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Hydrolysates of Citrus Plants Stimulate Melanogenesis Protecting Against UV-induced Dermal Damage

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The sun-tanning process occurs as a spontaneous response to ultraviolet (UV) irradiation. UV will induce tanning and DNA damage, processes that can lead to photoaging and skin disorders such as hyperpigmentation and cancer. The pigment melanin protects skin from UV damage; therefore, an efficient melanin-promoting suntan lotion could be highly beneficial. In this study, a process was developed to increase the content of naringenin in citrus extracts and to determine whether a higher naringenin content of citrus would induce melanogenesis. Melanin content and tyrosinase expression in mouse B16 melanoma cells were assayed after treatment with citrus plant extracts and their hydrolysates. The results indicate that hydrolysis increased the naringenin content in citrus extracts and that citrus preparations stimulated cellular melanogenesis and tyrosinase expression. It is suggested that this method is applicable to the industrial production of melanin-promoting suntan lotions with antiphotocarcinogenic properties derived from citrus rind and citrus products. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: citrus; naringenin; melanogenesis; tyrosinase; hydrolysate.

INTRODUCTION

Ultraviolet irradiation can damage DNA and other cellular molecules. This damage leads to mutagenesis, carcinogenesis, altered immunological responses and photoaging (Honigsmann, 2002; Garcia-Borron, 2008; Pinnell, 2003). The incidence of skin cancer (melanoma) is increasing at a rate of 3% to 4% per year, faster than any other type of cancer (Howe *et al*., 2001). Melanin plays an important role in the photoprotection of human skin. The pigment is produced via a process called melanogenesis that takes place within melanosomes, specialized intracellular organelles in melanocytes located in the basal layer of the epidermis. Once synthesized, melanin-containing melanosomes move from the perinuclear region to the dendrite tips where they are transferred to keratinocytes by a still not well-characterized mechanism. Melanogenesis is under complex regulatory control by multiple agents. Synthesis starts from the conversion of the amino acid L-tyrosine to dopaquinone by tyrosinase, an enzyme that catalyses the rate-limiting step for melanin biosynthesis (Hearing and Tsukamoto, 1991; Cooksey *et al*., 1997). Other melanogenic enzymes, such as tyrosinase-related proteins 1 (TRP-1) and tyrosinase-related proteins 2 (TRP-2), also regulate melanin synthesis (del Marmol and Beermann, 1996). In melanocytes or melanoma cells, melanogenesis is induced by α-melanocyte-stimulating hormone (α-MSH) (Thody and Graham, 1998). α-MSH binds to its specific receptor (MC1R), resulting in the activation of stimulatory GTP-binding proteins (Gs). This in turn stimulates adenylate cyclase to produce cyclic AMP (cAMP) (Thody and Graham, 1998), which mediates melanogenesis mainly via activation of microphthalmia-associated transcription factor (MITF), thereby inducing melanogenic enzyme expression (Busca and Ballotti, 2000). MITF is a melanocyte-specific transcription factor and plays a crucial role in melanocyte development by binding and activating melanogenic gene promoters, thereby resulting in melanin synthesis (Busca and Ballotti, 2000).

It is widely recognized that most forms of skin cancer can be prevented by limiting exposure to midday sun (10 am to 2 pm), wearing protective clothing and using sunscreen with a sun protection factor (SPF) of at least 15 (American Cancer Society, 2002). Dihydroxyacetone (DHA) is commonly used as an active ingredient in sunless tanning products. The tan is formed through a Maillard reaction between DHA and the amino acids in the dead layer on the skin surface. However, the tan is temporary and will fade gradually with desquamation over 3 to 10 days (Wu *et al*., 2006). An efficient melanin-promoting sunless tanning agent that can maintain a tan for a longer period of time and avoid skin damage due to UV irradiation could be highly beneficial.

Citrus peel contains abundant phenols with antioxidant activity (Xu *et al*., 2007). Naringenin (4′,5,7-trihydroxyflavanone), the aglycone of naringin (Fig. 1), is found in citrus plants and has been reported to inhibit lipopolysaccharide-induced production of NO in microglial cells (Benavente-Garcia and Castillo, 2008) and in endothelial cells (Liu *et al*., 2008). It has also been reported that naringenin can induce melanogenesis in

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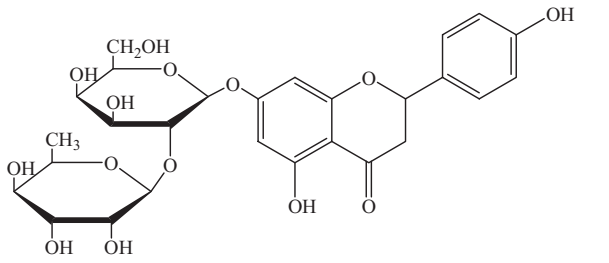
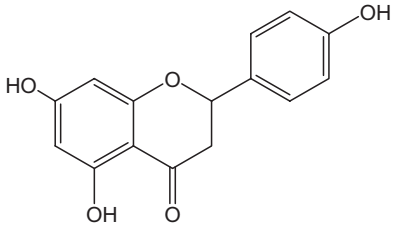


naringin



naringenin

Figure 1. Chemical structures of naringin and naringenin.

mouse B16 melanoma cells by increasing MITF expression (Ohguchi *et al*., 2006). This study attempted to increase the naringenin content in citrus plant extracts by using acid to hydrolyse and break the glycosidic bonds of naringin. The feasibility of this method will be tested using citrus hydrolysates instead of pure naringenin to increase cellular melanogenesis. The results suggest that our citrus hydrolysates, like pure naringenin, increase cellular melanogenesis; moreover, this hydrolysis method can be applied in the development of melanin-promoting suntan lotion for skin cancer prevention or alternative therapy for vitiligo.

MATERIALS AND METHODS

Sample preparation. The rinds from four Rutaceae plants, including *Citrus paradisi* (grapefruit), *Citrus grandis* (pomelo), *Fructus Aurantii Immaturus* and *Fructus Aurantii*, were used in this study. *Citrus paradisi* and *Citrus grandis* were purchased from traditional open-air markets. The edible flesh of *Citrus paradisi* and *Citrus grandis* were removed, and the rind was dried completely. *Fructus Aurantii Immaturus* and *Fructus Aurantii* were purchased from a Chinese herbal medicine retailer.

Dried *Citrus paradisi* and *Citrus grandis* rinds (20 g) were soaked in 800 mL water for 1 h. Dried material of *Fructus Aurantii Immaturus* and *Fructus Aurantii* (20 g) was soaked in 400 mL water for 1 h. Then all of the wetted materials were decocted to reach a volume of 200 mL. After filtration, decoctions were further concentrated to 50 mL in a vacuum under a low degree of heat. The decoction stocks (400 mg/mL) were stored at –20°C until used.

To prepare citrus hydrolysates, the decoctions were hydrolysed in 0.6 N hydrochloric acid. Briefly, the decoction stocks were mixed with an equal volume of 1.2 N HCl and heated at 80°C for 1 h. The hydrolysate was extracted twice with ethyl acetate. The mixtures were shaken vigorously for 10 min and then centrifuged at 9000× **g** for 10 min. The aqueous phase was removed, and the organic solvent layer was evaporated to dryness. The residue was dissolved in dimethyl sulphoxide (DMSO) to obtain citrus hydrolysate stocks (400 mg/mL) with 0.1% DMSO. The hydrolysate stocks (400 mg/mL) were also stored at –20°C until used.

High performance liquid chromatographic (HPLC) analysis. The HPLC system consisted of a Shimadzu LC-10AT pump, a Shimadzu SPD-10AVP photodiode array detector and a sample processor. Samples were separated by a reverse-phase column (Apollo C18, 4.6 × 250 mm, i.d., 5 μm particle size) with a guard column (4.6 × 50 mm, 5 μm particle size) maintained at ambient temperature. The mobile phase, comprising 12% acetonitrile, was filtered through a Millipore 0.45 μm filter and degassed prior to use. The flow rate was 1 mL/min. The detection wavelength was set at 287 nm. 5,7-Dimethoxycoumarin (5,7-DMC) was used as the internal standard. The elution was performed in gradient fashion, starting at 12% acetonitrile for 20 min, increasing acetonitrile to 55% at 45 min, reducing acetonitrile back to 12% at 55 min, and completing the gradient at 12% acetonitrile at the end of 60 min. The column was equilibrated with 12% acetonitrile before the next run.

Calibration standards were individually dissolved in methanol and diluted in series to 100, 50.0, 25.0, 12.5, 6.25 and 3.13 μg/mL, as calibrators. 5,7-DMC (10.00 mg/mL) was spiked into each calibrator as the internal standard. Based on the calibration curves, the linear regressions and correlative coefficients were determined. For validation, the intraday assays were determined by quantifying three replicates on the same day; the interday assays were carried out on three consecutive days. The real concentrations were calculated from standard curves and used to calculate the standard deviation (SD) as a measure of accuracy and coefficient of variation (CV), as a measure of precision.

Cell culture. B16 murine melanoma cells (purchased from the Food Industry Research and Development Institute, Taiwan) were cultured in DMEM containing 10% fetal bovine serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.25 μg/mL amphotericin B at 37°C in a humidified 95% air/5% CO_2 atmosphere. Drug treatment began 24 h after seeding. The cells were harvested 3 days later, and the melanin content was determined in triplicate for each treatment (Chiang *et al*., 2010).

MTT assay. The viability of cultured cells was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described previously (Mosmann, 1983). After treatment, the cells were washed twice with phosphate-buffered saline (PBS). Each well of a 96-well plate was seeded with 1 × 10^4 cells and incubated overnight. After attachment, 50 μL of sample was added and incubated for 24 h. MTT (100 μg/0.1 mL PBS) was then added to each well. After incubation at 37°C for 3 h, 75 μL of SDS solution (10% SDS in 0.1 N HCl) was added to dissolve the formazan crystals. The absorbance was then measured at 570 nm with an ELISA reader (Multiskan Ascent).

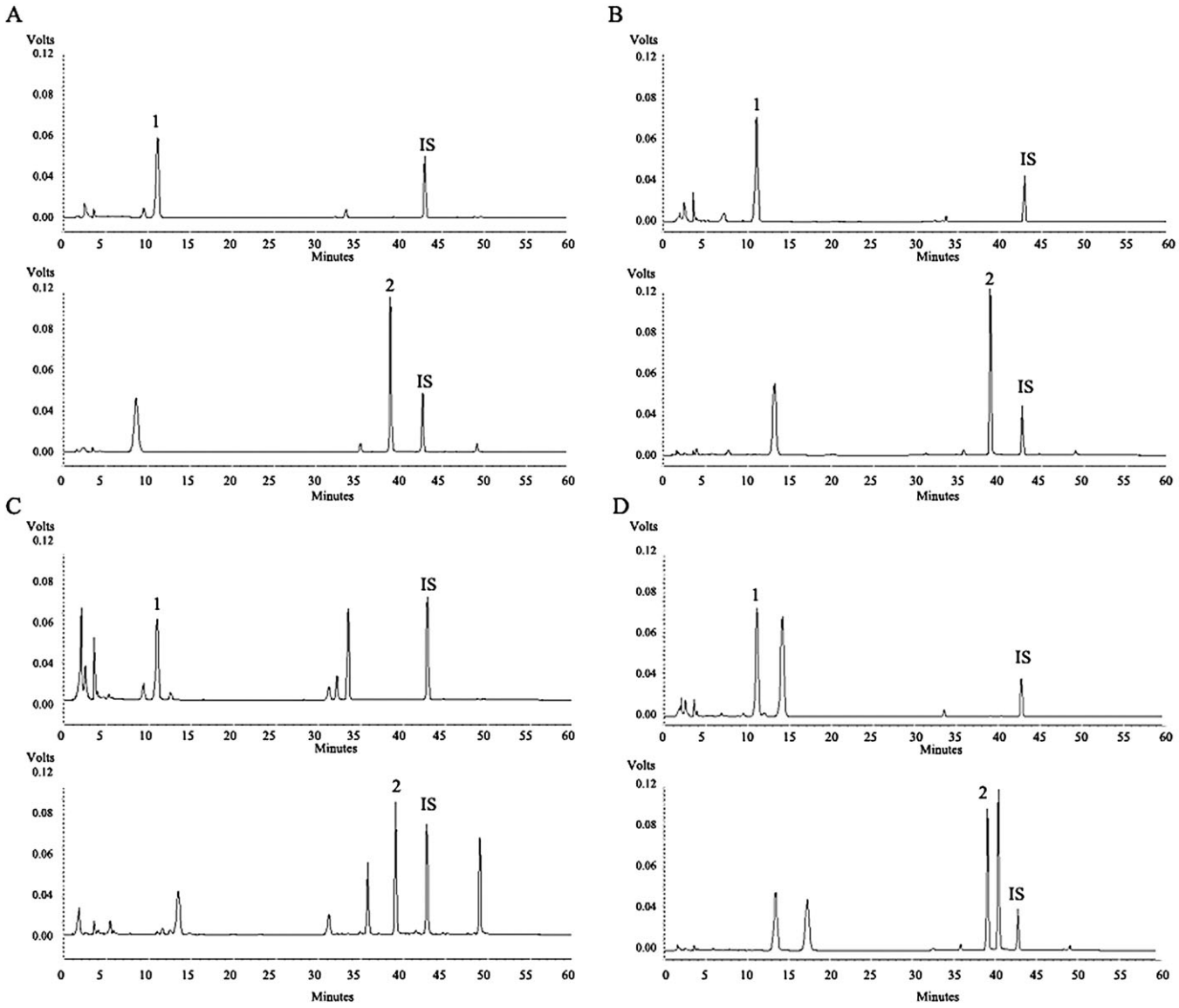


Figure 2. Typical chromatograms for the decoctions and their hydrolysate extracts of *Citrus paradisi* (A), *Citrus grandis* (B), *Fructus Aurantii Immaturus* (C) and *Fructus Aurantii* (D). In the panels, the top graphs are chromatograms for the citrus decoctions, and the bottom graphs are chromatograms for their hydrolysate extracts. The peak of naringin is labeled peak 1 (ca. 11 min). The peak of naringenin is labeled peak 2 (ca. 39 min). For the internal standard (IS), 5,7-dimethoxycoumarin (5,7-DMC) was used. Peak identification: IS, 5,7-DMC; 1, naringin; 2, naringenin.

Melanin content measurement. Each well of a 6-well plate was seeded with 5×10^4 cells and incubated overnight for cell attachment. After seeding, the medium was removed, and 3 mL of sample-containing medium was added and incubated at 37°C. Three days later, 0.4 mL trypsin was added to each well. The melanin content of the cultured B16 cells was measured as described previously (Jung *et al.*, 2001; Chiang *et al.*, 2010). The cells were washed twice with PBS and then collected. The melanin was precipitated with 10% trichloroacetic acid (TCA). The cell lysates were centrifuged at 3000× **g** for 5 min. After washing twice with 10% TCA, the pellets were air-dried, dissolved in 250 μL of 1 N NaOH, and incubated at 80°C for 30 min. After cooling, the absorbance was measured with a spectrophotometer at 405 nm. The amount of cellular melanin was corrected according to the protein content of the samples. The protein concentration was determined using the Bradford method (Bio-Rad, Hercules, CA, USA).

Western blot analysis. Seeding and drug treatment in the 6-well plate experiment were the same as described above. After 3 days of incubation with drugs, B16 cells were lysed in lysis buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 2 mM phenylmethylsulphonyl fluoride (PMSF)) and 10 mM ethylenediamine tetraacetic acid (EDTA) (Choi *et al.*, 2006). Lysates were then centrifuged at 20000× **g**, and supernatant fractions were collected. One hundred micrograms of protein per lane was separated in 10% SDS-polyacrylamide gels, transferred to PVDF membranes and then blocked with 5% dried milk in Tris-buffered saline (TBS) containing 0.1% Tween-20. Tyrosinase and actin were detected using Santa Cruz antibodies TYR (C-19) and actin (I-19), respectively. The blots were incubated with appropriate primary antibodies, followed by horseradish peroxidase-conjugated secondary antibody (sc-2378, Santa Cruz) and detected by enhanced chemiluminescence (ECL) (Amersham Biosciences).

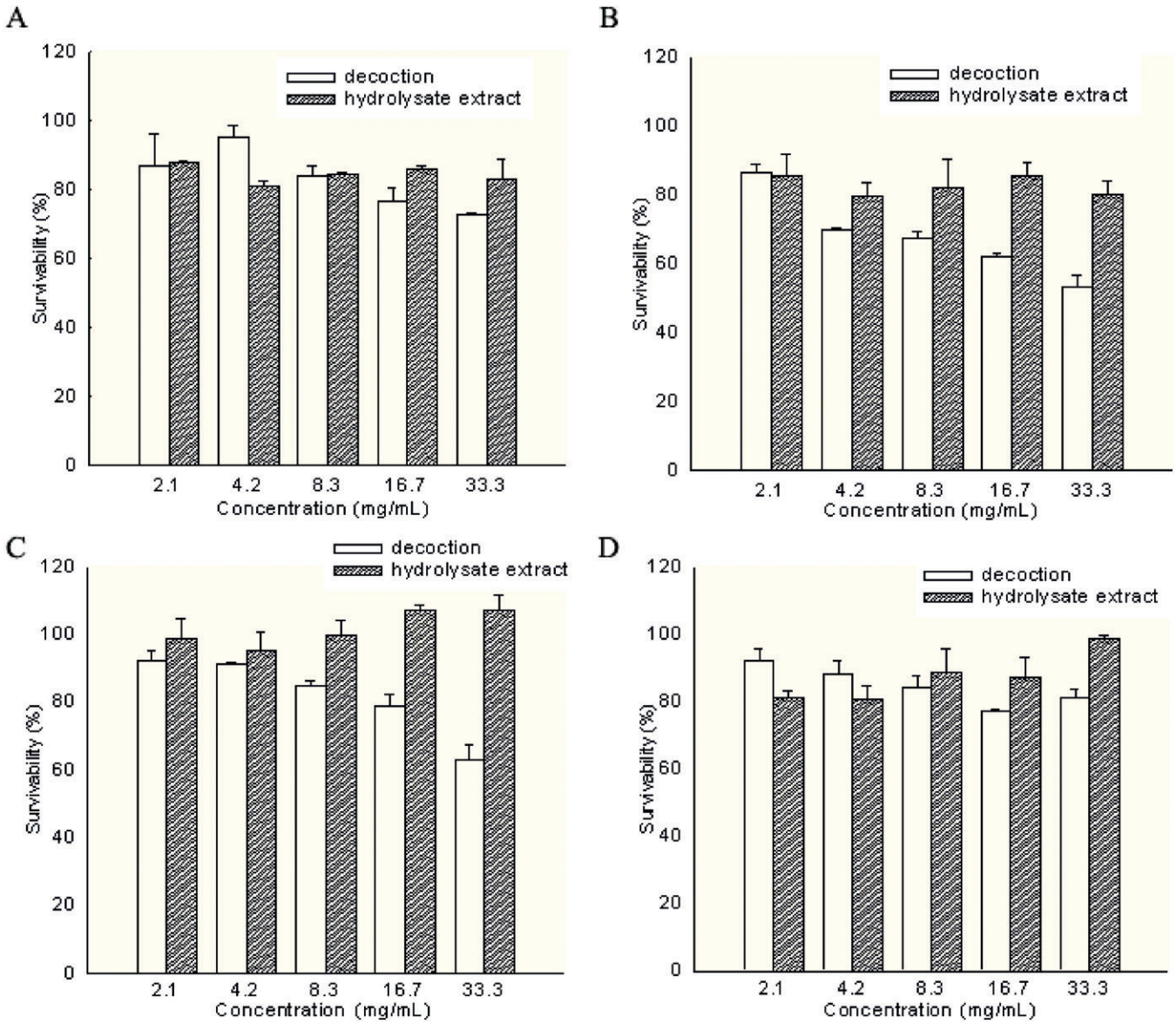


Figure 3. Effects of the citrus decoctions and their hydrolysate extracts on the survivability of B16 melanoma cells. Cells were treated with decoctions and hydrolysate extracts of *Citrus paradisi* (A), *Citrus grandis* (B), *Fructus Aurantii Immaturus* (C) and *Fructus Aurantii* (D) for 24 h. Cell survivability was determined by the MTT assay and the results are presented in the histograms. The values of cell survivability (%) were determined for the treatments of serial concentrations (mg/mL). The open columns represent decoction-treated cells, and the shadowed columns represent the hydrolysate extract-treated cells. Each point represents the mean ± SD, $n = 3$.

Table 1. The contents (μg/mL) of glycosides and aglycones in the four citrus decoctions and their hydrolysates

	Citrus paradisi		*Citrus grandis*		*Fructus Aurantii Immaturus*		*Fructus Aurantii*	
	Decoction	Hydrolysate	Decoction	Hydrolysate	Decoction	Hydrolysate	Decoction	Hydrolysate
Naringin	66.5 ± 1.6	–	118 ± 5.0	–	38.5 ± 1.1	–	116 ± 0.8	–
Naringenin	–	132 ± 3.5	–	169 ± 7.8	–	–	–	154 ± 4.0
Hesperidin	–	–	–	–	–	60 ± 2.1	5.6 ± 0.1	–
Hesperitin	–	–	–	–	–	–	–	175 ± 3.5

–, non-detected.

RESULTS

Acid hydrolysis of naringin

The HPLC chromatograms of naringin, naringenin and 5,7-DMC are shown in Fig. 2. Panels show the results of hydrolysis of naringin in *Citrus paradisi* (A), *Citrus grandis* (B), *Fructus Aurantii Immaturus* (C) and *Fructus Aurantii* (D). The upper and lower graphs illustrate the HPLC results from the decoction and the hydrolysate, respectively. According to the standard peaks (data not shown), the peak numbered 1 (retention time about 11 min) is naringin and the peak numbered 2 (retention

time about 39 min) is naringenin. Naringenin, naringin and the internal standard were well resolved within 1 h without any interference. The concentration–response relation of the method indicated that the linearity of these compounds (range 3.13–100 μg/mL) was excellent. Validation of this assay method indicated that all coefficients of variation for intraday and interday analysis were less than 10% and the relative errors were below 15% (data not shown). The contents of naringin and its cognate aglycone–naringenin, heperidin and its aglycone hesperitin in these four citrus decoctions and hydrolysates are shown in Table 1. Naringenin was not detectable in the decoction of *Citrus paradisi*; however, a strong naringenin peak was detected in the hydrolysate of *Citrus paradisi*. Similar results were obtained for *Citrus grandis*, *Fructus Aurantii Immaturus* and *Fructus Aurantii*. All the investigated decoction specimens contained naringenin under our detection limit, and their hydrolysates contained a considerable amount of naringenin.

Citrus hydrolysates have low cytotoxic activity

The MTT assay revealed that all the citrus hydrolysates (shadowed columns) were only slightly toxic to B16 cells (Fig. 3). The cell viability was greater than 80% in all citrus hydrolysate-treated cells (ca. 30 mg/mL). In contrast, the decoction cognates (open columns) were more toxic to the cells than the hydrolysates. The cell viability ranged from 50% to 80% in cells treated with decoction.

Citrus hydrolysates enhanced the synthesis of melanin in B16 cells

Briefly, B16 cells were treated with hydrolysate or decoction (5, 10 and 20 mg/mL) or 0.1% DMSO (blank control) for 3 days. The melanin content was measured thereafter and normalized to total protein (μg melanin/mg protein). The percentage of melanin synthesis relative to that of the blank control was calculated for both decoction-treated cells and hydrolysate-treated cells. The results are shown in Table 2 and Fig. 4. As expected, none of the citrus decoctions enhanced melanin synthesis at any concentration. In contrast, all of the citrus hydrolysates enhanced melanin synthesis. Citrus hydrolysates at 10 mg/mL enhanced melanin synthesis from 138% to 173% relative to the blank control (Table 2); however, there was no further enhancement at 20 mg/mL treatment.

Citrus hydrolysates increased tyrosinase expression in B16 cells

The effects of the citrus decoction and citrus hydrolysate extracts on melanin biosynthesis in mouse B16 cells are shown in Fig. 4. The levels of melanin stimulated by hydrolysates were higher than those stimulated by decoction. A specific anti-tyrosinase antibody was used to detect the level of tyrosinase expression in drug-treated (10 mg/mL) cells and in blank-treated cells. The housekeeping gene, β-actin, was used as the internal control. The level of β-actin expression did not change

Table 2. Effects of the four citrus decoctions and their hydrolysates on the synthesis of melanin

	Melanin levels											
	Citrus paradisi			*Citrus grandis*			*Fructus Aurantii Immaturus*			*Fructus Aurantii*		
	Decoction		Hydrolysate	Decoction		Hydrolysate	Decoction		Hydrolysate	Decoction		Hydrolysate
Concentration (mg/mL)	(μg melanin/mg protein)[a]	%[b]	(μg melanin/mg protein)	%	(μg melanin/mg protein)	%	(μg melanin/mg protein)	%	(μg melanin/mg protein)	%	(μg melanin/mg protein)	%	(μg melanin/mg protein)	%
0	11.7 ± 1.2	100	9.8 ± 0.2	100	13.9 ± 0.8	100	14.0 ± 1.0	100	16.6 ± 1.4	100	17.1 ± 1.2	100	11.1 ± 1.2	100	11.9 ± 0.5	100
5	13.5 ± 1.2	115	18.4 ± 0.9	187	15.1 ± 0.8	109	18.6 ± 2.4	133	13.5 ± 0.7	81	22.0 ± 1.4	129	10.5 ± 0.5	95	19.9 ± 1.3	167
10	11.8 ± 0.7	101	16.1 ± 0.4	164	11.8 ± 0.7	85	22.1 ± 2.0	158	12.4 ± 0.7	75	23.6 ± 1.1	138	10.0 ± 0.2	90	20.6 ± 0.7	173
20	9.0 ± 0.9	77	14.4 ± 1.0	147	11.5 ± 1.3	83	22.0 ± 0.2	157	13.4 ± 2.5	81	18.8 ± 1.1	110	8.7 ± 1.4	78	16.4 ± 1.4	138

[a] The levels of melanin were normalized to that of total proteins. The unit of melanin synthesis is represented as μg of melanin per mg of protein.
[b] The effect of treatments on melanin synthesis is represented as a percentage to the zero concentration of drug treatment.

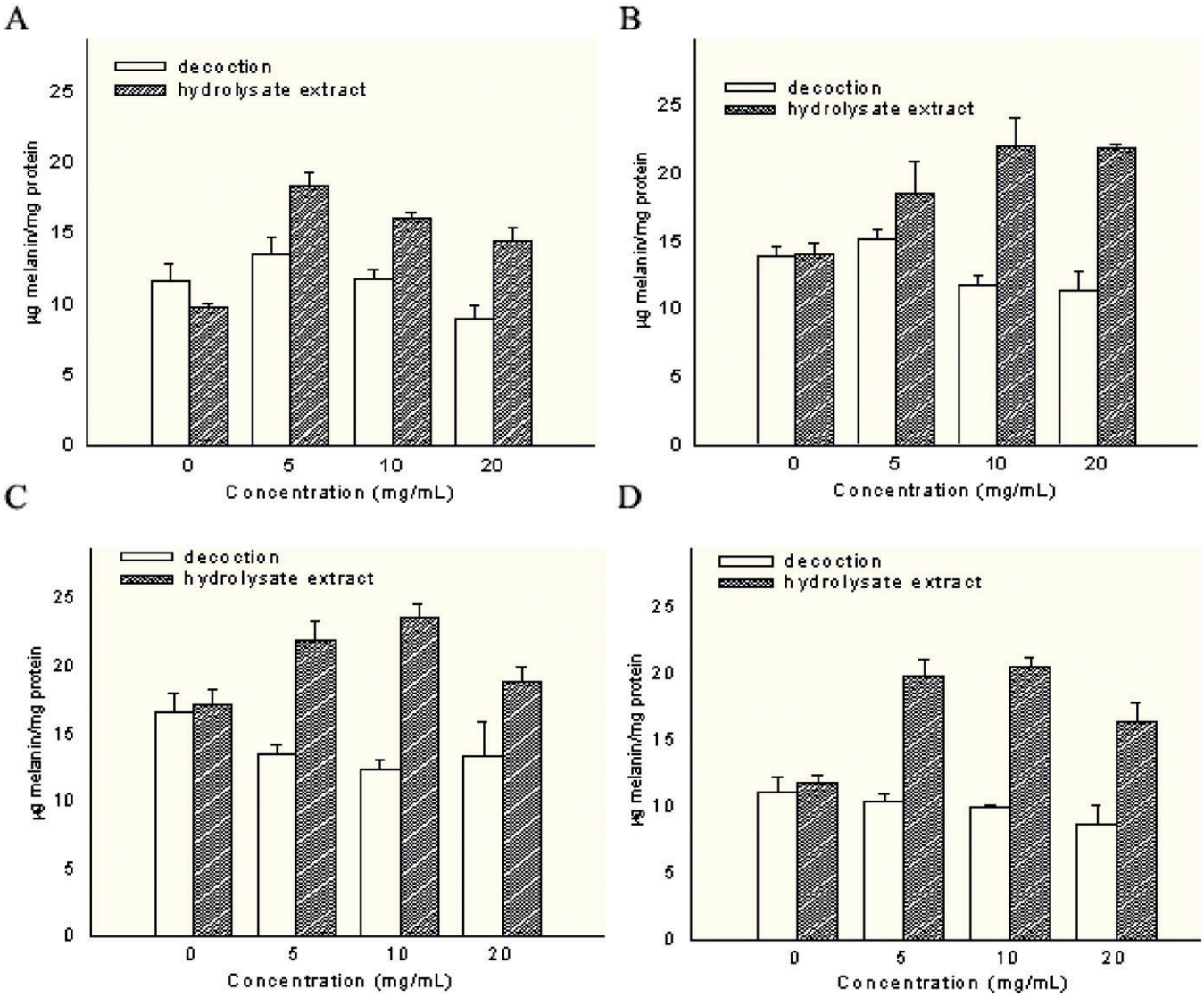


Figure 4. Effects of the citrus decoctions and their hydrolysate extracts on melanin biosynthesis in B16 cells. Cells were treated with decoctions and hydrolysate extracts of *Citrus paradisi* (A), *Citrus grandis* (B), *Fructus Aurantii Immaturus* (C) and *Fructus Aurantii* (D) for 3 days. The quantity of melanin was normalized to that of protein and presented in histograms. The melanin levels (μg melanin/mg protein) were determined for the treatment of serial concentrations (mg/mL). The open columns represent the citrus decoction-treated cells, and the shadowed columns represent the hydrolysate extract-treated cells. Each point represents the mean ± SD, $n = 3$.

in any of the treatment groups (Fig. 5). Several glycosylated forms (between 60 and 75 kDa) of tyrosinase were detected in the western blot, including a highly glycosylated form (the upper major band) and slightly glycosylated forms (lower doublet). Here, the sum of each form was used to represent the expression level of tyrosinase. Relative to the blank control (lane 1), all of the citrus hydrolysates stimulated the expression of tyrosinase. In cells exposed to hydrolysates of *Citrus paradisi*, the expression level increased by 217%; expression of tyrosinase in cells exposed to hydrolysates of *Citrus grandis* increased by 199%; in cells exposed to hydrolysates of *Fructus Aurantii Immaturus*, the expression level increased by 114%; and expression of tyrosinase in cells exposed to hydrolysates of *Fructus Aurantii* increased by 151%. Interestingly, in *Citrus grandis* decoction-treated cells, the proportion of the highly glycosylated form of tyrosinase decreased (Fig. 5, compare lanes 1 and 4), even though the total expression level was higher.

DISCUSSION

Four Rutaceae plants, including two edible fruits, grapefruit (*Citrus paradisi*) and pomelo (*Citrus grandis*) and two traditional Chinese herbs (*Fructus Aurantii Immaturus* and *Fructus Aurantii*) were investigated in this study. According to our previous studies (Chiang *et al*., 2007, 2009) and other reports (Kuo *et al*., 2006; Wu *et al*., 2007), the content of aglycones can be increased by using acid to hydrolyse cognate glycones. A hydrolysis reaction condition with 0.6 N HCl (final concentration) at 80°C for 1 h is optimal in terms of acid usage, energy consumption and time. The HPLC analysis showed the effectiveness of this hydrolysis condition Fig. 2 and Table 1. The same concentrations of decoctions and hydrolysates were analysed by HPLC. Naringenin was not detectable in these four citrus decoctions; however, a strong naringenin peak signal was detected in the hydrolysates. These results suggest that naringin,

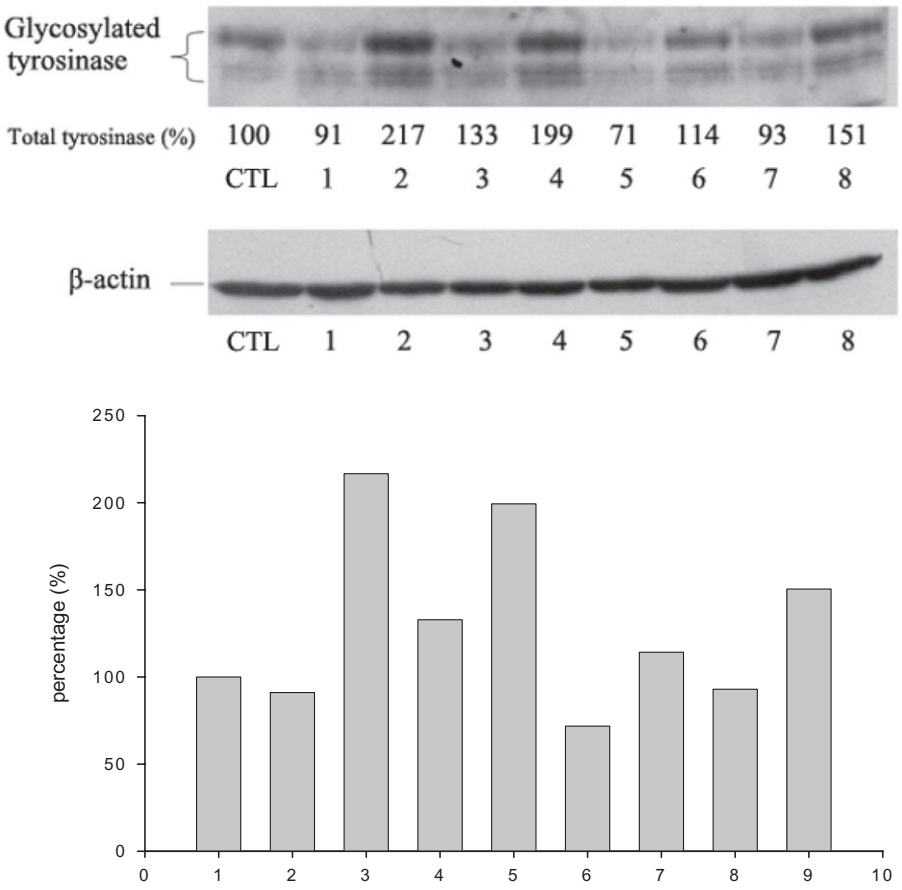


Figure 5. Effects of the citrus decoctions and their hydrolysate extracts on tyrosinase expression in B16 cells. Cells were treated with 10 mg/mL citrus decoction or hydrolysate extract for 3 days. As described in the materials and methods section, 100 μg of protein per lane was subjected to western blot analysis using the antibodies indicated at the left of each panel. Equal protein loadings were confirmed using anti-actin antibody. Odd lanes show the citrus decoction-treated cells (1, *Citrus paradisi*; 3, *Citrus grandis*; 5, *Fructus Aurantii Immaturus*; 7, *Fructus Aurantii*). Even lanes show the hydrolysate extract-treated cells (2, *Citrus paradisi*; 4, *Citrus grandis*; 6, *Fructus Aurantii Immaturus*; 8, *Fructus Aurantii*). CTL; blank control (0.1% DMSO).

rather than naringenin, dominates in these plants and that our hydrolysis method is efficient in increasing the naringenin content by hydrolysing naringin. The peak areas of naringenin, naringin, hesperitin and hesperidin standards showed no significant loss when stored in methanol at 4°C during the assay (almost 4 weeks). In addition, the extract was concentrated and stored at –20°C until used. The sample was kept at 4°C during the study. The peak area of the flavonoids in the extract showed 90% of the first day after 1 week. Xie *et al.* reported that naringenin extracted from rat plasma was stable for 2 weeks when stored at 4°C in the dark and that the processed samples experienced no significant loss at 4°C for 3 days (Xie *et al.*, 2004). Kanaze *et al.* showed that free hesperetin and naringenin in human urine samples were stable through three freeze–thaw cycles and at room temperature for 24 h. The loss was less than 8% (Kanaze *et al.*, 2004). These findings suggest that those flavonoids are stable and that some techniques (e.g. liposome, multi-layered microcapsules) may help to preserve the stability of these constituents in formula for topical use.

In addition to naringenin, citrus hydrolysates contain many other components that may be toxic to cells. To test whether these hydrolysates can be applied directly to cells without further purification, the survivability of the murine B16 cells exposed to each hydrolysate was determined by the MTT assay. The results revealed that all of the citrus hydrolysates possessed low cytotoxic activity and that the decoctions were more toxic than the hydrolysates. Overall, the results indicated that at least 30 mg/mL of citrus hydrolysates can be applied to B16 cells.

Naringenin, an aglycone of naringin found in citrus plants, has been reported to induce melanogenesis more effectively than naringin in mouse B16 melanoma cells by increasing the expression level of MITF, which modulates tyrosinase expression (Ohguchi *et al.*, 2006). Therefore, the melanin-promoting potential of these citrus hydrolysates was investigated. Briefly, B16 cells were treated with hydrolysates or decoctions (5, 10 and 20 mg/mL) and melanin synthesis was measured. No citrus decoctions enhanced melanin synthesis at any concentration, but all of the citrus hydrolysates enhanced melanin synthesis. Citrus hydrolysates at 10 mg/mL enhanced melanin synthesis; however, there was no further enhancement at 20 mg/mL treatment. This is probably due to the complexity of the components in these specimens. The results suggest that there is an optimal concentration (10 mg/mL) of a citrus hydrolysate to stimulate cellular melanogenesis.

The enhancement of cellular melanin synthesis by citrus hydrolysates (Fig. 4) could be due to the effect of naringenin. Since naringenin can enhance melanin synthesis in murine B16 cells by increasing the expression

level of tyrosinase (Ohguchi *et al*., 2006), a specific anti-tyrosinase antibody was used to detect the expression of tyrosinase in murine B16 cells. It has been reported that tyrosinase is glycosylated during its maturation process (Negroiu *et al*., 1999). In this study, several glycosylated forms of tyrosinase including a highly glycosylated form and a slightly glycosylated form were detected in the western blot. The sum of all forms of tyrosinase was used to represent the expression level of the tyrosinase. The results showed that all of the citrus hydrolysates stimulated total tyrosinase expression. However, the proportion of the highly glycosylated form of tyrosinase decreased in *Citrus grandis* decoction-treated cells, even though the total expression level was higher. These results suggest that these naringenin-containing hydrolysates can stimulate cellular melanin synthesis and that some citrus decoctions may slightly reduce the expression of tyrosinase or even affect the maturation of tyrosinase.

It was found that acid hydrolysis (0.6 N HCl) of naringin markedly increased the naringenin content in the decoctions of citrus plants. These naringenin extracts were shown to enhance melanin synthesis in B16 cells. Since naringenin, rather than naringin, is the form that is more absorbable by cells due to its more hydrophobic nature and therefore greater ability to penetrate the cell membrane, it can be inferred that the naringenin extract can be directly applied on the target area of the skin to penetrate into the cells beneath, where it leads to a UV-independent stimulation of melanogenesis. Besides, there is no photosensitivity concern caused by bergapten which is found in citrus essential oil, because the preparation of citrus plants soaked and decocted by water did not contain oil-soluble bergapten. We suggest that our method is applicable for an industrial-scale preparation of naringenin-enriched extract from waste materials such as grapefruit rind. Furthermore, citrus-containing products promote melanin synthesis by stimulating tyrosinase expression and, therefore, might be good candidates for developing agents with antiphotocarcinogenic properties or alternative therapy for vitiligo.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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