Melia azedarach extract stimulates melanogenesis through increase of tyrosinase-related protein 1 expression in B16F10 mouse melanoma cells

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Received August 27, 2014; Accepted March 26, 2015

DOI: 10.3892/ijmm.2015.2182

Abstract. *Melia azedarach* (MA) has been used in folk medicine in Asia for the treatment of several diseases. Several constituents from MA possess anti-herpetic, anti-angiogenic and anticancer properties. The aim of the present study was to investigate the effect of a 70% ethanol extract of MA on melanogenesis and the underlying mechanisms involved. A B16F10 mouse melanoma cell line was used in our experiments. Treatment of B16F10 cells with the MA extract (10, 20 and 40 μg/ml) increased melanin content in a concentration-dependent manner without cytotoxicity at 24 h. Further experiments indicated that the MA extract (20 μg/ml) increased melanin content as early as at 4 h after treatment. Additionally, although the MA extract did not affect intracellular tyrosinase activity and the protein levels of tyrosinase and tyrosinase-related protein-2 (TRP-2) at 2 and 4 h after treatment, the MA extract increased TRP-1 protein expression at both time points. However, no significant effect of the MA extract treatment on TRP-1 mRNA level at the time points measured was observed. In conclusion, the results from the present study demonstrate that the MA extract increases melanogenesis through the upregulation of TRP-1 protein expression by post-transcriptional control in B16F10 cells and suggest that the MA extract can be viewed as a rapid inducer of melanogenesis, thus rendering it a potential treatment for hypopigmentation diseases including vitiligo.

Introduction

In living systems, melanin offers many physiological functions. It can protect the human skin from damage, for example by UV irradiation (1). It also serves as the determinant of hair color. Melanogenesis is a complex process that results in the synthesis of melanin pigment. It occurs within specialized intracellular organelles known as melanosomes in melanocytes. UV irradiation increases transport of melanosomes from melanocytes to keratinocytes (2). Melanosomes in keratinocytes play protective roles from UV irradiation by scattering incoming light and UV radiation-generated free radicals in cells (3).

In melanocytes and melanoma cells, melanin synthesis is controlled by a cascade of enzymatic reactions. Melanin synthesis is regulated by melanogenic enzymes such as tyrosinase, tyrosinase-related protein-1 (TRP-1), and TRP-2 (4). First, tyrosinase converts tyrosine to 3,4-dihydroxyphenylalanine (DOPA). Second, tyrosinase catalyzes the oxidation of DOPA to DOPAquinone (5). DOPAquinone is then converted to DOPAchrome through auto-oxidation. TRP-2, which functions as DOPAchrome tautomerase, catalyzes the transition of DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (6). Subsequently, TRP-1 oxidizes DHICA to a carboxylated indole-quinone (7) to form brown-black pigment (eumelanin). Thus, tyrosinase, TRP-1 and TRP-2 have been recognized as three critical regulators in melanin biosynthesis.

As an essential factor for melanocyte growth and differentiation, microphthalmia-associated transcription factor (MITF) has been previously studied in melanogenesis. MITF is believed to activate the tyrosinase, TRP-1, and TRP-2 promoters by binding to the M- or E-box consensus motif (8,9).

Several signaling pathways have been identified to play positive or negative roles in melanogenesis. For instance, PKA activated by cyclic AMP (cAMP) translocates to the nucleus where it phosphorylates the cAMP responsive element-binding protein (CREB). The phosphorylated CREB then binds to the CRE site on the MITF promoter, interacts with CREB binding protein (CBP) to increase the expression of MITF, and eventually causes melanogenesis (10,11). However, the ERK and Akt signaling pathways have been shown to negatively regulate melanogenesis in melanocytes (12,13). Inhibition of ERK and PI3K/Akt can cause the stimulation of melanogenesis (14,15).

Exposure to the sun occurs with deliberate tanning for cosmetics purposes. However, UV-induced tanning may cause

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Key words: Melia azedarach extract, melanin, tyrosinase, tyrosinase-related protein 1

skin cancer, make skin age and wrinkle more rapidly, mutate DNA, and impair the immune system (16-18). Thus, a highly photoprotective tan that does not require skin damage may be highly beneficial.

Vitiligo is a condition that causes depigmentation of parts of the skin. It occurs when melanocytes die or are unable to function. The exact cause of vitiligo remains unknown, although studies suggest that it may arise from autoimmune, genetic, oxidative stress, or viral causes (19,20). Exposing the skin to UVB light from UVB lamps is the most common treatment for vitiligo (21). However, due to the higher risks of skin cancer by UVB, the usage of UVB for the treatment of vitiligo should be carefully applied. Thus, the development of new methods of treatment and new compounds exhibiting strong melanogenesis effects and fewer side effects is crucial.

Melia azedarach (MA) is a species of a deciduous tree that is native to Korea, China and Southeast Asia. It has been used in folk medicine for the treatment of several diseases. Many constituents including limonoids, triterpenoids, and steroids have been isolated from various parts of MA (22,23). In the present study, we investigated the effect of an ethanol extract of MA on melanin synthesis and the underlying molecular mechanisms in B16F10 cells.

Materials and methods

Reagents. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), forskolin and L-DOPA, were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Antibodies against tyrosinase, TRP-1, TRP-2 and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Preparation of the MA extract. MA collected from Korea was cut into 5-cm sections, dried in the dark at room temperature, and stored in a dark and cold room until needed. The dried plant material was then extracted with 70% (v/v) ethanol (5 times as much as the weight of the dried material) for 72 h at 25˚C. The plant extract was passed through 0.45-μm filter paper and then evaporated at 60˚C, after which the viscous residue was lyophilized to yield the product. In this experiment, DMSO was used to dissolve the product for making stock.

Cell culture. B16F10 mouse melanoma cells (from the Korean Cell Line Bank, Seoul, Korea) were cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM), supplemented with glutamine (2 mmol/l), penicillin (400 U/ml), streptomycin (50 g/l), and 10% FBS at 37˚C in a humidified atmosphere containing 5% CO_2.

Cell viability. An MTT assay was performed to examine cell viability following MA extract treatment. B16F10 cells were incubated overnight with DMEM (phenol red-free) containing 10% FBS. The cells were then treated with different concentrations of the MA extract for 24 h. Following treatment, MTT (dissolved in PBS to 0.5 g/l) was added. The cells were then incubated at 37˚C for an additional 4 h, the supernatant was removed and DMSO was added to dissolve the formazan crystals. Optical absorbance was determined at 570 nm with a microplate spectrophotometer from Molecular Devices, Inc. (Sunnyvale, CA, USA).

Measurement of melanin content. B16F10 cells were incubated overnight with DMEM (phenol red-free) containing 10% FBS. The cells were treated with the MA extract or forskolin at different time points. After incubation, the cells were collected and washed twice with PBS. Centrifugation (Centrifuge 5424R; Eppendorf, Hamburg, Germany) at 15,000 x g for 15 min was performed and the melanin pellets were dissolved in 1 N NaOH containing 20% DMSO for 30 min at 95˚C. The mixed homogenate (100 μl) was placed in a 96-well microplate and optical densities (ODs) were measured at 405 nm. The protein concentration of each sample was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Relative melanin production was calculated by normalizing the OD values with the protein concentrations (absorbance/μg protein).

Measurement of tyrosinase activity. B16F10 cells were incubated with the MA extract and forskolin at the indicated time points. Cell were then washed with ice-cold PBS, and lysed with PBS containing 1% Triton X-100. After centrifugation at 15,000 x g for 15 min, the supernatants were collected. The amount of each cell lysate was adjusted with lysis buffer to yield the same protein concentration. Then, 90 μl of each lysate and 10 μl of 10 mmol/l L-DOPA were added in the well of a 96-well plate. The control wells contained 90 μl of lysis buffer and 10 μl of 10 mmol/l L-DOPA. Absorbance was measured at 475 nm after the wells were incubated at 37˚C for 30 min.

Western blot analysis. For tyrosinase, TRP-1, TRP-2 and actin protein expression analysis, B16F10 cells were treated with MA extract, and collected at 2 and 4 h. B16F10 cells were then lysed in cell lysis buffer [20 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 1% Triton X-100, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l β-glycerophosphate, 1 mmol/l Na_3VO_4, 1 mmol/l dithiothreitol, 0.01 g/l leupeptin, and 1 mmol/l PMSF]. Total protein (30 μg) in sample buffer was loaded in 10% SDS-polyacrylamide gels. Separated proteins were transferred onto PVDF membranes (Roche, Mannheim, Germany), which were then saturated with 5% dry milk in Tris-buffered saline containing 0.4% Tween-20. The membranes were incubated with the appropriate primary antibodies against tyrosinase, TRP-1, TRP-2 and actin, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Blotting proteins were visualized by enhanced chemiluminescence (Amersham Bioscience, Buckinghamshire, UK).

Total RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) and 1 μg of total RNA was converted to cDNA using a First Strand cDNA Synthesis kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. Quantification of TRP-1 and endogenous reference GAPDH cDNA was performed using a 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The SYBR Premix Ex Taq™ (Takara Bio Inc., Shiga, Japan) was used in all the samples and reactions were carried out in a 10 μl final reaction volume. TRP-1 and GAPDH primer sequences were designed as follows: mTRP-1 forward, 5'-GCT GCA GGA GCC TTC

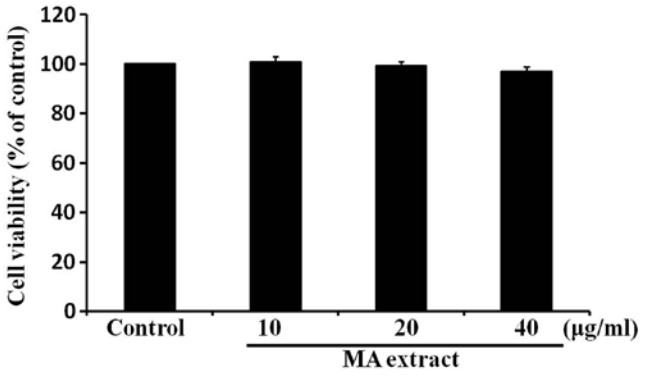

Figure 1. Effect of the *Melia azedarach* (MA) extract on cell viability in B16F10 cells. B16F10 cells were treated with the MA extract at the indicated concentrations. Cell viabilities were measured using MTT assay at 24 h after treatment. Control, vehicle only as a control. Data are presented as the means ± SD of three independent experiments carried out in triplicate.

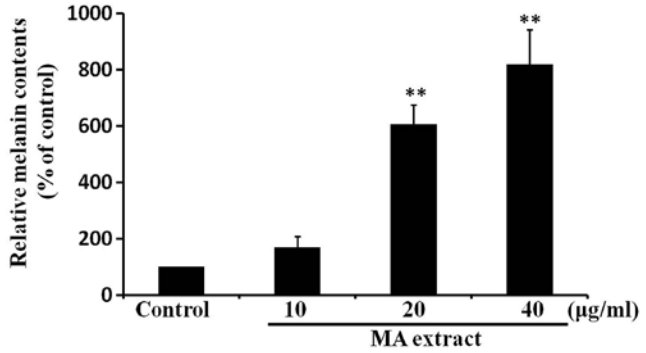

Figure 2. Effect of the *Melia azedarach* (MA) extract on melanogenesis in B16F10 cells. B16F10 cells were treated with the MA extract at the indicated concentrations. Relative melanin contents were measured at 24 h after treatment. Control, vehicle only as a control. Data are presented as the means ± SD of three independent experiments carried out in triplicate. **P<0.01 vs. control.

TTT CTC-3' and reverse, 5'-AAG ACG CTG CAC TGC TGG TCT-3'; mGAPDH forward, 5'-GAT GCC CCC ATG TTT GTG-3' and reverse, 5'-ACA ACC TGG TCC TCA GTG-3'. The PCR cycling conditions were 50˚C for 2 min and 95˚C for 2 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. Data were analyzed using the 2[-ΔΔC(T)] method (24). Data were presented as the means ± SD normalized to GAPDH and relative to the control sample. The experiments were carried out in triplicate.

Statistical analysis. Statistical significance was performed using the Student's t-test. The results are presented as the means ± SD. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

MA extract does not a cause cytotoxic effect on B16F10 cells. To determine whether the MA extract has a cytotoxic effect on B16F10 cells, we treated B16F10 cells with the MA extract for 24 h at various concentrations, ranging from 10 to 40 μg/ml. Cell viability was determined by the MTT assay. The result showed that the MA extract had no significant effect on cell viability (Fig. 1). This result indicated that the MA extract was not cytotoxic to B16F10 cells at the concentrations used in the present study.

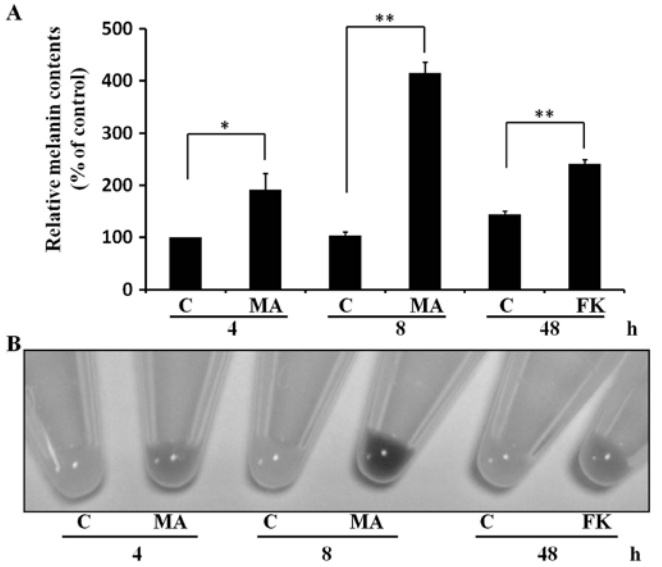

Figure 3. Effect of the *Melia azedarach* (MA) extract on melanogenesis in B16F10 cells. B16F10 cells were treated with the MA extract (20 μg/ml) and forskolin (FK) (20 μM). Relative melanin contents were measured at 4 and 8 h after the MA extract treatment and 48 h after the FK treatment. (A) Determination of relative melanin contents. (B) Images of pellets of B16F10 cells after harvest. C, vehicle only as a control. Data are reported as the means ± SD of three independent experiments carried out in triplicate. *p<0.05 and **p<0.01 vs. control.

MA extract induces melanogenesis in B16F10 cells. To assess the effects of the MA extract on melanogenesis, we measured intracellular melanin contents at 24 h after the treatment of B16F10 cells with the MA extract (10, 20 and 40 μg/ml). We found that melanin contents were significantly increased in a dose-dependent manner by the MA extract treatment (Fig. 2). Taken together, these results indicated that the MA extract induces melanogenesis in B16F10 cells.

MA extract induces melanogenesis as early as at 4 h after treatment in B16F10 cells. Since we found that the MA induced melanogenesis at 24 h after treatment, we determined whether MA can induce melanogenesis at early time points <24 h in B16F10 cells. To compare the melanogenic response ability, we treated cells with the well-known melanogenesis inducer, forskolin (20 μM). Of note, we found that the MA extract (20 μg/ml) induced melanogenesis as early as at 4 h, and the melanogenic effect was more obvious at 8 h (Fig. 3). We also observed that forskolin (20 μM) induced melanogenesis at 48 h (Fig. 3) but not at <24 h (data not shown). Thus, the data indicated that the MA extract induced melanogenesis rapidly in B16F10 cells.

MA extract does not affect tyrosinase activity in B16F10 cells. To investigate possible mechanisms responsible for the increase in melanogenesis after 4 h of treatment with the MA extract in B16F10 cells, the effect of the MA extract on tyrosinase activity was examined. We treated B16F10 cells with the

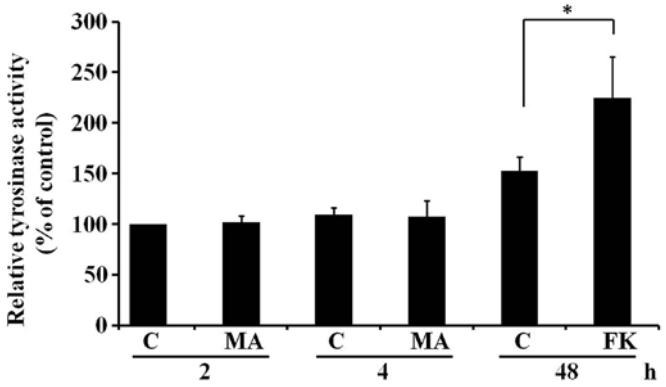

Figure 4. Effect of the *Melia azedarach* (MA) extract on tyrosinase activity in B16F10 cells. B16F10 cells were treated with the MA extract (20 μg/ml) and forskolin (FK) (20 μM). Intracellular tyrosinase activities were measured at 2 and 4 h after the MA extract treatment and 48 h after FK treatment. C, vehicle only as control. Data are presented as the means ± SD of three independent experiments carried out in triplicate. *P<0.05 vs. control.

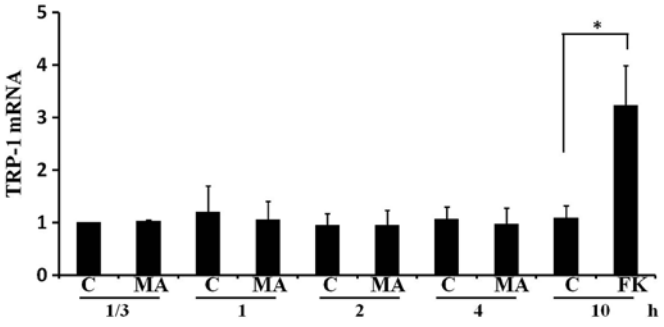

Figure 6. Effect of the *Melia azedarach* (MA) extract on tyrosinase-related protein-1 (TRP-1) mRNA levels in B16F10 cells. B16F10 cells were treated with 20 μg/ml of the MA extract and collected separately at the indicated times. Forskolin (FK) (20 μM) was used as a positive control. mRNA level of TRP-1 was measured by RT-qPCR. C, vehicle only as a control. Data are presented as the mean ± SD of three independent experiments carried out in triplicate.*P<0.05 vs. control.

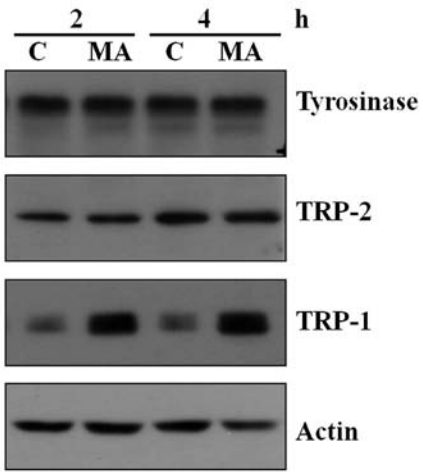

Figure 5. Effect of the *Melia azedarach* (MA) extract on the protein expression of tyrosinase, tyrosinase-related protein-1 (TRP-1) and TRP-2 in B16F10 cells. B16F10 cells were treated with the MA extract at 20 μg/ml. Protein expression of tyrosinase, TRP-1 and TRP-2 was analyzed at 2 and at 4 h after treatment by western blotting. Equal protein loadings were confirmed using anti-actin antibody. C, vehicle only as a control. Data shown are representative of three independent experiments.

MA extract at 20 μg/ml for 2 and 4 h. We then measured intracellular tyrosinase activities. Forskolin, which is known to induce intracellular tyrosinase activity, was used as a positive control. Fig. 4 shows that the MA extract had no effect on intracellular tyrosinase activity at 2 and 4 h. Thus, the results suggested that the MA extract induced melanogenesis at 4 h in B16F10 cells without affecting intracellular tyrosinase activity.

MA extract increases TRP-1 protein expression in B16F10 cells. Tyrosinase, TRP-1 and TRP-2 are known as three critical regulators in melanin biosynthesis. Since the MA extract did not affect tyrosinase activity in B16F10 cells, we examined the protein expression of the three factors after the MA extract treatment by western blotting. We found that treatment of the MA extract did not affect tyrosinase and TRP-2 protein expression in samples collected at 2 and 4 h (Fig. 5). However, the MA extract obviously increased TRP-1 protein expression at the two time points (Fig. 5).

MA extract does not increase TRP-1 mRNA levels in B16F10 cells. We examined the effect of the MA extract on the mRNA level of TRP-1 in B16F10 cells. Fig. 6 shows that the MA extract did not affect TRP-1 mRNA level in samples collected at 1/3, 1, 2 or 4 h. As a positive control, forskolin significantly increased the TRP-1 mRNA level at 10 h. The results suggested that the MA extract increased TRP-1 protein expression without affecting its mRNA level.

Discussion

Several compounds and plant extracts are capable of increasing melanogenesis in melanocytes and melanoma cells. It has been shown that human placental lipid (25), sesamin (26), quercetin (27), and extracts from *Erica multiflora* (28) and *Pyrostegia venusta* (29) can increase melanogenesis *in vitro*. Thus, these compounds and extracts can be considered as candidates for developing tanning cosmetics and medicines for vitiligo.

Many constituents derived from MA have been identified and studied. A limonoid isolated from MA has been reported to possess anti-feeding and insecticidal activities (30). Moreover, several other constituents from MA possess anti-herpetic (31), anti-angiogenic (32) and anticancer (33) properties. However, despite previous findings, the effect of MA on melanogenesis has not been reported. To identify new agents for developing tanning cosmetics and treating hypopigmentation disorders such as vitiligo, we investigated the effect of an extract from MA on melanogenesis in B16F10 cells.

The effects of the MA extract on melanogenesis were assessed using melanin content assay, intracellular tyrosinase activity assay, Western blotting, and PCR. First, we showed that the MA extract increased melanin synthesis in a concentration-dependent manner without cytotoxicity (10-40 μg/ml) in B16F10 cells. Second, we found that melanogenesis of B16F10 cells by the MA extract (20 μg/ml) was rapid and that

the pellet color of cells was enhanced within 4 h after treatment. To compare the melanogenic effect of MA with other agents, we used the well-known melanogenic inducer forskolin at a concentration (20 μM) that is higher than the concentrations (5 or 10 μM) usually used in other groups (34,35). After incubation with the MA extract (20 μg/ml) for 8 h, the pellet color was darker than that incubated with forskolin (20 μM) for 48 h (Fig. 3B). Since melanogenesis was observed within 4 h after the MA extract treatment, we focused on the mechanism that the MA extract induced melanogenesis within 4 h. Tyrosinase is an important enzyme involved in melanogenesis (5). We examined the activity and protein expression of tyrosinase. The MA extract did not affect tyrosinase activity within 4 h. Additionally, the MA extract did not affect tyrosinase protein expression. Tyrosinase is not the only important enzyme involved in melanogenesis, with TRP-1 and TRP-2 also playing essential roles (6,7). We observed that the protein expression of TRP-2 was not altered but that of TRP-1 was obviously increased after the MA extract treatment (Fig. 5). The function of TRP-1 in melanogenesis seems to be distinct with tyrosinase and TRP-2. The specific melanogenic function of TRP1 is the oxidation of DHICA to a carboxylated indole-quinone at the downstream point in the melanin biosynthetic pathway. TRP-1 activity is essential to further the metabolism of DHICA to a high molecular weight pigmented biopolymer (7). Despite the protein expression level of TRP-1 was obviously induced by the MA extract treatment, our results have shown that the mRNA level of TRP-1 was not affected. Thus, the overall results suggest that the MA extract increases melanogenesis via the upregulation of TRP-1 protein expression by post-transcriptional control in B16F10 cells.

In conclusion, the present findings have shown a rapid melanogenic effect of an ethanol extract of MA and the underlying mechanisms involved in the process in B16F10 cells. The results indicate that the MA extract may be useful for the development of self-tanning cosmetics products and the treatment of hypopigmentation disorders including vitiligo.

Acknowledgements

We would like to thank Bioland Co., Ltd. (Korea) for preparing reagents. This study was partially supported by a grant from the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (grant no. 2011-0029819) and by a grant of the Korean Health Technology R&D Project, Ministry of Health and Welfare, Republic of Korea (grant no. A121851).

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