Natural Product Sciences
22(3) : 168-174 (2016)
http://dx.doi.org/10.20307/nps.2016.22.3.168

Modulation of Melanin Synthesis by Amaranthus spp. L Seed Extract in Melan-a Cells

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Abstract – Anti-melanogenic effects of amaranth (AT), one of the key source of squalene, were investigated in melanocytes. Amaranth seed powder was extracted with water and melan-a cells were treated with various concentrations of AT. By using HPLC, content of *myo*-inositol, one of potential active components, was measured in the crude extract of AT.AT reduced the melanin content in melan-a melanocytes and down-regulated melanogenic enzyme activity such as tyrosinase, TRP-1 and TRP-2. By regulating melanogenic enzyme activity, AT may be a potential natural source for whitening agent. *Myo*-inositol was detected in AT by HPLC and may be one of the active compounds from AT involved in the regulation of anti-melanogenesis. In this study, we demonstrated that AT has anti-melanogenesis properties. This new function of amaranth may be useful in the development of new skin-whitening products and its value as food.
Keywords – Amaranth, *Myo*-inositol, Melan-a cells, Melanogenesis

Introduction

Melanosomes are biosynthesized in melanocytes in the basal layer of the epidermis. They contain melanin that protects the skin against harmful environments.[1-3] Melanosomes are transferred to keratinocytes through their dendrites and accumulate in the form of granules in the nucleus.[4] Although melanin plays important roles in melanogenesis, melanin overproduction can also cause hyper-pigmentation, melasma, freckles, and skin cancers. Therefore, the use of natural products that protect the skin is a common issue in dermatologic clinics and cosmetic research.[5]

Melanin is produced via several melanogenic enzymes, including tyrosinase, tyrosinase-related protein 1 (TRP-1), tyrosinase-related protein 2 (TRP-2), and microphthalmia-associated transcription factor (MITF).[6] MITF presents two binding sites, M-box and E box, stimulates the expression of melanogenic genes, and regulates the expression of the alpha melanocyte-stimulating hormone (α-MSH) and the cAMP pathway.[7,8] When α-MSH is stimulated in melanosomes, tyrosinase, TRP-1, and TRP-2 are activated.[9,10] Especially, tyrosinaseis an important enzyme in the melanin synthesis pathway because it triggers the melanogenic process.[11] It is responsible for the hydroxylation of L-tyrosine to L-β-3,4-dihydroxyphenylalanin (DOPA) and oxidation of DOPA to DOPA quinone.[12,13] DOPA quinone has the ability to transform melanin to pheomelanin or eumelanin.[14] TRP-2 serves as a dopachrometautomerase and inverts dopachrome to 5, 6-dihydroxyindole-2-carboxylic acid (DHICA). Subsequently, TRP-1 oxidizes DHICA to indole-5,6-quinone-2-carboxylic acid, a precursor of eumelanin.[8,14-16] Together with other melanogenic enzymes and factors, tyrosinase is one of the main enzymes for the development of skin whitening agents.[9]

Natural products cover traditional medicines and herbs. They garnered attention in the cosmetic market because they are safe and do not present side effects.[17] *Amaranthus* spp. L. (AT) has long been used as a dietary antioxidant throughout South America and Asia, including Korea, because it contains various amino acids and other important micro nutrients.[18-21] It is called the perfect food

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because it contains 60% starch, 8% fat with squalene, and 15% protein with a high concentration of lysine. Additionally, it is a gluten-free food.[22-24] Squalene is also widely used as an antioxidant, skin moisturizer, treating skin disorders like acne, psoriasis and atopic dermatitis. Therefore, it is needed to find alternative natural resources which explore the utility of squalene for skin cosmetics. Squalene, a highly unsaturated hydrocarbon, found as an important component of the liver oil of certain varieties of deep sea fish. Cosmetic company wants to use plant source to safe while actually deep sea products may not be a good source for cosmetics because of significant chemical contamination and toxicity such as mercury. Amaranth which contains about 7% lipids seems to be the key source of squalene.

Ultra violet radiation (UVR) stimulation induces the production of reactive oxygen species (ROS) and causes melanogenesis.[25] Therefore, anti-oxidative natural products may act as anti-melanogenic agents. The antioxidant activities of extracts from amaranth leaves, flowers, and seeds were previously reported.[26,27] We are to focus the research into the anti-melanogenic effects of amaranth for skin cosmetic product, which is still in its early stages. Therefore, our study was designed to investigate the anti-melanogenic activity of AT in melanocytes.

Experimental

General experimental procedures – RPMI 1640 was purchased from Gibco-BRL (Gaithersburg, MD, USA). Fetal bovine serum (FBS) and penicillin-streptomycin (PS) were purchased from Hyclone (Carlsbad, CA, USA). Phenylmethylsulfonyl fluoride (PMSF), 12-O-tetradecanoylphorbol-13-acetate (TPA), 1-phenyl-2-thiourea (PTU), kojic acid, triton X-100, aprotinin, mushroom tyrosinase, 3,4-dihydroxy-L-phenylalanin (L-DOPA), dimethyl sulfoxide (DMSO), anti-α-Tubulin antibody were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-MITF and anti-tyrosinase antibody were obtained from Cell signaling (Danvers, MA, USA). Anti-TRP-1, anti-TRP-2 antibody, Horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG and goat anti-rabbit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was purchased from Thermo scientific (Waltham, MA, USA). Santa Cruz Biotechnology)

Amaranth seed extract (AT) – Each variant of amaranth seed species was named as follows: *Amaranth uscruentus* (AA), *Amaranth uscruentus* var (AB), *Amaranth ushybridus* (AC), and the crossbreed between *Amaranth ushybridus* and *Amaranth ushypocondriacus* (AD). Amaranth seeds were air dried for 3 days at 40 °C to maintain their germinative power. Powdered amaranth seeds were extracted 3 times in distilled water. After freeze drying, the extract was fractionated with hexane and ethyl acetate using a separating funnel. The steps were present in Fig. 1.

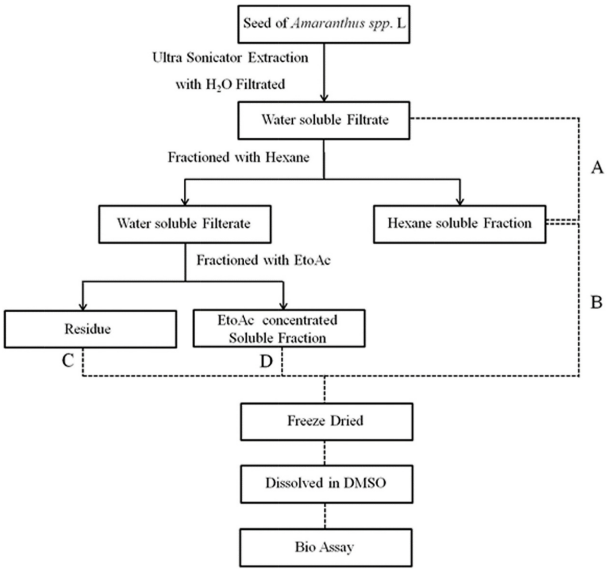


Fig. 1. Scheme of sample preparation. Simply present the steps of extraction of AT. A: *Amaranthus* spp. L Total extract, B: Hexane soluble fraction, C: Residue, D: EtoAc soluble fraction.

HPLC analysis – Analysis was carried out on a Waters system (Waters Corp., Milford, MA, USA), consisting of separation module (e2695). Evaporative light scattering detector (2424) was used to develop a method for content determination of *myo*-inositol in AD. Separation was carried out using an Asahipak NH2P-50 4E (250 × 4.6 mm; particle size, 5 um; SHOWA DENKO K.K., Japan) and Column temperature was maintained 30 °C. The mobile phase composed water and acetonitrile (25:75, v/v) and the flow rate was 1 ml/min. The temperature of drift tube was 80 °C with nitrogen as developing solvent.

Cell culture – Mouse melan-a cells were provided by Dr. Byeong Gon Lee at the Skin Research Institute, Amore Pacific Co. (Yongin, Korea). RPMI 1640 supplemented with 10% FBS, 1% PS, and 400 nM TPA was used to maintain melan-a cells. All cells were incubated at 37 °C in a humidified incubator with 5% CO_2.

Measurement of the melanin content – Melan-a cells (10,000/per well) were seeded in a 24-well plate and incubated for 72 h after being treated with AT. After 72 h, the melanin content was measured using the method reported by Hosoi *et al*. with slight modifications.[28] Briefly, after removing the medium, the cells were washed twice

with phosphate-buffered saline (PBS). One milliliter of sodium hydroxide solution (1 N) was then added to each well to dissolve the melanin content. The melanin absorbance was measured at 405 nm using a microplate reader.

Measurement of cell viability – Cell viability was determined by a 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Melan-a cells were seeded at 1×10^5 cells/well in a 24-well plate and incubated for 72 h after being treated with AT for 24 h. After 72 h, the medium was removed and 400 μL of DMSO was added and the plates were placed on a shaker for 10 min. The absorbance was then measured at 570 nm using a microplate reader.

Tyrosinase activity assay in melan-a cells – Melan-a cells were seeded at 10×10^5 cells in 100 mm dishes and incubated for 72 h. Cells were detached by using trypsin-EDTA and centrifuged. Tyrosinase buffer (80 mM phosphate buffer + 1% Triton-X 100 + 100 μg/mL PMSF) was added to the cell pellets for 1 h and centrifuged at 12,500 rpm for 20 min at 4 °C. The supernatant was used to assess the activity of tyrosinase. Cell lysate was measured to estimate the protein content using bovine serum albumin (BSA) as a standard; 150 μg of proteins were required for the reaction.

Tyrosinase activity was assessed by measuring the rate of L-DOPA oxidation according to the method reported in previous paper.[16] To estimate the inhibitory effects of AT on tyrosinase from melan-a cells, 0.1, 1, and 10 μg/mL of AT and kojic acid (positive control) were dissolved in methanol; 40 μL of each sample was then added to the wells of a 96-well plate with 120 μL of L-DOPA and 150 μg of protein. The samples were mixed, incubated for 15 min, and the absorbance was measured at 490 nm using a microplate reader.

Western blot analysis – 10×10^5 Melan-a cells were seeded in 100 mm dishes and treated with 0.1, 1, and 10 μg/mL of A.C and A.D for 3 days. Cells were washed with PBS and harvested using trypsin-EDTA. Detached cells in 1 mL of PBS were centrifuged at 7500 rpm for 5 min and PBS was removed. Cell pellets were lysed using lysis buffer (50 mM Tris-Cl, pH 8.0, 0.1% SDS, 150 mM NaCl, 1% NP-40, 0.02% sodium azide, 0.5% sodium deoxycholate, 100 μg/mL PMSF, and 1 μg/mL aprotinin) for 1 h in cold conditions. After centrifugation at 12,500 rpm for 20 min at 4 °C, the supernatant was used for western blot analysis. Protein contents were measured by using BSA as a standard. Forty micrograms of protein were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked for 1 h with 5% of skim milk in Tris-buffered saline-T (TBST) and incubated overnight with α-tubulin (1:3000 dilution), MITF (1:500 dilution), tyrosinase (1:500), TRP-1 (1:500 dilution), and TRP-2 (1:500 dilution) primary antibodies at 4 °C. After removing the primary antibody, membranes were washed 3 times with TBST and incubated with the appropriate secondary antibody for 1 h. The bands were detected with a ChemidocXRS + imaging system (Bio-Rad, Hercules, CA, USA).

Statistical Analysis – All experiments were repeated in at least three assay for ELISA and western blot. All values were presented as the mean + SD. The statistical significance of difference between the data from the dose-dependent assay was evaluated by student *t*-test, one-way analysis of variance (ANOVA). A p-value < 0.05 was considered statistically significant.

Result and Discussion

Amaranthus spp. L. may grow naturally or be cultivated. The plant is known to contain any nutrients, minerals, amino acids, and fibers. It shows anti-cancer properties, prevents cardiovascular diseases, and lowers the risk of diabetes.[29] Furthermore, there are many reports of its antioxidant activity.[19, 30-32] However, until now, there is no report on the whitening effect related to the antioxidant activity of amaranth plants. Currently, the *Amarantus* genus includes over 70 species, although the number of species is questionable due to the hybridization concept and technique. Our collaborator cultivated some amaranth geneous. For the purpose of expanding the use of amaranth cultivated in Korea, we first tried to develop amaranth products for the cosmetic industry.

In order to examine the skin-whitening effect of amaranth, we screened the effects of amaranth sub-species on the melanin content in melan-a cells. In an attempt to standardize amaranth samples, we measured *myo*-inositol in each sample by HPLC analysis. The linearity of *myo*-inositol was calculated by three concentrations. HPLC data for *myo*-inositol and the extract of Amaranthus seeds were shown in Fig. 2. The main component was *myo*-inositol and its highest amount was found in AD (49.036 ± 1.0325% ug/mg extract). *Myo*-inositol has been reported to inhibit melanin synthesis by reducing the activity of tyrosinase.[33] As shown in Fig. 2, the level of *myo*-inositol from sample is 5%. It means AT can be the potential inhibitor of tyrosinase because it highly contains *myo*-inositol. The main component of amaranth oil is squalene. It is composed of polyunsaturated lipids which is one of main component of skin surface. Therefore, amaranth oil may show advantage for usage as a natural base ingredient of cosmetics. Recently, it seems like the

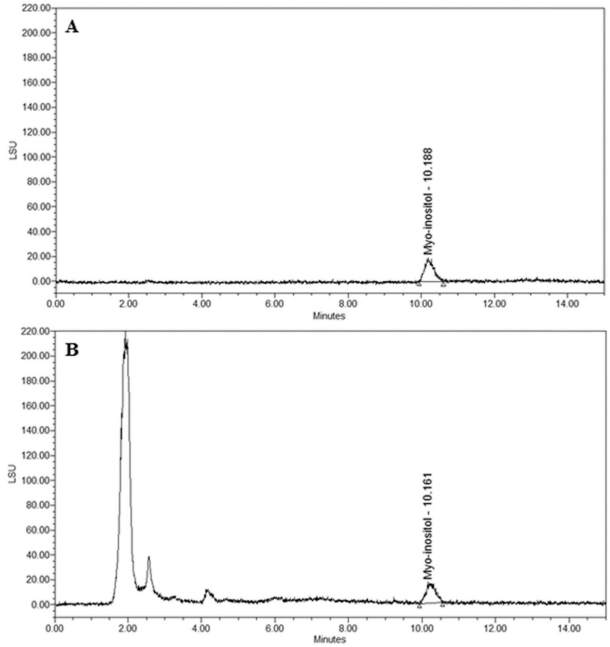


Fig. 2. HPLC chromatogram for the determination of *myo*-inositol in *Amaranth ushypocondriacus* (A.D). **A.** Chromatogram of *myo*-inositol **B.** Chromatogram of AD.

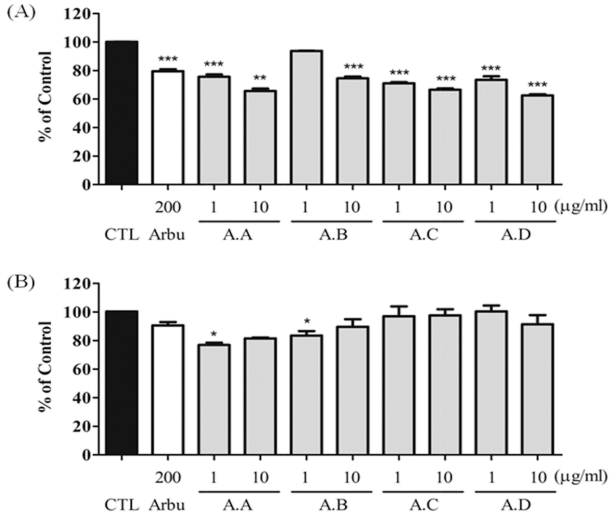


Fig. 3. Effects of Amaranth seed extract (AT) on melanin content and cell viability in melan-a cells. Melanin contents (A) and cell viability (B) were measured. CTL indicates normal conditions and Arbu indicate the use of arbutin as a positive control (μM). All sample (μg/mL) were treated for 72 h. Data are expressed as mean ± S.D. of three experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with control.

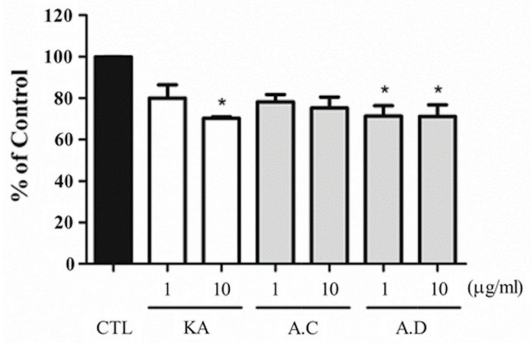


Fig. 4. Effects of AT on tyrosinase activity in melan-a cells. Melan-a cell originated tyrosinase activity was measured. CTL indicates normal conditions and KA indicate the use of kojic acid as a positive control (μM). Each samples were treated with μg/mL. The results are expressed as mean ± S.D. of three experiments (* $p < 0.05$).

cosmetic biotech are exploiting amaranth to use cosmetic products. Therefore, if anti-melanogenic action of amaranth turned, not simply providingan squalene's additional resource, it may be a potential good source for functional cosmetics.

To determine the effects of AT on melanogenesis, murine melanocytes were treated with different concentrations of AT for 72 h. Among various concentrations and samples, we selected AC and AD as representative samples and further experiments were performed.

Figure 3 indicates that AT inhibits melanin synthesis when compared with control conditions. Especially, 10 μM of AC and AD significantly reduced melanin content without cell toxicity in melan-a cells. Additionally, their anti-melanogenic effect was stronger than that of arbutin, a widely known whitening agent. These data suggest that AT affects melanogenesis and regulates melanin synthesis. Therefore, it is very interesting because it has possibility for skin whitening.

Tyrosinase is the main enzyme in melanin synthesis. It triggers the melanin synthesis pathway and affects melanogenic enzymes. It converts L-tyrosine to melanin polymers and stimulates TRP-1 and TRP-2 expression.[34] Therefore, tyrosinase activity was measured in cell or cell-free conditions.[35] To investigate whether AT inhibits tyrosinase activity, we estimated tyrosinase activity *in vitro*. Kojic acid, a well-known tyrosinase inhibitor, was used as a positive control. Compared with control, AC decreased the activity of mushroom tyrosinase, but not significantly. However, the AD treated group showed a significant decrease in tyrosinase activity. The level of inhibition of tyrosinase activity by A D was similar to that of Kojic acid (Fig. 4). Our results indicate that AD inhibits melanin formation by reducing tyrosinase activity. Additionally, its effect is similar to that of kojic acid, which is a well-known compound that decreases melanin synthesis by reducing tyrosinase activity.[36]

Interestingly, the effect of AT on the expression of

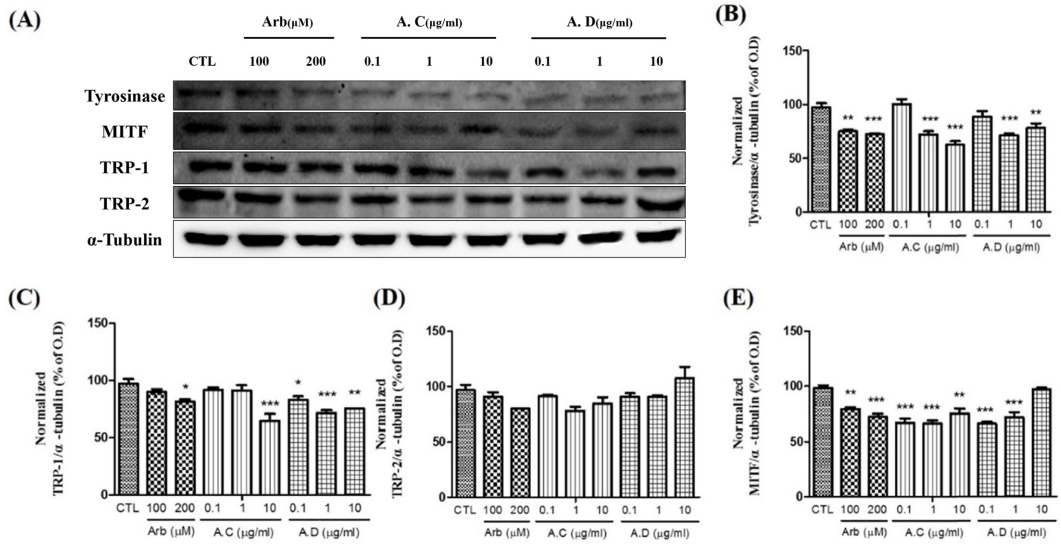


Fig. 5. Effects of *Amaranth ushybridus* (A.C) and *Amaranth ushypocondriacus* (A.D) on the expression of melanogenic enzymes in melan-a cells. To confirm the expression of melanogenic enzymes, melan-a cells were treated with 0.1, 1 and 10 μg/mL of A.C and A.D. Arbutin was used as a positive control (μM). (A) Western blot analysis. Densitometric analysis of Tyrosinase (B), TRP-1 (C), TRP-2 (D) and MITF (F). Each band was normalized to that of α-tubulin.

tyrosinase and/or TRP-2 was significantly stronger than that of arbutin.

To determine the mechanism by which AT affects melanin synthesis, we assessed the expression of melanogenic enzymes (tyrosinase, TRP-1, TRP-2, and MITF) by western blot analysis. We performed a western blot analysis after treatment with AC and AD for 3 days. As shown in Fig. 5, the expression of melanogenic enzymes decreased in a dose dependent manner after AT treatment (Fig. 5). AC and AD reduced the expression of melanogenic enzymes such as TRP-1, TRP-2, and tyrosinase, resulting in a decrease in melanogenesis.AT significantly decreased TRP-2 and tyrosinase levels when compared with control conditions. Especially, A.C reduced the expression level of TRP-2 to 35.2%. Although there was no effect on MITF expression, the expression of other melanogenic enzymes was decreased by AT treatment. Thus, we can conclude that AT decreases melanin synthesis by downregulating the expression of TRP-2 and tyrosinase. And, continuously, to determine an active component of AT, we tried to make subfraction of AT. Among them, hexane-soluble and water-soluble subfraction from AT showed the decrease of melanin in melan-a cells. It is well soluble in water, therefore, we can suggest that anti-melanogenic activity of water soluble fraction from AT is derived from *myo*-inositol.

This study was designed to determine the anti-melanogenic effects of AT. Among amaranth species, especially, *Amaranth ushybridus* significantly reduced the melanin level in melan-a cells by downregulating melanogenic enzymes such as tyrosinase and TRP-2.

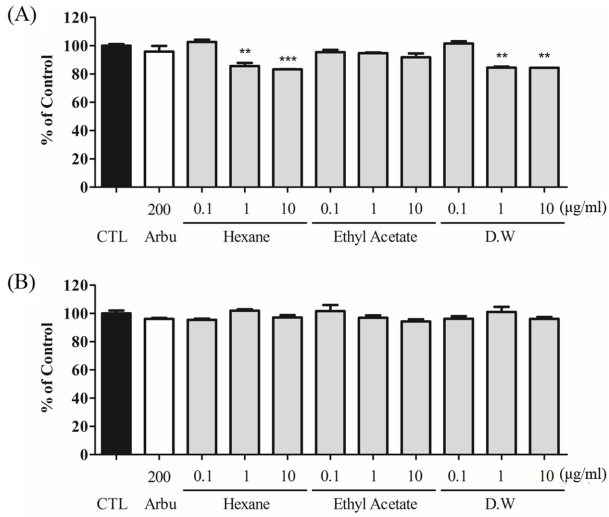
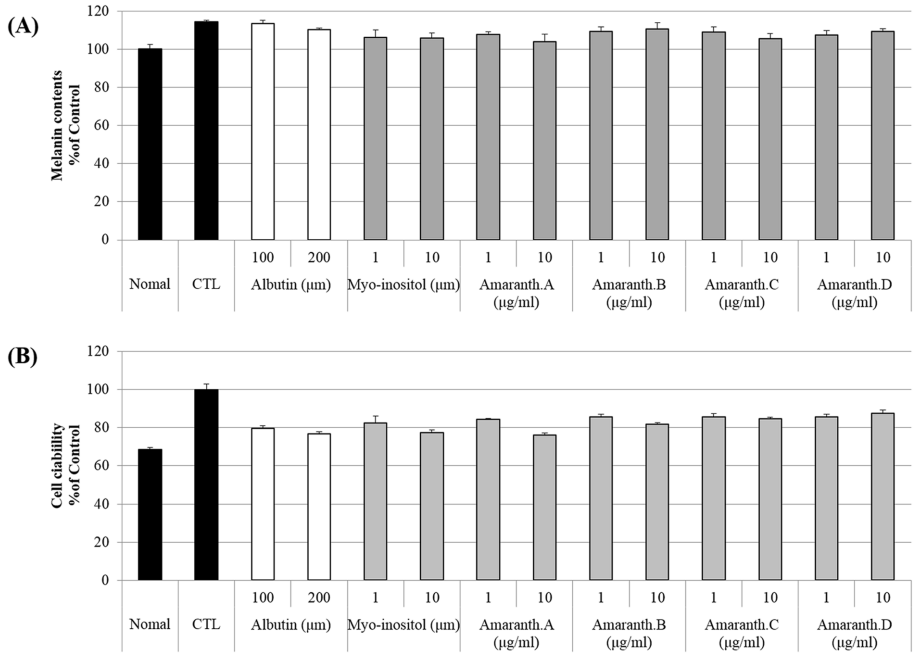


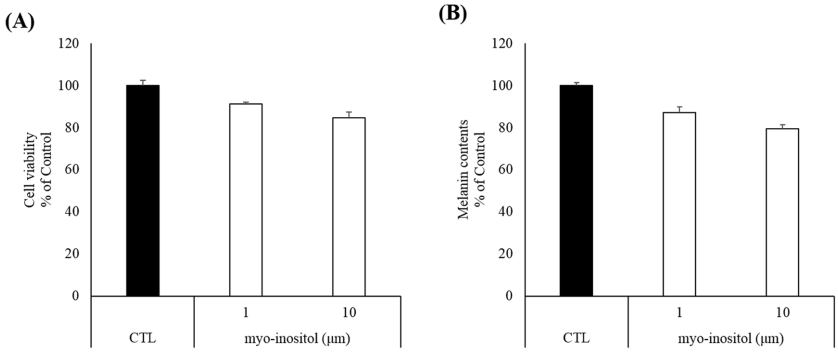
Fig. 6. Effects of each fraction of amaranth seed extract (AT) on melanin content and cell viability in melan-a cells Melanin contents (A) and cell viability (B) were measured. CTL indicates normal conditions and Arbu indicate the use of arbutin as a positive control (μM). All sample (μg/mL) were treated for 72 h. Data are expressed as mean ± S.D. of three experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with control.

Table 1. Contents (μg/mg) of *myo*-inositol in *Amaranth ushypocondriacus* (A.D).

	r^2	Content
Myo-inositol	0.9955	49.036 ± 1.032



Supplement 1. Effects of Amaranth species on melanin content and cell viability in mouse melanoma B16F10 cells. Cell viability (A) and melanin contents (B) were measured. B16F10 cells were pre-stimulated by α-MSH (100 ng/ml). After treatment of α-MSH for 30 min, all sample were treated for 72 h.



Supplement 2. Effects of *myo*-inositol on melanin content and cell viability in Melan-a cells. Cell viability (A) and melanin contents (B) were measured. *myo*-inositol was treated for 72 h.

Our results support the view that amaranth species may be used as a nutricosmetic food as well as a potential skin-whitening agent. Specially, the amaranth as a nutricosmetic agent has merits in respective of safety. In fact, amaranth is one of the major dietary sources in South America. However, further epidemiological studies are necessary to determine the safety and impact of amaranth on the skin. Therefore, further *in vivo* and clinical studies are warranted to determine the main active ingredient in amaranth, which is involved in its skin-whitening property.

In conclusion, this study suggests that amaranth may be a potential hypo-pigmentatory agent from natural products. Furthermore, it can be used for nutricosmetical resources.

Acknowledgments

This work was carried out with the support of "Cooperative Research Program for Agriculture Science & Technology Development (No. PJ01118803)" Rural Development Administration, Republic of Korea.

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Received November 5, 2015
Revised February 23, 2016
Accepted February 29, 2016