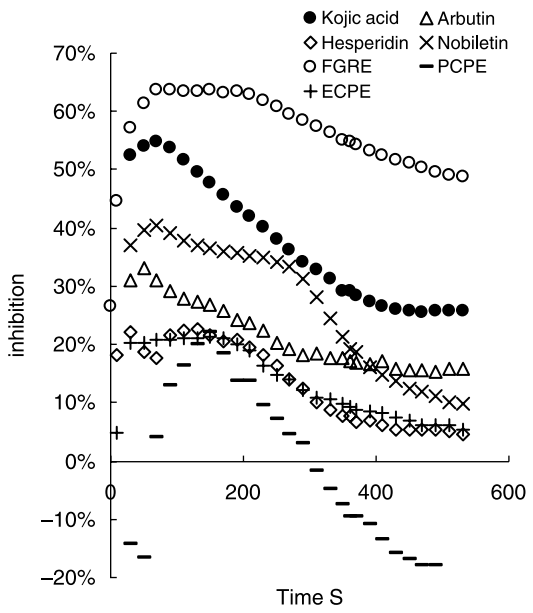

Figure 5. Effect of reaction time on tyrosinase inhibition, L-DOPA (10 mM) as substrate and inhibitors: kojic acid, 0.133 mM; arbutin, 28.29 mM; nobiletin, 1.29 mM; hesperidin, 4.81 mM; FGRE, 7.78 mg/mL; ECPE, 2.61 mg/mL; PCPE, 2.99 mg/mL.

appeared to have a turnover of inhibition with increasing time, which normally occured at the same time as the plateau appeared in Figure 4. It could result from the secondary reaction later in the oxygen consumed system. On the other hand, it cannot be ruled out that the test substances could be fast-binding inhibitors in the classical manner of tyrosinase activity [33].

The potency of tyrosinase inhibitors in citrus extracts, presented as 50% of inhibition (IC_{50}) of DMSO control in contrast with two positive controls, kojic acid and arbutin, is shown in Table I. As previously reported, the IC_{50} values of positive control samples, kojic acid and arbutin, are basically in the range of 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 including influence of DMSO [42], whereas it is still the strongest component from citrus extracts and a much more potent inhibitor than arbutin (IC_{50} 27.31 mM). Meanwhile, hesperidin, the major flavonoid in this cultivar, shows a mild inhibitory effect on tyrosinase, the weakest in citrus extracts, 16.08 mM.

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	

The type of by inhibition by 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 1 minute), the reaction for the mechanism study was under air-saturated conditions, and the Michaelis constant (Km) and the maximum velocity (Vm) were apparent. Lineweaver-Burk plots of hesperidin inhibition on diphenolase activity of tyrosinase were obtained through a series of reactions with various concentrations of substrate, L-DOPA and inhibitor (Figure 6). The set of straight lines by linear regression intersecting each other on the x-axis indicates that the inhibition mechanism of hesperidin is non-competitive type [43]. The inset secondary plots obtained from different intercepts and different slopes versus inhibitor concentration were used to estimated inhibition constant for free enzyme (K_i) and for the enzyme-substrate complex (K_{is}) respectively (inset of Figure 6). The estimated values shown in Table I indicate that the affinity of inhibitor, hesperidin, to free enzyme and complex of enzyme-substrate is the same ($K_i = K_{is} = 9.16$ mM).

Similarly, the Lineweaver-Burk plots of nobiletin inhibition of diphenolase activity of tyrosinase gave a

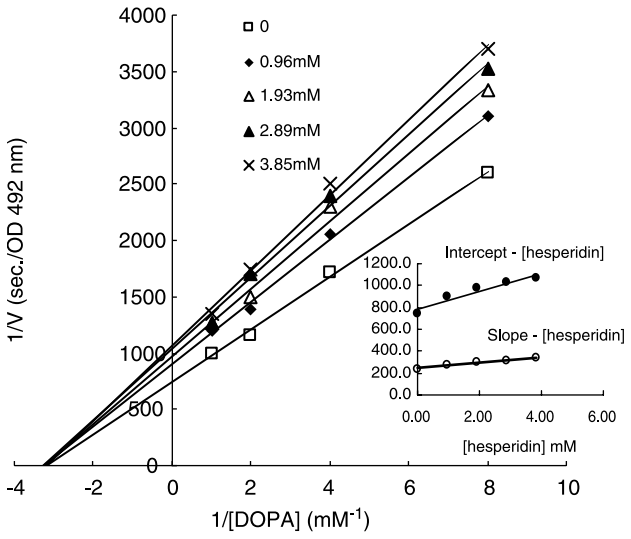

Figure 6. Lineweaver-Burk plots of hesperidin inhibition of diphenolase activity of tyrosinase with substrate, L-DOPA (0.125 mM, 0.25 mM, 0.5 mM, 1 mM). Five curves of five concentration of hesperidin are: 0, 0.96 mM, 1.93 mM, 2.89 mM and 3.85 mM, respectively. The inset is the secondary plots of the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

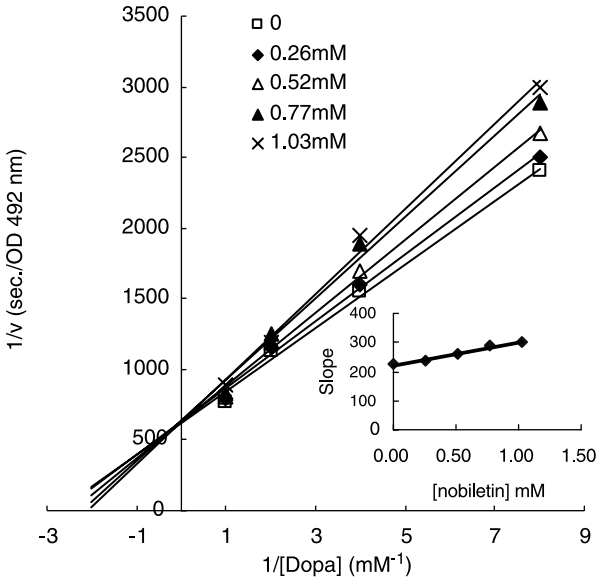

Figure 7. Lineweaver-Burk plots of nobiletin inhibition of diphenolase activity of tyrosinase with substrate, L-DOPA (0.125 mM, 0.25 mM, 0.5 mM, 1 mM). Five curves of five concentration of hesperidin are: 0, 0.26 mM, 0.52 mM, 0.77 mM and 1.03 mM, respectively. The inset is the secondary plot of the slope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

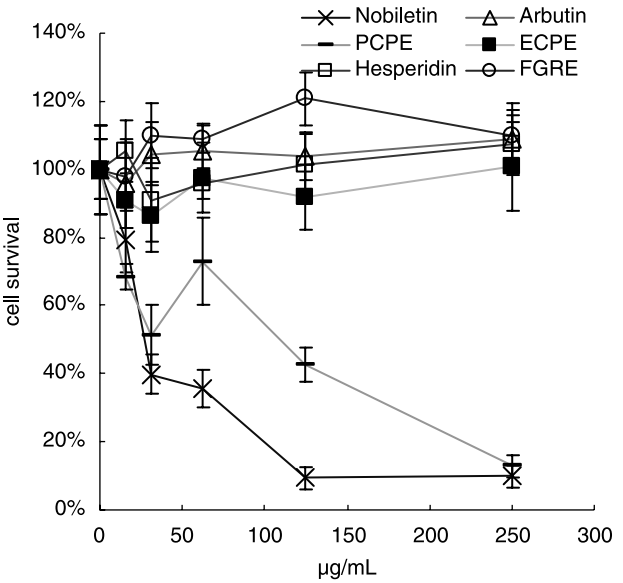

Figure 8. Cell viability determined by MTT assay after 72 h exposure in various concentrations of inhibitors: arbutin, nobiletin, hesperidin, FGRE, ECPE, PCPE, n = 4.

family of straight lines intersecting each other on the y-axis indicating a competitive type of inhibition mechanism (Figure 7). The plots of slope (apparent Km) versus concentration of inhibitor, nobiletin, are shown in the inset of Figure 7. K_i value of nobiletin is estimated as 2.82 mM (the inset of Figure 7, and Table I). This suggests that this 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 of flavonoids in that it is not a free 3-hydroxy group [4], not α-keto group [44] and not the 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 culturing, most of inhibitors showed no significant change in cell viability with the concentration ranging from 15.63 ~ 250 μg/mL while nobiletin and PCPE give significant antiproliferation activity to B16 melanoma cells (Figure 8). The estimated IC_{50} values of nobiletin and PCPE were 35.65 μg/mL (88.6 μM) and 62.96 μg/mL, respectively. A similar MTT test was carried out by another group on tangeretin, a 5-methoxylated flavone in citrus with an IC_{50} value, 11.2 μM [20].

Although this cell viability test was aimed at finding a relative safe dosage for further intracellular determination of melanin formation in B16 melanoma cells, nobiletin and the crude extract PCPE provided preliminary evidence that polymethoxylated flavones possess significant antiproliferation activity against melanoma cells. Previous reports 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 C2–C3 double 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 these above reports agree with our cell viability test results on the efficacy of nobiletin, which contains one more methoxylated radical than tangeretin, on B16 mouse melanoma cells.

Measurement of melanin content

Based on the results of the MTT test, concentrations of 31.25 μg/mL and 7.8 μg/mL of various test substances were chosen for determination of their effects on melanin content (Figure 9). The inhibitory potency of arbutin (12.31% at the level of 31.25 μg/mL, 0.12 mM) is consistent with a previous report (0.1 mM for ~18% inhibition) [31], where %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

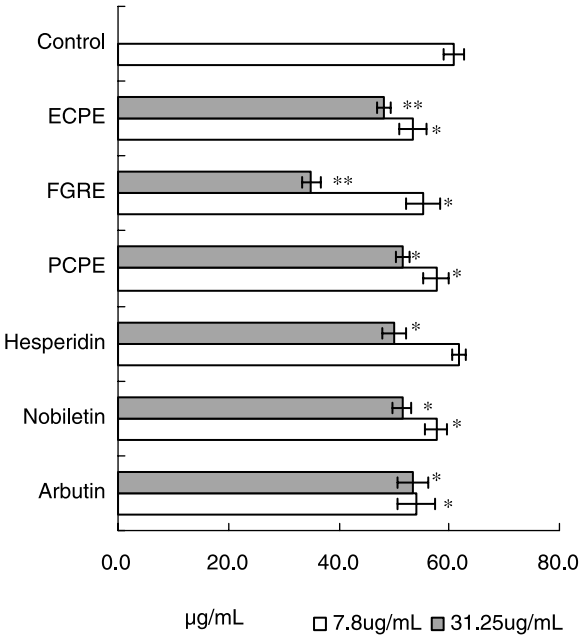

Figure 9. Melanin content (μg/mL 1N NaOH +10% DMSO), Concentration of test substances: 7.8 μg/mL, 31.25 μg/mL. n = 4, * $p < 0.05$, ** $p < 0.01$.

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 gave no inhibition of melanin formation in B16 cells. ECPE also showed a significant inhibitory effect (20.65% at 32.25 μg/mL, $p < 0.01$). Interestingly, FGRE showed significant strong inhibition of B16 melanin formation ($p < 0.01$), especially at the level of 31.25 μg/mL (42.48%). From these results it can be concluded that the presence of hesperidin in ECPE and FGRE does not play a key role in the tyrosinase inhibition. Although further work is needed the hypothesis could still apply, with respect to the effect of FGRE, that other constituents like polysaccharides might have an influence on tyrosinase inhibition.

Conclusion

In conclusion, citrus peel, a widely available industrial 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 for their tyrosinase inhibitory effect extracellularly and intracellularly. The crude extracts exhibit promising inhibition of mushroom tyrosinase, with even higher potency than arbutin. Although the effective inhibition of the enzyme by citrus crude extracts needs relative high dosage, it is still practical due to their low cytotoxicity. Furthermore, the fact that the crude extract FGRE shows significant inhibition on both mushroom tyrosinase and on 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 elucidated as noncompetitive and competitive respectively. Although nobiletin showed potent inhibition of mushroom tyrosinase, its intracellular activity is greater as an antiproliferation against B16 murine melanoma cells than inhibition of melanin formation. Moreover, nobiletin, the major polymethoxylated flavone in this citrus cultivar, gives an interesting exception to the existing flavonoid structure-activity relationship that polymethoxylated flavones including tangeretin, sinensetin may suppress tyrosinase activity by relying on their electron donating groups, methoxyl radical [4]. Therefore, it is necessary to carry out further research on other citrus flavonoids and elucidate their structure-activity relationship on tyrosinase inhibition.

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