Biochimica et Biophysica Acta 1850 (2015) 1389–1396

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

# Inhibitory effect of ephedrannins A and B from roots of *Ephedra sinica* STAPF on melanogenesis

Ik-Soo Kim [a,1,2], Sung-Jin Yoon [b,1], Young-Jun Park [b], Hee-Bong Lee [a,*]

[a] Department of Biochemistry, College of Natural Sciences, Kangwon National University, Chuncheon 200-701, Republic of Korea
[b] Cell Therapy Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Republic of Korea

ARTICLE INFO

*Article history:*
Received 26 December 2014
Received in revised form 30 March 2015
Accepted 1 April 2015
Available online 7 April 2015

*Keywords:*
*Epehdra sinica*
Ephedrannin A
Ephedrannin B
Tyrosinase activity
Anti-melanogenesis
Competitive inhibition

ABSTRACT

*Background:* Melanogenesis, a process producing the pigment melanin in human skin, eyes and hair, is a major physiological response against various environmental stresses, in particular exposure to ultraviolet radiation, and its pathway is regulated by a key enzyme, tyrosinase. In this study, we evaluated the effects of ephedrannins A and B, which are polyphenols from the roots of *Ephedra sinica*, commonly used in herbalism in oriental countries, on mushroom tyrosinase and melanogenesis in B16F10 melanoma cells.
*Methods:* Their effects on mushroom tyrosinase were determined via kinetic studies using a spectrophotometric analysis and those on melanin and tyrosinase production in melanoma cells treated with α-MSH (melanin stimulating hormone) were examined using PCR and ELISA.
*Results:* Both ephedrannins A and B exhibited concentration-dependent inhibitory effects on L-tyrosine oxidation by mushroom tyrosinase, and the inhibition mechanism was competitive and reversible with L-tyrosine as the substrate. In addition, melanin production in melanoma cells was also suppressed in a concentration-dependent manner by ephedrannins A and B without significant effects on cell proliferation at the concentrations tested. Both compounds showed inhibitory effects on melanin production by suppressing the transcription of tyrosinase in the cells.
*Conclusion:* Both compounds exhibited significant inhibitory effects, but the inhibition by ephedrannin B was much more effective than that by ephedrannin A. Both ephedrannins A and B may be good candidates for a whitening agent for skin.
*General significance:* This is the first report that describes effective inhibition of melanin production by ephedrannins A and B isolated from *Ephedra* roots.



## 1. Introduction

Skin diseases such as acne, contact dermatitis, urticaria, pigmentary disorders, and skin cancer are caused by chemicals, physical agents, biological agents, plants, mechanical forces, and the sun. Recent studies have focused on skin pigmentation disorders. The pigmentation disorders hyper- and hypopigmentation occur because the body produces either too much or too little melanin. Melanin is the pigment produced by melanocytes and deposited throughout the epidermis that determines skin color [1]. It is triggered by tyrosinase (EC 1.14.18.1). The enzyme is an oxidase and is the rate-limiting enzyme for regulating the production of melanin, known as melanogenesis. Tyrosinase catalyzes two distinct reactions in melanin biosynthesis; first, the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalaninine (DOPA) and, second, the subsequent oxidation of L-DOPA to Dopaquinone. Molecular oxygen is required for both of these reactions [2]. Dopaquinone is further oxidized to yield the mostly brown to black colored polymeric melanins that are responsible for the different colors of skin [3].

The melanin pigmentation of human skin is a major defense mechanism against ultraviolet light from the sun. However, disturbances in the amount and distribution of melanin pigmentation might ultimately provide clues to the etiology of several diseases. Albinism is caused by a genetic deficiency in melanin biosynthesis, which manifests as hypopigmentation of the skin. Hypopigmentation in the skin is associated with sensitivity to UV irradiation and a predisposition to skin cancer [4]. Conversely, hyperpigmentation includes melasma, freckles, and senile lentigines and is caused by the abnormal accumulation of melanin pigments. Hyperpigmentation can be a serious esthetic problem.

*Abbreviations:* α-MSH, melanin stimulating hormone; L-DOPA, L-3,4-dihydroxyphenylalaninine; ECG, epicathechin-3-O-gallate; DMEM, Dulbecco's modified Eagle's medium; FBS, Fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethylsulfoxide; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; OD, optical density; LDH, lactate dehydrogenase; ECL, enhanced chemiluminescence; EGCG, epigallocatechin-3-o-gallate; GCG, gallocatechin-3-o-gallate; MITF, microphthalmia-associated transcription factor; MAP, mitogen-activated protein (MAP).

* Corresponding author.
*E-mail address:* leehbong@kangwon.ac.kr (H.-B. Lee).
[1] Both of these authors contributed equally to this work.
[2] Present address: Hanwool Research Institute of Environment, Daejeon, 305-308, Republic of Korea.

http://dx.doi.org/10.1016/j.bbagen.2015.04.001
0304-4165/

Hence, nearly all studies related to pigmentation disorders are mainly focused on searching for the materials from natural and synthetic sources that inhibit melanin production and the activity of tyrosinase, the regulating enzyme in the melanin biosynthetic pathway, and on determining their potency and type of enzyme inhibition in an effort to understand the mechanism of action of the inhibitor [5]. Until now, it has been reported that depigmenting agents, such as ascorbic acid derivatives, kaempferol, hydroquinone, azelaic acid, retinoids, epicathechin-3-O-gallate (ECG), oxyresveratrol, hydroxystilbene compounds, arbutin, kojic acid, etc. show satisfactory improvements in treatment of hyperpigmentation disorders [6–10]. Kojic acid, an antibiotic and a good chelator of transition metal ions such as $Fe^{3+}$ and $Cu^{2+}$, is known to be a safe and mild agent for treating hyperpigmentation disorders and has been extensively used as a cosmetic agent with skin-whitening effects [11–14].

*Ephedra sinica*, also known by the common names Ma Huang or *Ephedra*, is a traditional Chinese medicinal herb commonly used for thousands of years. *Ephedra* is known to show enhanced pharmacological effects beyond its sympathomimetric activities such as anti-inflammatory, anti-anaphylactic, anti-microbial, anti-histaminic, and hypoglycemic effects [15–17]. Recent studies on the pharmacological effects of *Ephedra* have been focused on its active constituents rather than on the whole plant, including stems and roots, and on understanding its mechanism of action. The main active medicinal ingredients of *Ephedra* are the alkaloids ephedrine and pseudoephedrine [18,19]. It is known that ephedrine stimulates the central nerve system, dilates bronchial tubes, elevates blood pressure, and increases heart rate and that pseudoephedrine relieves nasal congestion. The stem of *Ephedra* contains 1–3% total alkaloids. In addition, other active constituents such as flavonoids [20], polysaccharides [20], tannins [21], and phenolic compounds [22] have also been reported. On the other hand, feruloylhistamine [23], ephedradines A–D [24,25] and ephedrannin A [26] have been identified to be active constituents in the root, which is used for antihydrotic and hypotensive effects almost opposite to the effects of the stem.

As described above, one major group of tyrosinase inhibitors from natural sources, particularly from plants, are the polyphenols, such as kaempferol, quercetin, and ECG, which are widely distributed in nature [5]. In previous study, we reported that ephedrannin A ($C_{30}H_{20}O_{11}$, 556 amu) and B ($C_{30}H_{20}O_{10}$, 540 amu) (Fig. 1), are A-type proanthocyanidines that have anti-inflammatory effects that have been isolated from *E. sinica* roots, and their structures and mechanisms of action have been defined [27]. These ephedrannins A and B, A-type proanthocyanidines are also polyphenols or more complex polyphenols known as tannins. In addition, it has been reported that extracts of *E. sinica* exhibited inhibitory effects on tyrosinase [28]. Hence, in this study we examined the inhibitory effects of the polyphenols ephedrannins A and B on mushroom tyrosinase using spectrophotometric analysis to determine the kinetics and the inhibition mechanism. We also elucidated the inhibitory action on melanin production and tyrosinase gene expression in B16F10 melanoma cells to clarify the depigmenting mechanism of these compounds.

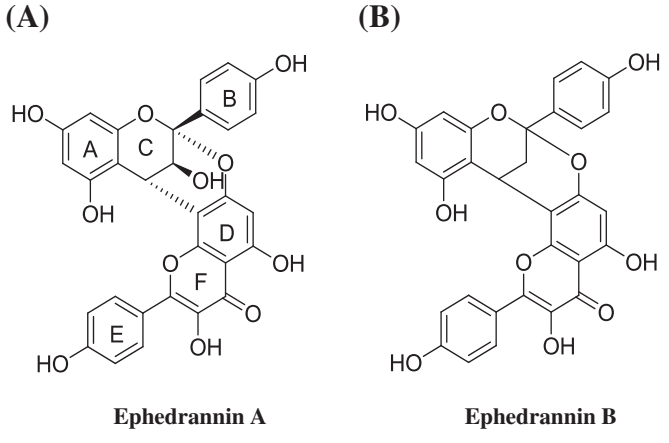



**Fig. 1.** Structures of ephedrannin A (A) and ephedrannin B (B).

## 2. Materials and methods

### 2.1. Materials

The roots of *E. sinica* STAPF were purchased from an herbalist. Ephedrannins A and B were purified from *Ephedra* roots as described in our previous paper [27]. Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), 0.25% trypsin-EDTA, penicillin, and streptomycin were purchased from Gibco BRL Co., USA. The RNeasy mini kit for RNA extraction and cDNA synthesis kit were purchased from Qiagen and Toyobo, Japan, respectively. LDH (lactate dehydrogenase) cytotoxicity detection kit was purchased from Clontech, USA. Nitrocellulose membrane was from Amersham, England. Polyclonal antibodies for tyrosinase and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Other chemicals, including mushroom tyrosinase, were from Sigma (USA) unless otherwise indicated.

### 2.2. Cell culture

B16F10 murine melanoma cells (from a Korean cell line bank) and human keratinocyte HaCat cells (from American Type Culture Collection, ATCC) were cultured in DMEM including 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% $CO_2$ in air at 37 °C. B16F10 cells ($5 \times 10^4$ cells per well) were cultured in 6-well plates for the investigation of melanin content and for western blot and RT-PCR experiments to determine protein and mRNA levels for tyrosinase in the presence and absence of ephedrannins A and B from *Ephedra* roots. B16F10 cells ($1 \times 10^4$ cells per well) were cultured in 96-well microtiter plates for the cell viability assay and human keratinocyte HaCat cells ($2 \times 10^4$ cells per well) in the same plates were cultured for examining the effect of ephedrannins A and B on toxicity for skin related cells. The cell-based assays were carried out in triplicate at least three times.

### 2.3. Measurement of cell viability

To examine the effect of ephedrannins A and B on cell viability, a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was performed [29], with minor modifications. B16F10 melanoma cells ($1 \times 10^4$ cells in 198 μl media per well) were transferred to 96-well plates and incubated overnight in a humidified atmosphere containing 5% $CO_2$ in air at 37 °C to allow the cells to attach to the wells. Dissolved ephedrannin A (2 μl) at a final concentration of 18.0, 36.0, 54.0, or 72.0 μM corresponding to 10, 20, 30, or 40 μg/ml, respectively, or dissolved ephedrannin B (2 μl) at final concentration of 1.85, 3.70, 5.55, or 7.40 μM corresponding to 1, 2, 3, or 4 μg/ml, respectively, in a stock solution was added to each well and the plate was shaken at a speed of 150 rpm for 5 min for thorough mixing. Stock solution (2 μl), which was prepared by mixing dimethylsulfoxide (DMSO) and DMEM containing 1% FBS in the ratio of one to four, was used as a control. The plate was incubated for 48 h in a humidified atmosphere containing 5% $CO_2$ in air at 37 °C. After incubation, 20 μl of freshly made MTT solution [5 mg/ml in phosphate buffered saline (PBS), pH 7.5] was added to each well, and the wells were shaken at 150 rpm for 5 min on a shaker. The plate was incubated for 3 h in a humidified atmosphere containing 5% $CO_2$ in air at 37 °C. After incubation, the media were aspirated and the plate was washed twice with PBS buffer then dried on paper towels. The insoluble MTT metabolic product formazan in each well was dissolved in 200 μl propan-2-ol. The absorbance of each well was read on a microplate reader (VERSAmax,

Molecular Devices, USA) at 570 nm. The optical density (OD) of formazan formed by control cells cultured in the absence of ephedrannin A or B was taken to be 100%. Cell viability tests were primarily performed prior to all experiments using the B16F10 melanoma cells supplemented with ephedrannin A or B.

Human keratinocyte HaCat cells ($2 \times 10^4$ cells per well) were cultured for 24 h in 96-well plates for the detection of cytotoxicity. The detection of LDH activity was performed using an LDH cytotoxicity detection kit as recommended by the manufacturer.

### 2.4. Tyrosinase activity assay

Tyrosinase activity was measured spectrophotometrically with L-tyrosine or L-DOPA as a substrate and mushroom tyrosinase as the enzyme using a modification of the method described by Im et al. [30]. The reaction was initiated by the addition of 100 μl of mushroom tyrosinase solution (400 units/ml for L-tyrosine or 200 units/ml for L-DOPA) to a mixture of 100 μl of 18 mM L-tyrosine or L-DOPA, 100 μl of 80 mM sodium phosphate buffer (pH 6.7), 100 μl of 50% DMSO solution, and 600 μl of $H_2O$ at 37 °C. The initial velocity of DOPAchrome formation was determined by monitoring the change in absorbance per min at 475 nm ($\Delta A_{475}$/mim). In each experiment, 100 μl of $H_2O$ was added to the reaction mixture instead of enzyme solution as a control. Measurements were carried out at least three times. Inhibition by ephedrannin A or B was determined by the addition of 100 μl ephedrannin A or B (final concentration: 5, 10, or 15 μg/ml 50% DMSO solution) instead of 100 μl of 50% DMSO solution. The Michaelis–Menten constant ($K_m$) and maximum velocity ($V_{max}$) of mushroom tyrosinase were determined using a Lineweaver–Burk plot with various concentrations of L-tyrosine or L-DOPA as the substrate in the absence and presence of ephedrannin A or B.

### 2.5. Determination of melanin content

To examine the effect of ephedrannin A or B on melanin production in B16F10 cells, the melanin content was determined using the modified method of Hosoi et al. [31] and No et al. [32]. B16F10 cells ($5 \times 10^4$ per well) were incubated overnight in 6-well plates in a humidified atmosphere containing 5% $CO_2$ in air at 37 °C to allow the cells to attach to the wells. After incubation, the media from each well were aspirated and exchanged with new media: 2 ml of DMEM including 5% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin in the absence and presence of different concentrations of ephedrannin A (18.0, 36.0, 54.0, and 72.0 μM) or ephedrannin B (1.85, 3.70, 5.55, and 7.40 μM) in a stock solution of 100 mg / 1 ml DMSO plus 4 ml DMEM containing 1% FBS. The positive control was 200 nM α-MSH. And also arbutin (2 mM) known well as one of positive agents for antimelanogenesis was used for comparison [33]. The cells were incubated in a humidified atmosphere containing 5% $CO_2$ in air at 37 °C. After incubation for 48 h, the medium from each well was aspirated and 1 ml of medium was collected into a microcentrifuge tube to compare the apparent color as affected by each concentration of ephedrannins A and B. After aspiration, the remaining cells were rinsed twice with PBS buffer. The rinsed cells were dislodged from well and harvested via trypsinization with 200 μl of 0.25% trypsin-EDTA, transferred to microcentrifuge tubes, and centrifuged at 2800 rpm for 3 min. The precipitated cells were washed twice with PBS buffer and then dissolved in 200 μl of 1 M NaOH containing 10% DMSO. The dissolved solutions were incubated at 80 °C for 1 h to solubilize the melanin. The amount of melanin in each microcentrifuge tube was determined spectrophotometrically by measuring the absorbance at 475 nm.

### 2.6. Western blot analysis

To examine the effect of ephedrannin A or B on the level of tyrosinase protein in the B16F10 cells, a western blot analysis was performed. The B16F10 cells ($5 \times 10^4$ per well) were incubated overnight in 6-well plates in a humidified atmosphere containing 5% $CO_2$ in air at 37 °C to allow the cells to attach to the wells. After incubation, the media from each well were aspirated and exchanged with new media: 2 ml of DMEM including 5% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin in the absence and presence of different concentrations of ephedrannin A (18.0, 36.0, 54.0, and 72.0 μM) or ephedrannin B (1.85, 3.70, 5.55, and 7.40 μM) in a stock solution of 100 mg/1 ml DMSO plus 4 ml DMEM containing 1% FBS and 200 nM α-MSH. The cells in the 6-well plate with new media containing ephedrannin A or B were incubated in a humidified atmosphere containing 5% $CO_2$ in air at 37 °C. After incubation for 48 h, the media from each well were aspirated and the cells were rinsed twice with PBS buffer. The rinsed cells were dislodged from well and harvested via trypsinization with 200 μl of 0.25% trypsin-EDTA, transferred to microcentrifuge tubes, and then centrifuged at 2800 rpm for 3 min. The precipitated cells were washed twice with PBS buffer then lysed with cell lysis buffer: 50 mM Tris·HCl (pH 7.5) containing 150 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 2 mM EDTA. After lysis, a cell extract was obtained from supernatant after centrifugation at 12,000 *g* at 4 °C for 20 min. Proteins in the cell extract (50 μg of protein) were separated on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The transfer membrane was blocked with 5% non-fat dried-milk proteins in PBS with 0.1% Tween 20 (PBST) and then probed with tyrosinase antibody in PBST containing 1% bovine serum albumin. After washing three times with PBST, the membrane was hybridized with horseradish peroxidase-coupled secondary antibody for 1 h and washed five times with PBST. After washing, the membrane was incubated with enhanced chemiluminescence (ECL) reagent for 2 min and exposed to X-ray film. The same membranes were stripped in the stripping buffer [63 mM Tris–HCl (pH 6.8) containing 2% SDS and 100 mM 2-mercaptoethanol], reprobed with a specific antibody for β-actin, and then treated with the same reagents as described above for the control experiments.

Protein concentration was determined using the method of Bradford [34] using bovine serum albumin as the standard.

### 2.7. RT-PCR

The expression level of tyrosinase mRNA from the cell extract of B16F10 cells was evaluated via RT-PCR using total RNA and β-actin as an internal control. From the cell extract of B16F10 cells obtained as described above for the western blot analysis experiment, total RNA was extracted using an Rneasy mini kit (Qiagen) as recommended by the manufacturer. Total RNA (1 μg) was reverse-transcribed using a cDNA synthesis kit (Toyobo) according to the manufacturer's protocol and analyzed using RT-PCR with a Dice™ TP 800 Thermal Cycler (Takara Bio). Single strand cDNA was amplified using the tyrosinase specific primers 5′-GGCCAGCTTTCAGGCAGAGGT-3′ (forward) and 5′-TGGTGCTTCATG GGCAAAATC-3′ (reverse) or β-actin specific primers 5′-GACAGGATGC AGAAGGAGATTACT-3′ (forward) and 5′-TGATCCACATCTGCTGGAAG GT-3′ (reverse). The resulting PCR products were electrophoresed on a 1.2% agarose gel containing ethidium bromide.

### 2.8. Statistical analysis

The results for all experiments are presented as the means ± standard deviation of at least three replicates. Student's t-test was used for the statistical analysis of the differences noted.

## 3. Results

### 3.1. Effect of ephedrannins A and B on mushroom tyrosinase activity

To investigate whether the polyphenols ephedrannins A and B inhibited tyrosinase activity like other polyphenols, such as kaempferol, quercetin, ECG, and epigallocatechin-3-o-gallate (EGCG), a kinetic study of L-tyrosine or L-DOPA oxidation catalyzed by mushroom tyrosinase

was performed using spectrophotometry in the absence and presence of ephedrannin A or B. The results are shown in Fig. 2 with L-tyrosine as a substrate. The $V_{max}$ value ($\Delta A_{475}$/mim) of mushroom tyrosinase activity for L-tyrosine was $6.53 \times 10^{-2}$, and the $K_m$ value was 0.266 mM L-tyrosine. However, the addition of ephedrannin A to this reaction mixture increased the $K_m$ for the substrate L-tyrosine in a concentration-dependent manner but did not change the $V_{max}$ value (Fig. 2A). The $K_m$ values in the presence of ephedrannin A at 5, 10, and 15 μg/ml (corresponding to 8.99, 17.99, and 26.98 μM, respectively) were 0.430, 0.833, and 1.199 mM, respectively. This result suggested that ephedrannin A was a competitive inhibitor of mushroom tyrosinase. Similarly, ephedrannin B also increased the $K_m$ value for L-tyrosine in a concentration-dependent manner but did not change the $V_{max}$ (Fig. 2B). The $K_m$ values for L-tyrosine in the presence of ephedrannin B at 5, 10, and 15 μg/ml (corresponding to 9.26, 18.52, and 27.78 μM, respectively) were 0.478, 0.823, and 1.160 mM, respectively. These results indicate that like other polyphenols, ephedrannins A and B are reversible competitive inhibitors of mushroom tyrosinase with L-tyrosine as the substrate. Together with these results, it was examined that the effects on L-tyrosine oxidation by the mushroom tyrosinase were 88.2% inhibition at 53.96 μM and an $IC_{50}$ value of 21.6 μM by ephedrannin A and 89.3% inhibition at 54.5 μM and an $IC_{50}$ value of 22.2 μM by ephedrannin B, respectively (data not shown).

In addition, the results with L-DOPA as the substrate were similar to those for L-tyrosine (Fig. S1). The $V_{max}$ ($\Delta A_{475}$/mim) for mushroom tyrosinase activity for L-DOPA was $1.28 \times 10^{-1}$, and the $K_m$ was 0.464 mM L-DOPA. Both ephedrannins A and B increased the $K_m$ for L-DOPA, as for L-tyrosine, in a concentration-dependent manner but did not alter the $V_{max}$. The $K_m$ values for L-DOPA in the presence of ephedrannin A at 5, 10, and 15 μg/ml were 1.041, 1.247, and 1.586 mM, respectively (Fig. S1A), and for ephedrannin B at the same concentrations, the values were 0.918, 1.121, and 1.289, respectively (Fig. S1B). These results indicate that, like other polyphenols, ephedrannins A and B are reversible competitive inhibitors of mushroom tyrosinase with L-DOPA as the substrate. Together with these results, it was investigated that the effects on L-DOPA oxidation by the mushroom tyrosinase were 99.2% inhibition at 53.96 μM and an $IC_{50}$ value of 13.7 μM by ephedrannin A and 98.6% inhibition at 54.5 μM and an $IC_{50}$ value of 20.0 μM by ephedrannin B, respectively (data not shown).

These new findings on the inhibitory effects of ephedrannins A and B toward mushroom tyrosinase triggered an investigation of the effects of ephedrannins A and B on melanin production and tyrosinase gene expression in B16F10 melanoma cells.

### 3.2. Effects of ephedrannins A and B on cell viability of B16F10 cells and on cytotoxicity of human keratinocyte HaCat cells

To determine the effects of ephedrannins A and B on cell viability, B16F10 melanoma cells were exposed for 48 h to various concentrations of ephedrannin A or B. The concentrations of ephedrannin A or B used for the cell viability tests were chosen based on preliminary tests for their effects on melanin production in the melanoma cells. As shown in Fig. S2, concentrations of ephedrannin A ranging from 10 to 40 μg/ml and ephedrannin B ranging from 1 to 4 μg/ml did not have significant effects on the viability of B16F10 melanoma cells compared with untreated control after 48 h. These results suggest that the concentrations of ephedrannins A and B used in this study did not significantly affect cell viability and that the data obtained from the experiments on the effects of these compounds on melanin and tyrosinase production are reliable.

In addition, to determine the effects of these ephedrannins A and B on skin related cells, the cytotoxicity of both compounds toward human keratinocyte HaCat cells was examined under the same conditions with B16F10 melanoma cells. As shown in Fig. S3, concentrations of ephedrannins A and B used in this study had no cytotoxic effect on human keratinocyte HaCat cells. These results indicate that both ephedrannins A and B are safe for human skin cells under these concentrations.

### 3.3. Effect of ephedrannins A and B on melanin content

To examine the effect of ephedrannin A or B on melanin production in B16F10 melanoma cells, cells treated with 200 nM α-MSH, which stimulates melanin production, were incubated for 48 h in media with and without ephedrannin A (10, 20, 30, or 40 μg/ml, corresponding to 18.0, 36.0, 54.0, or 72.0 μM, respectively) and B (1, 2, 3, or 4 μg/ml, corresponding to 1.85, 3.70, 5.55, or 7.40 μM, respectively). The amounts of ephedrannins A and B used for the inhibitor studies on melanin production were chosen based on preliminary tests. As shown in Fig. 3A and B, melanin production was considerably up-regulated greater than 1.75-fold in the B16F10 cells by α-MSH. However, the α-MSH-induced melanin production was significantly decreased in a concentration-dependent manner by the addition of ephedrannin A or B. Approximately 60% of the α-MSH-induced melanin production was inhibited by both of 72.0 μM ephedrannin A and 7.40 μM ephedrannin B. This inhibitory effect by ephedrannin A or ephedrannin B was superior to that of arbutin at 2 mM used as a positive control. These results indicate that both ephedrannins A and B exhibited strong anti-melanogenesis effects; however, the inhibitory effect of ephedrannin B was approximately 10-fold lower than that of ephedrannin A.

These results of the inhibitor studies of ephedrannins A and B on melanin production were expected from the difference in the colors of the media after incubation of the cells for 48 h in the absence and presence of ephedrannin A or B with α-MSH. As shown in Fig. 4, the strength of the color of the media was proportional to the melanin content in cells. The media color from cells stimulated by α-MSH became a much darker brown than that of the untreated control. However, its color was lightened in a concentration-dependent manner after the addition of ephedrannin A or B. Although the color was also lightened by the

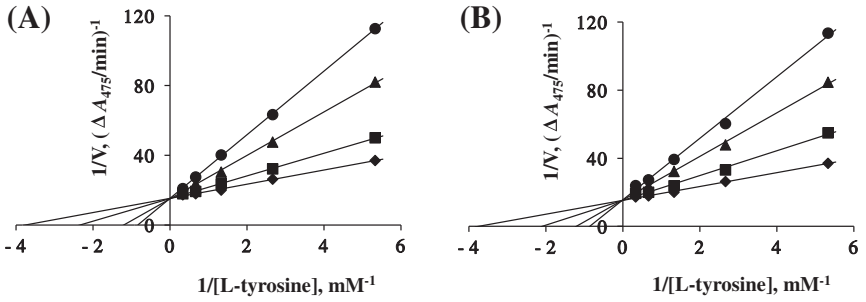



**Fig. 2.** Lineweaver–Burk plots for mushroom tyrosinase in the absence and presence of ephedrannin A (A) and ephedrannin B (B). Data were obtained as the mean values of 1/V, inverse of the increase of absorbance at the wavelength 475 nm per min ($\Delta A_{475}$/min), of three independent tests with different concentrations of L-tyrosine as a substrate. Inhibitors of the enzyme were ephedrannin A (A) and B (B) with 0 μg/ml (♦), 5 μg/ml (■), 10 μg/ml (▲), or 15 μg/ml (●).

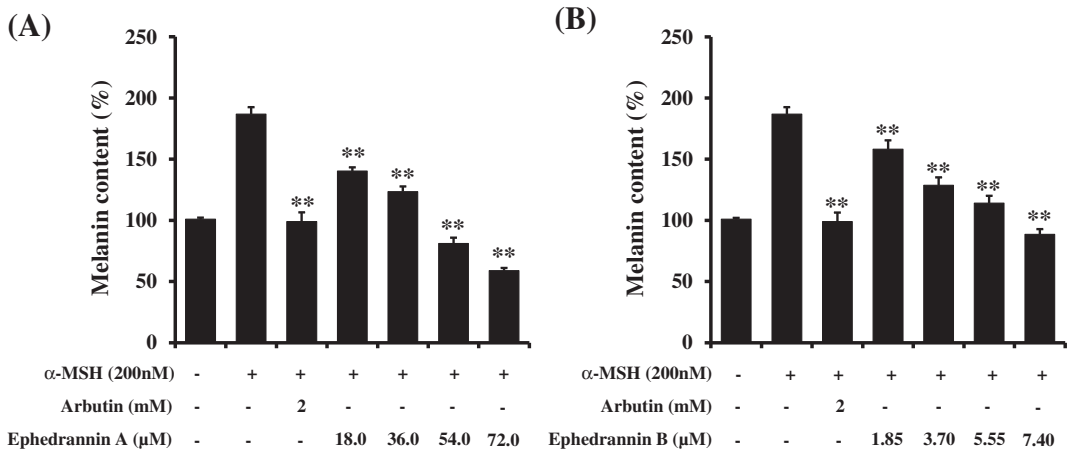



**Fig. 3.** Effects of ephedrannin A (A) and B (B) on melanin production in α-MSH-stimulated B16F10 melanoma cells. The cells were exposed to α-MSH (200 nM) in the presence of ephedrannin A (18.0, 36.0, 54.0, and 72.0 μM) or ephedrannin B (1.85, 3.70, 5.55, and 7.40 μM) for 48 h. Arbutin (2 mM) was used as a positive control under the same conditions. The melanin content was determined by measuring the absorbance at 475 nm using a spectrophotometer. **P < 0.001 compared with the control.

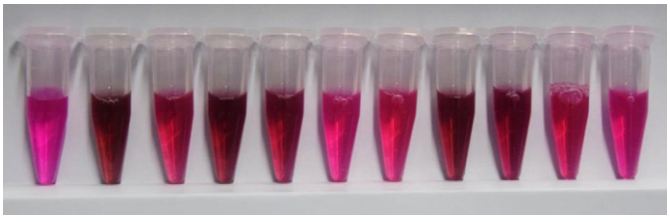
addition of arbutin used as a positive control, the color changed by the addition of 72.0 μM ephedrannin A or 7.40 μM ephedrannin B was much lighter than that by arbutin at 2 mM. The result showed that the inhibitory effects of both ephedrannins on melanin production in B16F10 melanoma cells were much greater than that of arbutin. From these results, ephedrannin A or B reduced melanin production in cells and could induce depigmentation of the skin.

### *3.4. Effect of ephedrannins A and B on tyrosinase gene expression*

From the results of the inhibitor studies of ephedrannins A and B on melanin production in B16F10 cells (Fig. 3A and B), to determine whether ephedrannin A or B inhibited melanin production via the suppression of tyrosinase gene expression, the changes in tyrosinase protein and mRNA levels at the same concentrations of ephedrannin A (18.0, 36.0, 54.0, and 72.0 μM) or B (1.85, 3.70, 5.55, and 7.40 μM) were examined. As shown in the western blot (Fig. 5A and B) and the RT–PCR (Fig. 6A and B) analyses, both ephedrannins A and B significantly decreased the levels of tyrosinase protein and mRNA in the α-MSH-stimulated cells in a concentration-dependent manner. The increased synthesis of tyrosinase induced by α-MSH was completely inhibited by the addition of 72.0 μM ephedrannin A and was almost completely inhibited by the addition of 7.40 μM ephedrannin B (Fig. 5A and B). As shown in Fig. 6A and B, the increased tyrosinase mRNA levels induced by α-MSH were decreased almost to the control level in the absence of α-MSH by the addition of 72.0 μM ephedrannin A or 7.40 μM ephedrannin B. The inhibition of tyrosinase protein and mRNA levels by ephedrannin B was much more effective than that of ephedrannin A. In conclusion, these results suggest that both ephedrannins A and B inhibit melanin production by down-regulating tyrosinase mRNA transcription.

## 4. Discussion

Interest in skin pigmentation has been gradually increasing. In particular, many efforts have been focused on (1) the development of treatments for hyperpigmentation, which is caused by the abnormal accumulation of the melanin pigment, because hyperpigmentation could be a serious esthetic problem, and (2) discovering inhibitors of tyrosinase activity because melanin biosynthesis is governed by that enzyme. Agents such as arbutin, hydroquinone, kojic acid, EGCG-like tannins, vitamin C and its derivatives, as well as placenta extract are known topical skin-lightening agents [9,32,35–38]. Most of these agents inhibit tyrosinase activity and melanin production. In addition, recent studies have focused on active constituents with tyrosinase inhibitory activity isolated from herbs such as *Atractylis Rhizoma* [39], *Heterotheca inuloides* [40], green teas [32], and *Euphorbiae Lathyridis* semen [41], and on understanding their mechanisms of action [10,32,40,42]. In this study, we showed for the first time that ephedrannins A and B, recently isolated from *Ephedra* roots, inhibit mushroom tyrosinase activity in a concentration-dependent manner and also inhibit the melanin production in B16F10 melanoma cells. These substances suppress the transcriptional level of tyrosinase in a concentration-dependent manner as well. In addition, we suggest that ephedrannins A and B are possible skin whitening agents.



| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| α-MSH (200nM) | - | + | + | + | + | + | + | + | + | + | + |
| Arbutin (mM) | - | - | 2 | - | - | - | - | - | - | - | - |
| Ephedrannin A (μM) | - | - | - | 18.0 | 36.0 | 54.0 | 72.0 | - | - | - | - |
| Ephedrannin B (μM) | - | - | - | - | - | - | - | 1.85 | 3.70 | 5.55 | 7.40 |

**Fig. 4.** Color changes in the media resulting from the effects of ephedrannins A and B on melanin biosynthesis in α-MSH-stimulated B16F10 melanoma cells. The cells were exposed to α-MSH (200 nM) in the absence or presence of ephedrannin A (18.0, 36.0, 54.0, and 72.0 μM) or ephedrannin B (1.85, 3.70, 5.55, and 7.40 μM) for 48 h. Arbutin (2 mM) was used as a positive control under the same conditions.

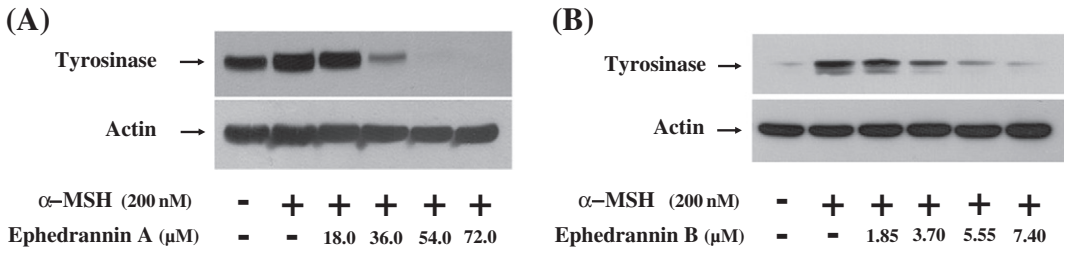



**Fig. 5.** Effects of ephedrannin A (A) and B (B) on the expression of the tyrosinase gene in α-MSH-stimulated B16F10 melanoma cells. The cells were exposed to α-MSH (200 nM) in the presence of ephedrannin A (18.0, 36.0, 54.0, and 72.0 μM) or ephedrannin B (1.85, 3.70, 5.55, and 7.40 μM) for 48 h. The tyrosinase expression levels were investigated by western blot analysis as described in Materials and methods. Equal protein loading was confirmed by actin expression.

Both ephedrannins A and B are polyphenols/tannins that showed concentration-dependent inhibition of L-tyrosine oxidation by mushroom tyrosinase and exhibited a typical competitive inhibition mechanism with L-tyrosine as the substrate, displaying no change in $V_{max}$ value but increasing $K_m$ values with an increase in the concentration of ephedrannin A or B, as shown in Lineweaver–Burk plots (Fig. 2). The inhibitory effects and the typical competitive inhibition mechanism of ephedrannins A and B on tyrosinase with L-tyrosine as substrate were the same as those of polyphenols such as kaempferol from saffron flower [42], quercetin from *Heterotheca inuloids* [43], and ECG, GCG (gallocatechin-3-o-gallate), and EGCG from green tea [32]. Differently, the polyphenols kurarinone from *Sophora flavescens* roots [44] and oxyresveratrol from *Morus alba* Linne [10] were also shown to inhibit mushroom tyrosinase but exhibit noncompetitive inhibition with L-tyrosine as substrate.

The inhibitory effects of ephedrannins A and B on L-tyrosine oxidation by the mushroom tyrosinase were 88.2% at 53.96 μM and 89.3% at 54.5 μM, respectively, and also $IC_{50}$ values of them were 21.6 μM and 22.2 μM, respectively. On the other hand, oxyresveratrol reported previously with L-tyrosine exhibited 97.3% inhibition of the mushroom tyrosinase activity at 100 μM and an $IC_{50}$ value of 1.2 μM, and resveratrol and kojic acid exhibited 63.8% inhibition at 100 μM and an $IC_{50}$ value of 54.6 μM, and 76.7% at 100 μM and an $IC_{50}$ value of 40.1 μM, respectively [10]. In addition, the inhibitory effect of arbutin as a positive control in this study on L-tyrosine oxidation by mushroom tyrosinase showed 66% inhibition at 10 mM and an $IC_{50}$ value of 6 mM [45].

In addition, because ephedrannins A and B are competitive inhibitors of mushroom tyrosinase with L-tyrosine and L-DOPA as substrates (Fig. S1A and S1B), like other polyphenols such as quercetin and kaempferol, their structures resemble the substrates whose reactions they inhibit, and they compete with the substrate for the same binding site on mushroom tyrosinase because of their structural similarities. The tyrosinase inhibitory activity of quercetin and kaempferol was shown to arise from their ability to chelate copper in the enzyme due to a 3-hydroxy-4-keto moiety in their structures [40]. Hence, the tyrosinase inhibitory activity of ephedrannins A and B could be caused by the ability to chelate copper in the mushroom tyrosinase because both ephedrannins A and B have kaempferol moieties in their structure (Fig. 1), designated as ent-epiafzelechin-[2(α) → *0* → 7, 4(α) → 8]-kaempferol [46] and 5,7,4′-trihydroxyflavan-[2(α) → *0* → 7, 4(α) → 8]-kaempferol [47], respectively.

Melanin biosynthesis has been described as the oxidation of L-tyrosine by tyrosinase using molecular oxygen followed by a series of non-enzymatic steps resulting in the formation of melanin. Tyrosinase is the pivotal, rate-limiting enzyme for this biosynthetic pathway [48]. Hence, it has been reported that the melanogenesis induction in B16F10 melanoma cells was characterized by the stimulation of tyrosinase activity resulting from an increase in the tyrosinase protein expression [49]. Englaro et al. suggested that the increase of tyrosinase activity stimulated melanin synthesis [50]. On the contrary, there are many studies that show that the inhibition of melanin production was caused by the inhibition of tyrosinase activity resulting from a decrease in tyrosinase protein expression [30,51]. In this study, to obtain more direct evidence about whether ephedrannins A and B lead to the melanogenesis inhibition, the melanin content of B16F10 melanoma cells cultured under various concentrations of ephedrannins A and B was determined. The results in Fig. 3A and B show that ephedrannins A and B were considerably effective in reducing melanin formation after 48 h. These findings indicate that the ephedrannins A and B might promote the suppression of melanin synthesis. In addition, the results (Fig. 3A and 3B) that melanin production in B16F10 melanoma cells was inhibited in a concentration-dependent manner by ephedrannins A and B agree well with those of the apparent color of the medium after 48 h incubation (Fig. 4). These color changes in the media seem to be caused by exosomes containing melanin released from melanoma cells during incubation under various conditions. In the present study, a western blot analysis showed that ephedrannins A and B significantly decreased the amount of tyrosinase protein (Fig. 5A and B). As shown in Fig. 6A and B, the results showed that both ephedrannins A and B decreased the level of tyrosinase mRNA. In conclusion, these results clearly demonstrate that both ephedrannins A and B display anti-melanogenesis activity by suppressing tyrosinase mRNA transcription. These results can be deduced from following reports. Lin et al. suggested that the expression of tyrosinase is regulated by the transcriptional

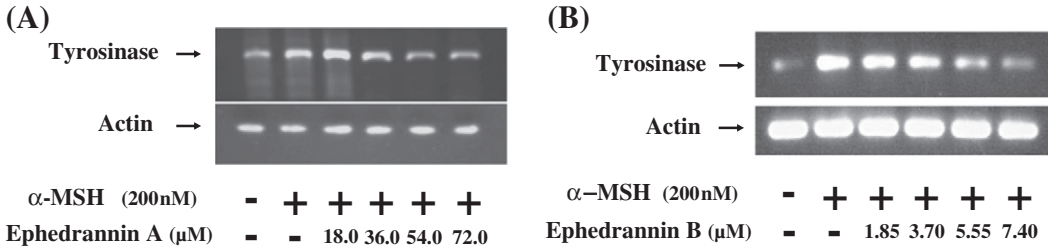



**Fig. 6.** Effects of ephedrannin A (A) and B (B) on the tyrosinase mRNA level in α-MSH-stimulated B16F10 melanoma cells. The cells were exposed to α-MSH (200 nM) in the presence of ephedrannin A (18.0, 36.0, 54.0, and 72.0 μM) or ephedrannin B (1.85, 3.70, 5.55, and 7.40 μM) for 48 h. The tyrosinase mRNA levels in the cell extract were evaluated by RT-PCR analysis. Actin was used as an internal control.

regulator microphthalmia-associated transcription factor (MITF) in skin pigmentation [52]. In relation to this MITF, Fujita et al. proposed that the transcriptional activity of MITF is regulated by p38 mitogen-activated protein (MAP) kinase in osteoclasts [53]. In addition, we reported in our previous study that ephedrannins A and B inhibit lipopolysaccharide-induced inflammatory mediators by suppressing the translocation of nuclear factor-κB (NF-κB) and the phosphorylation of p38 MAP kinase in RAW 264.7 macrophages [27]. To sum them up, the down-regulation of the tyrosinase mRNA level by ephedranin A and B might be induced by the down-regulation of this p38-MITF pathway in B16F10 melanoma cells. The results that both ephedrannins A and B exhibit anti-melanogenesis activity by suppressing tyrosinase mRNA transcription were the similar to those obtained with *Radix trichosanthis* extract [30]. However, it was reported that oxyresveratrol from *M. alba* Linne showed reversible inhibition of tyrosinase activity but not suppression of the expression and synthesis of the enzyme in murine B16F10 melanoma cells [10]. A similar result that linoleic acid decreased melanin synthesis but did not alter mRNA levels of melanogenic enzymes including tyrosinase has also been reported [54].

In addition, although both ephedrannins A and B inhibited melanin production in B16F10 melanoma cells by suppressing tyrosinase gene expression, inhibition by ephedrannin B was more effective than that by ephedrannin A. This result indicates that ephedrannin B could be a better candidate than ephedranin A as a whitening agent for the skin.

## Transparency document

The Transparency document associated with this article can be found, in the online version.

## Acknowledgements

This study was supported by 2013 Research Grant from Kangwon National University (No. 120131795), by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (RBM0221211), and by the KRIBB Research Initiative Program, Republic of Korea.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2015.04.001.

## References

[1] A.A. Mills, Changing colors in mice: an inducible system that delivers, Genes Dev. 15 (2001) 1461–1467.
[2] V.J. Hearing Jr., Mammalian monophenol monooxygenase (tyrosinase): purification, properties, and reactions catalyzed, Methods Enzymol. 142 (1987) 154–165.
[3] G. Prota, Natural and synthetic melanins, in: G. Prota (Ed.), Melanins and melanogenesis, Academic Press Inc., San Diego, CA 1992, pp. 63–87.
[4] W.S. Oetting, R.A. King, Molecular basis of albinism: mutations and polymorphisms of pigmentation genes associated with albinism, Hum. Mutat. 13 (1999) 99–115.
[5] S.-Y. Seo, V.K. Sharma, N. Sharma, Mushroom tyrosinase: recent prospects, J. Agric. Food Chem. 51 (2003) 2837–2853.
[6] I.L. Guevara, A.G. Pandya, Melasma treated with hydroquinone, tretinoin, and a fluorinated steroid, Int. J. Dermatol. 40 (2001) 212–215.
[7] Q.H. Nguyen, T.P. Bui, Azelaic acid: pharmacokinetic and pharmacodynamic properties and its therapeutic role in hyperpigmentary disorders and acne, Int. J. Dermatol. 34 (1995) 75–84.
[8] G. Okan, C. Baykal, Nevoid hyperkeratosis of the nipple and areola: treatment with topical retinoic acid, J. Eur. Acad. Dermatol. Venereol. 13 (1999) 218–220.
[9] K. Maeda, M. Fukuda, Arbutin: mechanism of its depigmenting action in human melanocyte culture, J. Pharmacol. Exp. Ther. 276 (1996) 765–769.
[10] Y.M. Kim, J. Yun, C.K. Lee, H. Lee, K.R. Min, Y. Kim, Oxyresveratrol and hydroxystilbene compounds, J. Biol. Chem. 277 (2002) 16340–16344.
[11] T. Yoshimoto, K. Yamamoto, D. Tsuru, Extracellular tyrosinase from *Streptomyces* sp. KY-453: purification and some enzymatic properties, J. Biochem. 97 (1985) 1747–1754.
[12] G. Battaini, E. Monzani, L. Casella, L. Santagostini, R. Pagliarin, Inhibition of the catecholase activity of biomimetic dinuclear copper complexes by kojic acid, J. Biol. Inorg. Chem. 5 (2000) 262–268.
[13] J. Cabanes, S. Chazarra, F. Garcia-Carmona, Kojic acid, a cosmetic skin whitening agent, is a slow-binding inhibitor of catecholase activity of tyrosinase, J. Pharm. Pharmacol. 46 (1994) 982–985.
[14] J.T. Lim, Treatment of melasma using kojic acid in a gel containing hydroquinone and glycolic acid, Dermatol. Surg. 25 (1999) 282–284.
[15] H. Hikino, C. Konno, H. Takana, M. Tamada, Anti-inflammatory principle of *Ephedra* herbs, Chem. Pharm. Bull. (Tokyo) 28 (1980) 2900–2904.
[16] C. Konno, T. Mizuno, H. Hikino, Isolation and hypoglycemic activity of ephedrans A, B, C, D and E, glycans of *Ephedra distachya* herbs, Planta Med. 51 (1985) 162–163.
[17] E.A. Abourashed, A.T. El-Alfy, I.A. Khan, L. Walker, Ephedra in perspective — a current review, Phytother. Res. 17 (2003) 703–712.
[18] R. Andraws, P. Chawla, D.L. Brown, Cardiovascular effects of ephedra alkaloids: a comprehensive review, Prog. Cardiovasc. Dis. 47 (2005) 217–225.
[19] G. Ma, S.A. Bavadekar, Y.M. Davis, S.G. Lalchandani, R. Nagmani, B.T. Schaneberg, I.A. Khan, D.R. Feller, Pharmacological effects of ephedrine alkaloids on human {alpha}1- and {alpha}2-adrenergic receptor subtypes, J. Pharmacol. Exp. Ther. 322 (2007) 214–221.
[20] L.L. Ding, S.S. Shi, J. Cui, S.C. Wang, Z.T. Wang, Advances in research of chemical constituents and pharmacological activities of *Ephedra*, Zhongguo Zhong Yao Za Zhi 31 (2006) 1661–1664.
[21] H. Friedrich, H. Wiedemeyer, Quantitative determination of the tannin-precursors and the tannins in *Ephedra Helvetica*, Planta Med. 30 (1976) 223–231.
[22] F. Cottiglia, L. Bonsignore, L. Casu, D. Deidda, R. Pompei, M. Casu, C. Floris, Phenolic constituents from *Ephedra nebrodensis*, Nat. Prod. Res. 19 (2005) 117–123.
[23] H. Hikino, Y. Kiso, M. Ogata, C. Konno, K. Aisaka, H. Kubota, N. Hirose, T. Ishihara, Pharmacological actions of analogues of feruloylhistamine, an imidazole alkaloid of *Ephedra* roots, Planta Med. 50 (1984) 478–480.
[24] M. Tamada, K. Endo, H. Hikino, C. Kabuto, Structure of ephedradine A, a hypotensive principle of *Ephedra* roots, Tetrahedron Lett. 20 (1979) 873–876.
[25] H. Hikino, K. Ogata, C. Konno, S. Saito, Hypotensive actions of ephedradines, macrocyclic spermine alkaloids of *Ephedra* roots, Planta Med. 48 (1983) 290–293.
[26] H. Hikino, M. Takahashi, C. Konno, Structure of ephedrannin A, a hypotensive principle of *Ephedra* roots, Tetrahedron Lett. 23 (1982) 673–676.
[27] I.S. Kim, Y.J. Park, S.J. Yoon, H.B. Lee, Ephedrannin A and B from roots of *Ephedra sinica* inhibit lipopolysaccharide-induced inflammatory mediators by suppressing nuclear factor-κB activation in RAW 264.7 macrophages, Int. Immunopharmacol. 10 (2010) 1616–1625.
[28] Y.-G. Yoo, M.-S. Joung, J.-W. Choi, J.-H. Kim, The study on the whitening effect of *Ephedra sinica* extract, J. Soc. Cosmet. Sci. Korea 31 (2005) 153–159.
[29] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[30] S.-J. Im, K.-N. Kim, Y.-G. Yun, J.-C. Lee, Y.-J. Mun, J.-H. Kim, W.-H. Woo, Effect of *Radix ginseng* and *Radix trichosanthis* on the melanogenesis, Biol. Pharm. Bull. 26 (2003) 849–853.
[31] J. Hosoi, E. Abe, T. Suda, T. Kuroki, Regulation of melanin synthesis of B16 mouse melanoma cells by 1 alpha, 25-dihydroxyvitamin D3 and retinoic acid, Cancer Res. 45 (1985) 1474–1478.
[32] J.K. No, D.Y. Soung, Y.J. Kim, K.H. Shim, Y.S. Jun, S.H. Rhee, T. Yokozawa, H.Y. Chung, Inhibition of tyrosinase by green tea components, Life Sci. 65 (1999) 241–246.
[33] K.C. Wen, C.S. Chang, Y.C. Chien, H.W. Wang, W.C. Wu, C.S. Wu, H.M. Chiang, Tyrosol and its analogues inhibit alpha-melanocyte-stimulating hormone induced melanogenesis, Int. J. Mol. Sci. 14 (2013) 23420–23440.
[34] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[35] K. Jimbow, H. Obata, M.A. Pathak, T.B. Fitzpatrick, Mechanism of depigmentation by hydroquinone, J. Investig. Dermatol. 62 (1974) 436–449.
[36] G. Imokawa, Y. Mishima, Loss of melanogenic properties in tyrosinases induced by glucosylation inhibitors within malignant melanoma cells, Cancer Res. 42 (1982) 1994–2002.
[37] M. Masuda, T. Tejima, T. Suzuki, G. Imokawa, Skin lighteners, Cosmet. Toilet. 111 (1996) 65–77.
[38] E.V. Curto, C. Kwong, H. Hermersdoerfer, H. Glatt, C. Santis, V. Virador, V.J. Hearing, T.P. Dooley, Inhibitors of mammalian melanocyte tyrosinase: in vitro comparisons of alkyl esters of gentisic acid with other putative inhibitors, Biochem. Pharmacol. 57 (1999) 663–673.
[39] B.W. Choi, B.H. Lee, K.J. Kang, E.S. Lee, N.H. Lee, Screening of the tyrosinase inhibitors from marine algae and medicinal plants, Kor. J. Pharmacogn. 29 (1998) 237–242.
[40] I. Kubo, I. Kinst-Hori, S.K. Chaudhuri, Y. Kubo, Y. Sanchez, T. Ogura, Flavonols from *Heterotheca inuloides*: tyrosinase inhibitory activity and structural criteria, Bioorg. Med. Chem. 8 (2000) 1749–1755.
[41] C.T. Kim, M.H. Jung, H.S. Kim, H.J. Kim, S.J. Kang, S.H. Kang, Inhibitors of melanogenesis from *Euphorbiae Lathyridis* Semen, Kor. J. Pharmacogn. 31 (2000) 168–173.
[42] I. Kubo, I. Kinst-Hori, Flavonols from saffron flower: tyrosinase inhibitory activity and inhibition mechanism, J. Agric. Food Chem. 47 (1999) 4121–4125.
[43] Q.X. Chen, I. Kubo, Kinetics of mushroom tyrosinase inhibition by quercetin, J. Agric. Food Chem. 50 (2002) 4108–4112.
[44] T.J. Ha, M.S. Yang, D.S. Jang, S.U. Choi, K.H. Park, Inhibitory activities of flavanone derivatives isolated from *Sophora flavescens* for melanogenesis, Bull. Kor. Chem. Soc. 22 (2001) 97–99.
[45] S.Y. Jun, K.M. Park, K.W. Choi, M.K. Jang, H.Y. Kang, S.H. Lee, K.H. Park, J. Cha, Inhibitory effects of arbutin-β-glycosides synthesized from enzymatic transglycosylation for melanogenesis, Biotechnol. Lett. 30 (2008) 743–748.

[46] A.R. Bilia, I. Morelli, M. Hamburger, K. Hostettmann, Flavans and A-type proanthocyanidines from *Prunus prostrate*, Phytochemistry 43 (1996) 887–892.
[47] H. Tao, L. Wang, Z. Cui, D. Zhao, Y. Liu, Dimeric proanthocyanidines from the roots of *Ephedra sinica*, Planta Med. Lett. 74 (2008) 1823–1825.
[48] V.J. Hearing, K. Tsukamoto, Enzymatic control of pigmentation in mammals, FASEB J. 5 (1991) 2902–2909.
[49] A. Winder, T. Kobayashi, K. Tsukamoto, K. Urabe, P. Aroca, K. Kameyama, V.J. Hearing, The tyrosinase gene family—interactions of melanogenic proteins to regulate melanogenesis, Cell. Mol. Biol. Res. 40 (1994) 613–626.
[50] W. Englaro, C. Bertolotto, R. Busca, A. Brunet, G. Pages, J.P. Ortonne, R. Ballotti, Inhibition of the mitogen-activated protein kinase pathway triggers B16 melanoma cell differentiation, J. Biol. Chem. 273 (1998) 9966–9970.
[51] M. Martínez-Esparza, C. Jiménez-Cervantes, F. Solano, J.A. Lozano, J.C. García-Borrón, Mechanisms of melanogenesis inhibition by tumor necrosis factor-alpha in B16/F10 mouse melanoma cells, Eur. J. Biochem. 255 (1998) 139–146.
[52] J.Y. Lin, D.E. Fisher, Melanocyte biology and skin pigmentation, Nature 445 (2007) 843–850.
[53] K. Fujita, M. Iwasaki, H. Ochi, T. Fukuda, C. Ma, T. Miyamoto, K. Takitani, T. Negishi-Koga, S. Sunamura, T. Kodama, H. Takayanagi, H. Tamai, S. Kato, H. Arai, K. Shinomiya, H. Itoh, A. Okawa, S. Takeda, Vitamin E decreases bone mass by stimulating osteoclast fusion, Nat. Med. 18 (2012) 589–595.
[54] H. Ando, Y. Funasaka, M. Oka, A. Ohasi, M. Furumura, J. Matsunaga, N. Matsunaga, V.J. Hearing, M. Ichihashi, Possible involvement of proteolytic degradation of tyrosinase in the regulatory effect of fatty acids on melanogenesis, J. Lipid Res. 40 (1999) 1312–1316.