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감귤 추출물의 미백효능 및 항산화 효능에 관한 연구

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The Study on the Whitening Effects and Antioxidant Activity of Various Citrus Fruits

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요 약: 본 연구에서는 제주도 자생 감귤 17종에 대하여 탈색소효과(depigment effect)를 연구하였다. B16 멜라닌 생성세포(B16 melanoma cell)를 이용하여 tyrosinase 및 멜라닌 생성억제 효과를 연구한 결과 대조군 대비하여 농도 의존적으로 감소하는 3종의 미성숙 온주밀감(immature *Citrus unshue*), 팔삭(*Citrus hassaku*), 진지향(*Citrus sinensis* × *reticulata*)을 도출하였다. 또한, 이와같이 도출된 감귤 추출물을 이용하여 멜라닌 세포 생성(melanogenesis)에 영향을 미치는 tyrosinase, DOPAchrome tautomerase (TRP-2)와 DHICA oxidase (TRP-1) 단백질 발현을 실험한 결과 tyrosinase, TRP-1, TRP-2 단백질 발현이 감소함을 나타내었다. 특히 이들 중 미성숙 온주밀감은 tyrosinase와 TRP-1 단백질 발현에서 유의적으로 감소하는 결과를 보였다. 본 연구 결과로써 미성숙 온주밀감은 모든 실험에서 우수한 효과를 나타내어 UV에 의해서 야기되는 피부 색소 침착을 방지할 수 있는 물질로서 기대할 수 있다.

Abstract: We examined the depigmentation effect on Korean traditional citrus 17 species. With B16 melanoma cells, we have seen inhibition of the tyrosinase and melanin formation, which eventually were dose dependently decreased by three citrus fruits, immature *Citrus unshiu, Citrus hassaku*, and *Citrus sinensis* × *reticulata* as compared with positive control. Also, we examined expression of tyrosinase, DOPAchrome tautomerase (TRP-2), and DHICA oxidase (TRP-1) which affect melanin synthesis. Especially, immature *Citrus unshiu* decreased the protein levels of tyrosinase and TRP-1. In conclusion, immature *Citrus unshue* showed the strongest activity in all the experiments mentioned above and we expect that it can be used for preventing UV-induced pigmentation.

Keywords: *malanin, antioxidative, TRP-1, TRP-2, depigment, immature Citrus unshiu, Citrus hassaku*

1. Introduction

Melanin is a major pigment for color of human skin. The melanin synthesis inhibitors have been of interest as target molecules of natural product chemistry because they are related to localized hyperpigmentation in humans such as lentigi, nevus, epheils, post-inflammatory state and melanoma of pregnancy. Epidermal and dermal hyper-pigmentation can be dependant on ethier an increased number of melanocytes or activity of melanogenic enzymes[1].

Melanin biosynthesis is regulated by melanogenic enzymes such as tyrosinase, tyrosinase-related protein 1 (TRP-1), and tyrosinase-related protein 2 (TRP-2)[2]. Tyrosinase is bifunctional enzyme that plays a pivotal role in the modulation of melanin production first by catalyzing the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and second by catalyzing

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the oxidation of DOPA to DOPAquinone[3]. TRP-2, which functions as DOPAchrome tautomerase, catalyzes the rearrangement of DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA), and TRP-1 oxidizes DHICA to a carboxylated indole-quinone [4,5].

Melanin biosynthesis can be inhibited by avoiding ultraviolet (UV) exposure, by inhibition of melanocyte metabolism and proliferation, by inhibition of tyrosinase, or by removal of melanin by corneal ablation. Apart from avoiding UV exposure, application of tyrosinase inhibitors may be the least invasive procedure for maintaining skin whiteness; such agents are increasingly used in cosmetic products[6,7].

Oxidative stress may be induced by increasing generation of ROS and other free radicals. UV radiation can induce formation of ROS in skin such as singlet oxygen and superoxide anion, promoting biological damage in exposed tissues via iron-catalyzed oxidative reactions[8]. These ROS enhance melanin biosynthesis, DNA damage, and may induce proliferation of melanocytes. A previous study also found evidence for a role of oxidative stress in pathogenesis of skin disorders[9]. It is known that ROS scavengers or inhibitors such as antioxidant may reduce hyper-pigmentation[10].

Citrus fruits are a dietary source of abundant antioxidant and have been used in traditional medicine in Korea, Japan and China. Many of the chemical contained in citrus fruits have been reported to possess biological activities. In particular, the citrus fruit peel has been shown to be more efficient than corresponding juice-containing portions at eliminating free radical species[11]. This study is continuation of our efforts to evaluate natural products for possible radical scavenging activity that could be used in pharmaceutical and cosmetic formulation (mainly whitening).

2. Materials and methods

2.1. Reagents

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, sodium nitroprusside (SNP), xanthine, xanthine oxidase (XO) grade I from buttermilk (EC 1.1.3.22), phenazine methosulfate (PMS), nicotinamide adenine dinucleotide (NADH) and nitroblue tetrazolium chloride (NBT), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), trypsin-EDTA, synthetic melanin, melanocyte stimulating hormone (MSH), theophylline, sodium phosphate, pyrocatechol, Triton X-100, sodium hydroxide were purchased from Sigma-Aldrich (USA). Fetal bovine serum (FBS), penicillin-streptomycin was purchased from Gibco BRL Div. of Invitrogen (USA) and Dulbecco's modified Eagle's medium (DMEM), BCA protein assay reagent kit were purchased from Cambrex Bioscience Walkersville (USA), Pierce Biotech (USA) respectively.

All organic solvents were used of analytical grade and purchased from Fisher scientific (Loughborough, Leics, UK)

2.2. Plant Materials and Preparation Extracts

The various citrus fruits were collected from the Jeju High-Tech Industry Development Institute of Jeju-island (South Korea). The dry powdered pees of fruits were pulverized in a grinder and extracted with 70 % ethanol solution at room temperature for 3 days and then filtered. The procedure was repeated two times. The filtrates were combined and were concentrated under reduced pressure, freeze-dried, and stored in a closed container until use.

2.3. Assay of Cellular Tyrosinase Activity

B16 melanoma cells were cultured for 3 days with various concentrations of citrus fruits extract. The cells (about 1.5×10^6 cells/well) were collected by centrifugation and washed with ice-cold PBS, resuspended in 1 mL of homogenization buffer (50 mM sodium phosphate, pH 6.8, 1 % Triton X-100, 2 mM PMSF) and homogenized by ultrasonication. The cell lysate was centrifuged at $15{,}000 \times g$ for 30 min at 4 ℃ and resulting supernatants were used for tyrosinase assay and protein determination. Tyrosinase activity was measured as follows. The reaction mixture contained 50 mM sodium phosphate (pH 6.8), 0.3 mL of 1.5 mM tyrosine, 30 μL of 0.06 mM DOPA and 0.3 mL of cell lysate. The reaction mixtures were incubated at 37 ℃ for 30 min and absorbances were measured at 475 and 490 nm using an ELISA (Benchmark, BIO-RAD, Japan) reader.

Inhibition rate of tyrosinase activity (%)

$$= \{(A-B) / A\} \times 100$$

A : mean O.D. value of negative control

B : mean O.D. value of sample

2.4. Measurement of Melanin Content in Melanocytes

We replaced the medium with new DMEM medium containing citrus extracts of various concentration. After 5 days, we washed the cells with phosphate buffer saline (PBS) and collected the cells by trypsinization and centrifugation. We separated melanin from the pellet of the cells using 5 % trichloroacetic acid and dissolved the melanin in 1 N NaOH solution. We determined the melanin contents with an absorbance at 475 and 490 nm. The optical density (O.D.) of each supernatant was measured at 475 and 490 nm using an ELISA reader. A standard curve for melanin determination was prepared using synthetic melanin. The cell number was determined with the counter. Our citrus fruit extracts have an inhibitory effect on melanogenesis.

Inhibition rate of melanin formation (%)

$$= \{(A-B) / A\} \times 100$$

A : mean O.D. value of negative control
B : mean O.D. value of sample

2.5. Western Blot Analysis

The protein was extracted using cold RIPA buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 % NP40, 5 mM NaF, 0.1 mM PMSF, proteinase inhibitor cortail) by separating melan a cell from test phase and control phase. And separated protein from 10 % SDS-PAGE and transferred to nitrocellulose membrane and the primary antibody was diluted in 1,000 fold and conjugated in 3 % BSA reagent (1 × TBS; 0.05 % Tweens, 20 mM Tris-HCl, 150 mM NaCl, pH 7.5) overnight. The treatment of second antibody which HRP is conjugated was diluted in 5 % milk reagent in 2,000 fold for 1 h at a room temperature. And the membrane was cleansed using No. 3 TBS reagent and the band was traced using ECL kit. On the moment, the x-ray film was contacted to membrane for 1 to 3 min and developed in the automatic film development device. For the primary antibody, anti-tryrosinase and TRP-1, 2 were used and for band detection, ECL detection kit was used (Amersham, UK).

2.6. Scavenging Effects on DPPH Radicals

DPPH radical-scavenging assay was carried out the following procedure. The reaction mixture containing various concentrations of the test samples and DPPH methanolic solution (0.2 mM) was incubated at room temperature for 30 min and the absorbance was measured at 517 nm. The scavenging activity was expressed as a percent compared to control DPPH solution (100 %). The trolox and L-ascorbic acid were included in experiments as a positive control.

2.7. Scavenging Effects on Hydroxyl Radical

The hydroxyl radical was generated by the Fenton reaction using the luminescence method as modified by Cheng *et al.* The reaction mixture contained 40 mM luminol, 4.17 mM phosphate buffer (pH 7.5), 4.6 mM Fe(III) 2.3 mM EDTA, test preparation, and 96 mM H_2O_2. The chemiluminescent reaction proceeded in KH_2PO_4-NaOH buffer solution (pH 7.5) at room temperature. Initiation of reaction was achieved by adding Fe(III) EDTA and then H_2O_2 into the mixture. Luminescence intensity was monitored in the wavelength range of 200 ~ 900 nm.

2.8. Scavenging effects on Nitrite Oxide

The scavenging effects of citrus fruit extracts on NO were measured according to the method of Marcocci *et al.* A total of 4 mL of citrus fruit extract solution was added in the test tubes to 1 mL of SNP solution (25 mM) and the tubes were incubated at 37 ℃ for 150 min. An aliquot (0.5 mL) of the incubation solution was removed and diluted with 0.3 mL of Griess reagent (1 % sulfanilamide in 5 % H_3PO_4 and 0.1 % naphthylethylenediaminedihydro chloride (NEDD)). The absorbance of the chromo phore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with NEDD was immediately read at 550 nm and referred to the absorbance of standard solutions of sodium nitrite salt treated in the same way with Griess reagent.

Scavenging effect (%) = [1 - (nitrite concentration of the sample with 5 mM SNP / nitrite concentration of the control)] × 100.

3. Results

Therefore, we evaluated their effects on tyrosinase and melanin content in B16 melanoma cells, as well as their antioxidant activities. The 17 selected citrus fruit were extracted with 70 % ethanol. In the present study, we used B16 melanoma cells as an *in vitro* model because of the need to measure cytotoxic effects. An MTT assay for cytotoxicity was employed before further *in vitro* testing in B16 melanoma cells was done to test tyrosinase inhibition and melanin content. Among the 17 tested extracts showed relatively lower cytotoxicity, with cell viability above 80 % with a concentration of 50 μg/mL.

3.1. Effect of Citrus Fruit Extracts on Tyrosinase Activity in B16 Melanoma Cells

Recently, natural substances have been in increased demand in the global market for new agents for depigmenting, cosmeceutical and skin-lightening purposes. Tyrosinase inhibitors are important constituents of cosmetics and skin-lightening agents. Among extracts, *Citrus hassaku* (20.53 %) and *Citrus erythrosa* (20.88 %) showed potent tyrosinase inhibitory effects. In comparison, arbutin a naturally occurring cosmetic vehicle and whitening agent. Thus, our tested extracts exhibited greater inhibitory activity than arbutin.

3.2. Effect of Citrus Fruit Extracts on Melanin Formation of B-16 Melanoma Cells

In melanogenesis, the proximal pathway consists of the enzymatic oxidation of tyrosine or *l*-DOPA to its corresponding *o*-DOPAquinone catalyzed by tyrosinase. After multi-biosynthesis steps, further polymerization yields melanin. In the present study, normal human melanocytes were used for determination of extract effects and cellular melanin content (Table 1). The most active anti-tyrosinase extracts, immature *Citrus unshiu* (34.68 %), *Citrus hassaku* (33.28 %), and *Citrus sinensis* × *reticulata* (35.49 %) show inhibition of melanin formation. Thus, our tested extracts exhibited greater inhibitory activity than arbutin.

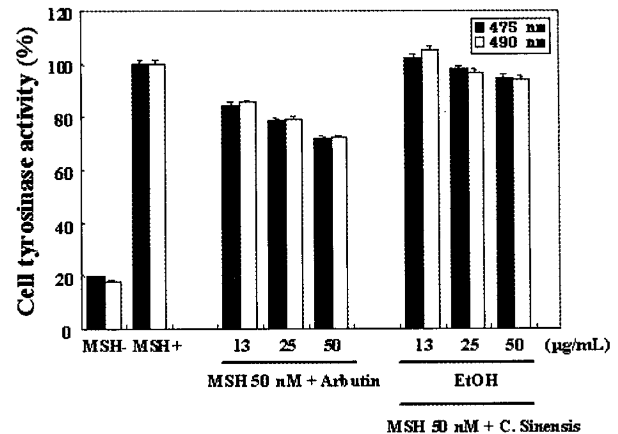

Figure 1. Cell tyrosinase activity assay of *Citrus sinensis* × *reticulata* extracts in B16/F10 melanoma cells.

3.3. Effect of Protein Expression Inhibition of Tyrosinase, ERK 1/2, TRP-1, 2 and α-MSH Induced Pigmentation

To scrutinize furtherthe role of tyrosinase in B16 cells treated with three citrus fruit extracts (13 ~ 50 μg/mL), the level of protein expression was determined. To examine the action of three citrus fruits extract on skin pigmentation, we investigated its effects in B16 cells stimulated with α-MSH which are the best characterized biologically relevant agents inducing melanigenesis. After 48 h incubaton with α-MSH, tyrosinase activity increased dose-dependently, respectively, compared with controls.

As a result of conditioning 50 μg/mL of citrus fruit ethanol extract, it did not show the whitening effect at the level of albutin (Figure 2). Also, it was analyzed of the expression of ERK 1/2, tyrosinase, TRP-1, 2 proteins using western blot and as a result the tyrosinase and TRP-1 protein showed reduced in the citrus fruit extract-treated cells (Figure 3).

The cell tyrosinase activity was compared in *Citrus hassaku* ethanol extract. As a result, it showed similar effect as albutin conditioning concentration at ethanol extract test phase (Figure 4). Also, the expression of ERK 1/2, tyrosinase, TRP-1, 2 proteins were analyzed using western blot. As a result, millet article in the phase of tyrosinase and TRP-1 protein showed significant reduction (Figure 5). As a result of comparing cell tyrosinase activation in immature *Citrus unshiu* ethanol extract, it showed similar effect with albutin conditional concentration from the *Citrus unshiu* ethanol

Table 1. Inhibition of Tyrosinase Activity and Melanin Formation in B16 Melanoma Cells by the Selected Citrus Fruit Extracts

Samples	Concentration (μg/mL)	Inhibition rate of tyrosinase activity (%)	Inhibition rate of melanin formation (%)
Arbutin	62.5	10.80	13.20
	125	28.20	28.00
	250	40.20	35.20
	500	48.00	53.20
Citrus natsudaidai Hay	50	-	4.81
(Citrus unshiu × *Citrus sinensis)* × *Citrus reticulate*	50	-	2.81
Citrus unshiu × *Citrus sinensis*	50	2.81	-
Citrus natsudaidai Hay	50	-	-
Citrus sulcata Hort. Ex. Tan	50	-	9.62
Citrus grandis × *Citrus tangerina*	50	3.03	-
Citrus sinensis × *Citrus reticulata*	50	9.25	35.49
Immature *Citrus unshiu*	50	11.38	34.68
Citrus unshiu	50	-	21.85
Citrus hassaku Hort. Ex Y. Tan	50	20.53	33.28
Citrus grandis Osbecle	50	-	-
Citrus erythrosa Hort. et Tanaka	50	20.88	23.55
Citrus paltymanna Hort. et Tanaka	50	17.01	24.21
Citrus grandis Osbeck	50	17.84	23.70
Citrus leiocarpa Hort. et Tanaka	50	19.99	13.30
Citrus pseudogulgat Hort. et Tanaka	50	14.25	30.25
Citrus aurantium L.	50	-	26.09

- : not available

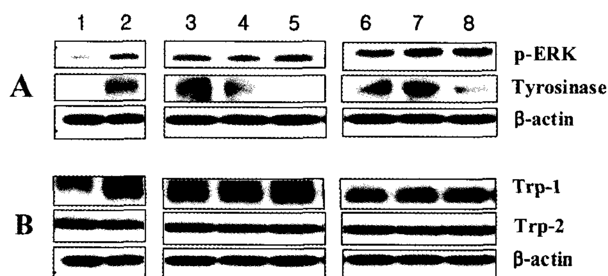

Figure 2. Immunoblot analysis of p-ERK, tyrosinase, Trp-1, Trp-2 and -actin in the B16/F10 melanoma cells after a treatment with extract of *Citrus sinensis* × *reticulata* for a dose-dependent. B16/F10 melanoma cells were cultured for 72 h inducing melanogenesis with α-MSH 50 nM. When it passed for 36 h, media were exchanged and samples were retreated. 1: without MSH, 2: MSH 50 nM addition, 3 ~ 11: MSH 50 nM and sample (ug/mL) addition 3: arbutin 13, 4: arbutin 25, 5: arbutin 50, 6: *Citrus sinensis* × *reticulata* extract 13, 7: *Citrus sinensis* × *reticulata* extract 25, 8: *Citrus sinensis* × *reticulata* extract 50.

extract (Figure 6). Also, the expression of ERK 1/2, tyrosinase, TRP-1, 2 proteins were analyzed using western blot. As a result, the expression of tyrosinase and TRP-1 protein showed reduced in the immature *Citrus unshiu* extract-treated cells (Figure 7).

3.4. DPPH Scavenging Activities of Citrus Fruit Extracts

DPPH is a stable radical that is used in a popular method for screening free radical-scavenging ability of compounds or antioxidant activity of plant extracts. The three extracts immature *Citrus unshiu*, *Citrus hassaku*, and *Citrus sinensis* × *reticulata* showed dose-response curves for DPPH radical-scavenging activity. The IC_{50} values were calculated and are presented in Table 2. The results imply that these active extracts may contain constituents with strong proton-donating abilities.

Table 2. Radical Scavenging Activity of Citrus Fruits

Samples	DPPH scavenging IC_{50} (μg/mL)	Superoxide scavenging IC_{50} (μg/mL)	Nitric oxide scavenging IC_{50} (μg/mL)
Immature *Citrus unshiu*	132.2 ± 16.4	67.12 ± 16.9	214.8 ± 23.0
Citrus hassaku Hort. Ex Y. Tan	104.4 ± 15.8	197.1 ± 84.9	> 1000
Citrus sinensis × *Citrus reticulata*	609.8 ± 84.2	241.4 ± 77.0	785.5 ± 50.0
Trolox	11.2 ± 0.6	59.5 ± 15.5	> 1000
Butylated hydroxy anisole	6.8 ± 0.0	-	> 1000
Allopurinol	-	1.2 ± 0.8	-
Quercetin	-	1.4 ± 0.9	> 1000

IC_{50} was calculated from regression line using thirteen different concentrations in triplicate experiments
- : not available

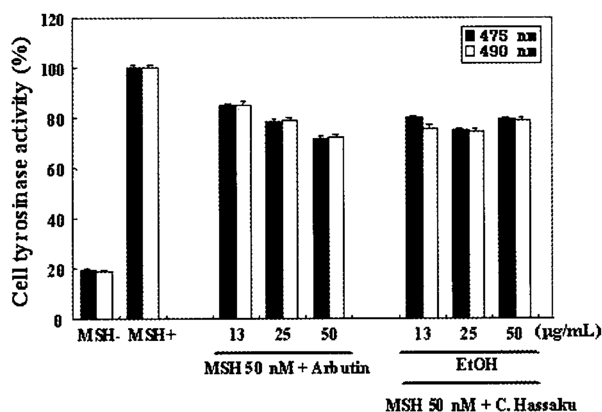

Figure 3. Cell tyrosinase activity assay of *Citrus hassaku* extracts fraction in B16/F10 melanoma.

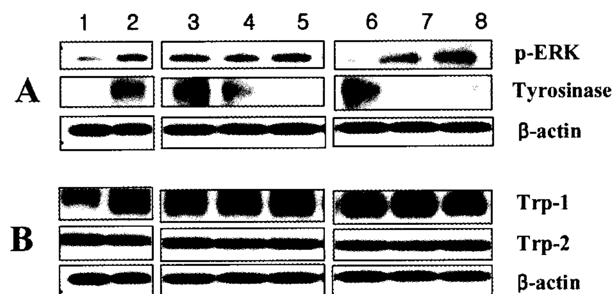

Figure 4. Immunoblot analysis of p-ERK, tyrosinase, Trp-1, Trp-2 and β-actin in the B16/F10 melanoma cells after a treatment with extract of *Citrus hassaku* for a dose-dependent. B16/F10 melanoma cells were cultured for 72 h inducing melanogenesis with α-MSH 50 nM. When it passed for 36 h, media were exchanged and samples were retreated. 1: without MSH, 2: MSH 50 nM addition, 3~8: MSH 50 nM and sample (ug/mL) addition, 3: arbutin 13, 4: arbutin 25, 5: arbutin 50, 6: *Citrus hassaku* extract 13, 7: C-M1 Citrus hassaku extract 25, 8: *Citrus hassaku* extract 50.

3.5. Superoxide Scavenging Activity of Citrus Fruit Extracts

The hydroxyl radical is one of the most reactive radicals generated from biologic molecules and can damage living cells. Some plant extracts have the ability to scavenge hydroxyl radicals and may protect cellular lipids against free radical reactions. The data in Table 2 show that immature *Citrus unshiu*, *Citrus hassaku*, and *Citrus sinensis* × *reticulata* can effectively inhibit formation of OH in a concentration-dependent manner.

3.6. Nitric Oxide Scavenging Activity of Citrus Fruit Extracts

It is well-known that NO has an important role in the various types of physiological processes. In the present study, the citrus fruit extracts were evaluated for their scavenging effects on NO derived from SNP. The compound SNP is known to decompose in aqueous solution at physiological pH, producing NO. Under aerobic conditions, NO reacts with oxygen to produce the stable products nitrate and nitrite, which can be determined using Griess reagent. Data in Table 2 show that the scavenging effects of citrus fruit extracts on NO.

With all test findings taken together, we saw that immature *Citrus unshiu*, *Citrus hassaku*, and *Citrus sinensis* × *reticulata* exhibited low cytotoxicity, potent tyrosinase inhibitory activity, and the ability to reduce melanin content. They also good hydroxyl radical

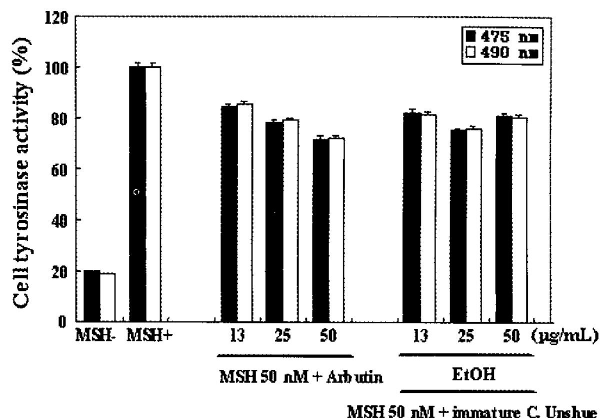

Figure 5. Cell tyrosinase activity assay of immature *Citrus unshiu* extracts in B16/F10 melanoma.

scavenging activities. In previous papers, antioxidants may prevent or delay pigmentation by different mechanisms such as by scavenging ROS and reactive nitrogen species (RNS) or by reducing *o*-quinones or other intermediates in melanin biosynthesis thus delaying oxidative polymerization[12,13].

4. Discussion

The purpose of the present study was to investigate the inhibitory effects of citrus fruits on melanin formation, which is closely related to pigmentation of the skin.

Melanogenesis a major differentiated function of melanocytes plays an important role in protecting skin from sun-related injuries and is principally responsible for skin color[14,15]. Tyrosinase plays a critical regulatory role in melanin biosynthesis and it is suggested that tyrosinase activity is pivotal[16]. Thus numerous studies have focused on characterizing the melanogenic pathways that regulate tyrosinase activity in melanocytes[17].

However, the effects of Korean traditional citrus fruits associated with various *in vitro* on tyrosinase activity are currently not being investigated. Especially, immature *Citrus unshiu* is the first report of the antioxidant and depigment agent. Our study demonstrated that melanin formation and tyrosinase activity were cleary suppressed by citrus fruits at dose-dependently.

ROS scavengers or inhibitors such as antioxidants may reduce hyper-pigmentation while ROS enhances

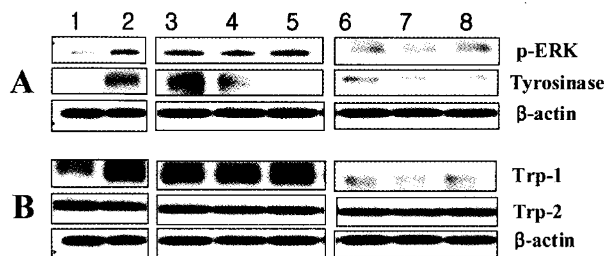

Figure 6. Immunoblot analysis of p-ERK, tyrosinase, Trp-1, Trp-2 and β-actin in the B16/F10 melanoma cells after a treatment with extract of immature *Citrus unshiu* for a dose-dependent. B16/F10 melanoma cells were cultured for 72 h inducing melanogenesis with α-MSH 50 nM. When it passed for 36 h. media were exchanged and samples were retreated. 1: without MSH, 2: MSH 50 nM addition, 3 ~ 8: MSH 50 nM and sample (ug/mL) addition, 3: arbutin 13, 4: arbutin 25, 5: arbutin 50, 6: immature *Citrus unshiu* extract 13, 7: immature *Citrus unshiu* extract 25, 8: immature *Citrus unshiu* extract 50.

melanin biosynthesis, damage DNA and may induce proliferation of melanocytes. UV radiation can increase the melanization and proliferation of melanocytes and induce the formation of ROS in the skin. Endogenously produced NO has a remarkably diverse range of biological function. From the previous research, the effect of melanogenesis on skin due to NO from UV is recently reported in many times[18]. In this study, immature *Citrus unshiu* is expected to affect the melanogenesis due to the excellent NO scavenging activity along with other antioxidant effects.

A positive relationship between tyrosinase activity and tyrosinase mRNA levels in murine melanoma cells has been reported although no correlation between them was observed in human melanocytes or in human/murine melanoma cells [19-21]. Using melanogenic regulatory agents, 12-*O*-tetradecanoylphorbol-13-acetate was shown to decrease melanogenesis and to down-regulate the abundance of tyrosinase mRNA, while cyclic adenosine monophosphate was shown to increase melanogenesis and to up-regulate tyrosinase mRNA levels. Induction of melanogenesis in B16 cells was characterized by the stimulation of tyrosinase activity resulting from an increase in tyrosinase protein expression. In this study, we observed that tyrosinase activity and depigment related protein (tyrosinase, TRP-1, TRP-2, ERK 1/2) expression level are sig-

nificantly suppressed by citrus fruits.

Our results clearly demonstrate that citrus fruits are effective inhibitor of tyrosinase activity and depigment related protein expression in melanoma cells, which eventually slow melanin biosynthesis. These results indicate that citrus fruits may be a useful inhibitor of melanogenesis and suggest that it may have beneficial effects in the treatment of hyperpigmentation disorders such as ephelis and melasma.

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