

## Original Article

**Inhibitory effects of *Taraxacum mongolicum* with phreatic water on melanin synthesis**Moon-Hee Jang<sup>a,b</sup>, Taek-Won Ahn<sup>a,b,\*</sup><sup>a</sup> Department of Sasang Constitutional Medicine, Cheonan Oriental Hospital of Daejeon University, Cheonan, Korea<sup>b</sup> Hot Spring Medicine Laboratory, Daejeon University, Cheonan, Korea

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## ABSTRACT

**Background:** Recently, people have begun showing heightened interest in skin whitening. Melanin is an important factor that determines skin color. The purpose of this study is to investigate the inhibitory effect of *Taraxacum mongolicum* (TAM) with phreatic water (PW) from Dogo Hot Springs on melanin synthesis.

**Methods:** We assessed the inhibitory effects of TAM on melanin synthesis in B16F10 mouse melanoma cells. The mRNA levels of tyrosinase related protein (TRP)-1, TRP-2, tyrosinase, MITF, ERK, and PKA protein were analyzed with reverse transcription polymerase chain reaction and Western blot analysis. We also assessed the inhibitory effects of TAM with PW on melanin synthesis in HRM-2 melanin-possessing hairless mice. After UVB irradiation, differences in melanin were analyzed with an image analysis software between the left dorsal skin (untreated part) and the right dorsal skin (treated part). The mRNA levels of TRP-1, TRP-2, and matrix metalloproteinase (MMP)-9 were analyzed with real-time quantitative polymerase chain reaction. The dorsal skins were analyzed with histological test by hematoxylin and eosin staining.

**Results:** TAM inhibited the TRP-1, TRP-2, tyrosinase, MITF mRNA gene expression, and PKA protein expression on the concentration-dependent B16F10 cell. Moreover, TAM increased the ERK mRNA gene expression in the B16F10 cell. After UVB irradiation, TAM with PW increased the differences in melanin between the left dorsal skin (untreated part) and the right dorsal skin (treated part) in HRM-2 mice. TAM with PW inhibited the TRP-1, TRP-2, and MMP-9 mRNA gene expression in HRM-2 mice. TAM with PW decreased the epidermal thickness, around the cell deformation, keratinization, and infiltration in HRM-2 mice.

**Conclusion:** These results indicate that TAM with PW has the inhibitory effect of decreasing the melanin synthesis.

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## 1. Introduction

As people are increasingly exposing their skin to UV radiation, skin damage and photoaging by UV rays have also been increasing because of environmental pollution, and consequently people are taking a heightened interest in skin whitening.<sup>1</sup>

The major elements determining human skin color are blood volume and hemoglobin distributed in the blood vessels, melanin, and carotene. Moreover, keratin layer thickness, personal difference, seasonal status, and health status also affect skin color.<sup>2</sup> Among these factors, melanin, which is synthesized by the melanosome of the melanocyte residing in the basal skin layer, moves to the skin keratinocyte through the dendrite and exhibits skin color as well as demonstrates positive functions in skin protection from the damaging effects of UV exposure. However, excessively generated melanin creates abnormal hyperpigmentation such as melasma, freckles, and skin spots.<sup>3,4</sup> Therefore, to verify the whitening effect, it is important to figure out the inhibitory degree of melanin pigment generation in the melanin synthesizing cell.<sup>2</sup>

Recent studies on whitening effect include the study on tyrosinase activation inhibition, the study on action control of the messenger ordering melanin synthesis to the melanocyte, the study on the material promoting keratin layer removal and blocking UV rays, and the study on the active oxygen removing material.<sup>2,5</sup> However, materials (such as hydroquinone, kojic acid, arbutin, vitamin C, glutathione, salicylic acid, and resorcin) known to be effective through these studies exhibit limitations such as cytotoxicity, safety problems, and skin penetration. Consequently, studies are widely conducted on using more effective and stable medicinal herbs to prove their whitening effect and to use them as whitening cosmetics and medical substances.<sup>5-7</sup>

For whitening studies of medicinal herbs, there are studies on Radix glycyrrhizae Water Extract ( ),<sup>8</sup> Schizandrae Fructus ( ),<sup>9</sup> and Belamcandae Rhizoma ( ).<sup>10</sup> Among herbal medicine prescriptions, the whitening effects of Kakamseosikyong-san ( ),<sup>11</sup> Kamigwibi-tang ( ),<sup>12</sup> and Dokhwalkisaeng-tang ( )<sup>13</sup> have been studied.

*Taraxacum mongolicum* ( , TAM) is the turning of dandelion, a perennial herb that belongs to the Compositae family. Its nature is cool, and its taste is bitter and sweet. It is used for the hepatogastric pill. It also detoxifies rashes caused by heat and removes carbuncles, making it a useful medicine commonly used on all kinds of surgical rashes, carbuncles, and rash symptoms.<sup>14</sup>

Studies on TAM have been continuously conducted to determine its anti-inflammatory efficacy,<sup>15</sup> antiallergenic efficacy,<sup>16</sup> and antioxidant efficacy.<sup>17</sup> However, to date only a few studies have focused on the whitening effect of TAM.<sup>18,19</sup>

Accordingly, to verify the whitening effect of TAM, this study identified its whitening content using high-performance liquid chromatography (HPLC) analysis, and using B16F10 mouse melanoma cells, we identified the inhibitory effect of TAM on melanin generation by verifying the gene expression inhibition of the principal enzymes

involved in melanin generation and the signal transfer mechanism associated with melanin generation. Although the whitening effect of phreatic water (PW)<sup>20,21</sup>—which is widely used for the treatment of skin disorders such as psoriasis, atopic dermatitis, pimples, and xeroderma—has not been studied yet, this study tried to verify if PW, together with TAM, enhances the inhibitory effect on melanin generation. For PW, we used the water from Dogo Hot Springs, whose sulfuric content is known to effectively control allergic inflammatory responses (see the recent study of Jang et al.<sup>22</sup>). UVB was irradiated on HRM-2 melanin-possessing hairless mice. Here, we report that significant results were obtained from the study using the image analysis software on pigmentation, the analysis on gene expression of principle enzymes associated with melanin generation, and the histological skin analysis.

## 2. Methods

### 2.1. Cell strain and cell culture

B16F10 mouse melanoma cells (ATCC, Manassas, VA, USA) were used as experimental cell strains. These were floated in Dulbecco's modified Eagle medium, to which 10% fetal bovine serum and 1% penicillin/streptomycin (100 U/ml) were added, and adapted in the 37 °C, 5% CO<sub>2</sub> incubator for subculture.

### 2.2. Herb

The TAM used in this experiment was purchased from Omniherb ([www.omniherb.com](http://www.omniherb.com)), Daegu, Korea and carefully selected. Its origin is Gyeongsangbuk-do in Korea.

### 2.3. TAM extract separation

First, 200 g of TAM powder used in this experiment was immersed in 30% ethanol (1.5 l) in the ASE300 Accelerated Solvent Extractor (Dionex Co., Sunnyvale, CA, USA) for 3 days, and the extract liquid was extracted for 20 minutes at 50 °C. The extract filtrate liquid obtained from the extract liquid through filtration and decompressed drying was frozen and dried for 24 hours at -70 °C for cold trap and at 40 °C of the chamber temperature in the freezing dryer (Biotron Co., Bucheon, Gyeonggi, Korea) to obtain 18 g of extract.

### 2.4. Animal

The animals used in this experiment consisted of 20–22 g HRM-2 melanin-possessing hairless male mice parceled from Hoshino Laboratory Animals Co. Ltd. (Saitama, Japan). They were fully fed with antibiotic-free pellet feed (Samyang Feed, Seoul, Korea) and water, and adapted for a week at the temperature 22 ± 2 °C and relative humidity of 55 ± 15% in the animal breeding room where the 12-hour light–dark cycle was maintained prior to use.

For ethical and scientific feasibility study and efficient control of animal experiments, this experiment was approved by the Institutional Animal Care and Use Committee of Daejeon University, Cheonan, South Korea (approval no. DJUARB2012-019).

## 2.5. PW

The natural PW was collected from Paradise Spa Dogo, Korea. Component analysis on the PW of Dogo Hot Springs had been conducted in the study of Jang et al.<sup>22</sup>

## 2.6. Experimental drugs and instruments

Experimental drugs used for the whitening effect measurement were tyrosinase mushroom, dimethyl sulfoxide and L-3,4-dihydroxy-phenyl-alanine (L-DOPA) purchased from Sigma Chemical Co. (St. Louis, MO, USA). Those used for polymerase chain reaction (PCR) were extracellular regulated kinase (ERK), cAMP responsive element binding protein (CREB), microphthalmia-associated transcription factor (MITF), tyrosinase, tyrosinase related protein-1 (TRP-1), tyrosinase related protein-2 (TRP-2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), primary antibody, and secondary antibodies such as mouse-antigoat and rabbit-antimouse purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

For cell culture, Dulbecco's modified Eagle medium, fetal bovine serum, phosphate buffered saline, penicillin/streptomycin, and trypsin were purchased from Thermo Scientific Hyclone (Logan, UT, USA) and Gibco BRL Co. (Grand Island, NY, USA). For cytotoxicity measurement, a Haemacytometer (Marienfeld-Superior, Lauda-Königshofen, Germany) and 3-[4,5-dimethylthiazol]-2-yl]-2,5-diphenyl-tetrazoliumbromide (MTT) were purchased from Sigma Chemical Co.. In addition, diethyl pyrocarbonate (DEPC), chloroform, trichloroacetic acid, isopropanol, Tris-HCl, KCl, and MgCl<sub>2</sub> were purchased from Sigma Chemical Co.. For the HPLC reference sample, the Luteolin product was purchased from Sigma Chemical Co.. For other experimental drugs, first-class experimental drugs were used.

The TAM analysis HPLC (Waters 2695 system; Waters Co., Milford, MA, USA) was the Agilent 1200 series and used the autosampler, the column oven, the binary pump, the DAD detector (Agilent Technologies, Santa Clara, CA, USA), and the degasser (Agilent Technologies, Santa Clara, CA, USA). The software used was Chemstation software (Agilent Technologies, Santa Clara, CA, USA).

Instruments used in this experiment included the hot water extractor (DWT-1800T; Daewoong, Seoul, Korea), the reduced pressure distiller [EYELA (Tokyo, Japan)/BUCHI B-480 (Flawil, Switzerland)], the centrifuge (Hanil Science Industrial, Incheon, Korea), the freeze dryer [ilShinBioBase, (Dongduchun, Gyeonggi, Korea)/EYELA FDU-540], the microscope (Olympus, Tokyo, Japan), the CO<sub>2</sub> incubator (Vision Scientific, Daejeon, Korea), the vortex (Scientific Industries, Inc., Bohemia, NY, USA), the pH meter (Mettler-Toledo AG, Greifensee, Switzerland), the BOD incubator (Daihan Scientific, Wonju, Gangwon, Korea), the autoclave (JS Research Inc., Gongju, Chungnam, Korea), the ELISA Reader (Tecan, Männedorf, Switzerland), the PCR (C-100, Bio-Rad Laboratories, Hercules, CA, USA), the plate shaker (Lab-Line, San Diego, CA, USA), the spectrophotometer (Shimadzu, Kyoto, Japan), the biofreezer (Sanyo Electric Co., Osaka, Japan), the Applied Biosystems® real-time quantitative (RQ) PCR (Life

**Table 1 – TAM cream formulation**

	2% TAM + DW cream	2% TAM + PW cream	0.5% SPF 47/PA <sup>++</sup> cream
Water phase (%)			
Glycerine	10.00	10.00	10.00
Silicon derivatives	0.20	0.20	0.20
Microcide-C	0.20	0.20	0.20
EDTA-2Na	0.01	0.01	0.01
Keltrol F (1%)	5.00	5.00	5.00
Sepiplus 400	0.30	0.30	0.30
Oil phase (%)			
Lanette O	2.00	2.00	2.00
Olive M 1000	1.00	1.00	1.00
Tego care 450	2.00	2.00	2.00
Puresyn 4	2.00	2.00	2.00
TCG-M	3.00	8.00	8.00
DC 200/6cs	2.50	2.50	2.50
Lipex Shea	1.00	1.00	1.00
Vitamin E	0.50	0.50	0.50
D-P	0.10	0.10	0.10
BHT	0.02	0.02	0.02
Stabilizer (%)			
DW	2.00	–	–
PW	–	2.00	–
Additives (%)			
2% TAM + DW	2.00	–	–
2% TAM + PW	–	2.00	–
0.5% SPF 47/PA <sup>++</sup>	–	–	0.5

DW, distilled water; PW, phreatic water; TAM, *Taraxacum mongolicum*.

Technologies, Grand Island, NY, USA), and the HPLC (Waters Co., Milford, MA, USA).

## 2.7. TAM cream formation

The TAM cream form was made by blending 2% TAM + distilled water (DW), 2% TAM + PW and 0.5% SPF 47/PA<sup>++</sup> additives over water phase and oil phase stabilizers (Table 1).

## 2.8. Effective HPLC component analysis on TAM

HPLC conditions applied were 25 °C for the column oven temperature, 348 nm for the analysis wavelength, and 1.0 mL/minute for the flow rate. The column used was the Luna C18 column (250 mm × 4.6 mm, particle size 5 μm; Phenomenex, Torrance, CA, USA). For Solvent A and Solvent B, 0.1% formic acid was added to 100% of the third DW and 100% acetonitrile, respectively. Solvent gradient eluents for solvents are as shown in Table 2. Luteolin was used as the

**Table 2 – Solvent gradient condition for HPLC-DAD**

Final time (min)	Flow rate (mL/min)	A	B
0	0.3	85	15
10	0.3	80	20
30	0.3	65	35
35	0.3	65	35
40	0.3	85	15

A, water with 0.1% formic acid; B, acetonitrile with 0.1% formic acid; HPLC-DAD, high-performance liquid chromatography with diode array detection.

index component, and components of the reference sample and TAM were analyzed.

## 2.9. Effect of TAM on melanin generation in B16F10 mouse melanoma cell

### 2.9.1. Cytotoxicity measurement by MTT assay

The cytotoxicity was measured in accordance with various methods including the Carmichael et al<sup>23</sup> method. A total of 0.18 mL of B16F10 cells was separately seeded on the 96-well plate by  $5 \times 10^4$  cells/well. Samples were prepared by concentrations, and 0.02 mL of each sample was added into each well and the plate was placed and cultured in the 5% CO<sub>2</sub> incubator for 24 hours. The same amount of DW as that used in the sample group was added to the control group, and it was cultured under identical conditions. The MTT solution prepared in the 5 mg/mL concentration was added (at 0.02 mL each), and then both groups were cultured for 4 hours. Next, 0.15 mL of dimethyl sulfoxide was added to each for reaction for 30 minutes. The optical absorbance was measured at 540 nm using the ELISA reader. The cytotoxicity was indicated by the optical absorbance reduction ratio of the indication solution added group and the nonadded group.

Cytotoxicity(%)

$$= \left( 1 - \frac{\text{Optical absorbance of the sample added group}}{\text{Optical absorbance of the nonadded group}} \right) \times 100$$

### 2.9.2. Tyrosinase inhibitory activity measurement of TAM

The tyrosinase inhibitory activity was measured in accordance with various methods including the Yagi et al<sup>24</sup> method. In the reactor, 200 U/mL mushroom tyrosinase (40  $\mu$ L) was added to the mixture of the deposited solution (40  $\mu$ L), in which 10 mM L-DOPA was dissolved over 67 mM sodium phosphate buffer (pH 6.8; 80  $\mu$ L), and the sample solution (40  $\mu$ L). It was reacted for 10 minutes at 37 °C. The DOPA chrome generated in the reaction solution was measured at 492 nm. The tyrosinase inhibitory activity was indicated by the optical absorbance reduction ratio of the indication solution added group and the nonadded group.

Inhibitory ratio (%)

$$= \left( 1 - \frac{\text{Optical absorbance of the sample added group}}{\text{Optical absorbance of the nonadded group}} \right) \times 100$$

### 2.9.3. B16F10 cell experimental group classification

To identify the effect of TAM when inducing melanin generation on B16F10 cells, the experimental groups were classified as shown in Table 3.

### 2.9.4. mRNA expression measurement by PCR

(1) Total RNA extraction and cDNA synthesis. B16F10 cells were seeded on the 100-mm culture dish and cultured for 24 hours. These were treated with a-melanocyte stimulating hormone (a-MSH; 100 nM), TAM (5  $\mu$ g/mL, 25  $\mu$ g/mL, and 50  $\mu$ g/mL), and kojic acid (50 ppm) and cultured for 24 hours. Then, the culture medium was removed. The

**Table 3 – Classification of experimental groups (in B16F10 cell)**

Group	Treatment
Normal	Nontreatment
Negative control	a-MSH (100nM) treatment
Positive control	a-MSH (100nM) + kojic acid (50 ppm) treatment
Experimental	a-MSH (100nM) + TAM (5 $\mu$ g/mL, 25 $\mu$ g/mL, 50 $\mu$ g/mL) treatment

a-MSH, a-melanocyte stimulating hormone.

trizol lysis buffer was planted in each well by 1 mL for cell lysis, chloroform (200 mL) was added to each, and the dish was shaken up and down for 20 seconds. It was centrifuged for 20 minutes at 16,100 rpm  $\times$  g. The supernatant was collected and mixed in the tube that contained isopropanol (500 mL). Then, it was centrifuged for 20 minutes at 16,100 rpm  $\times$  g again. The supernatant was removed. Next, 75% EtOH-diethylpyrocarbonate water was added in each tube by 1 mL. It was centrifuged for 5 minutes at 13,200 rpm. The supernatant was removed then dried at room temperature. The DEPC was seeded in the 96-well plate by 50  $\mu$ L/well and melted. RNA (5  $\mu$ L) and sterile water (195  $\mu$ L) were added in each well. Total RNA quantities were measured by measuring the optical absorbance at 260 nm and 280 nm, respectively. Overall, 10  $\mu$ L was made with Oligo (dT) 15 primer (500  $\mu$ g/mL) 1  $\mu$ L, extracted RNA (2  $\mu$ g), and nuclease-free water. It was reacted for 5 minutes at 75 °C. Then, 5  $\times$  reaction buffer, MgCl<sub>2</sub>, PCR neucleotide mix, rnasin inhibitor, reverse transcriptase, and nuclease-free water were added. It was reacted for 5 minutes at 25 °C, for 60 minutes at 42 °C, and for 15 minutes at 70 °C to synthesize cDNA.

(2) Reverse transcription (RT) PCR. Go Flexi DNA polymerase, primer, and synthesized cDNA was added in the PCR tube and fully blended, and PCR was conducted. The primer sequence used in the experiment is listed in Table 4. The GAPDH was kept for 30 seconds at 94 °C, for 45 seconds at 55 °C, and for 45 seconds at 72 °C for 35 times. The TRP-1, TRP-2, and MITF were kept for 30 seconds at 94 °C, for 45 seconds at 58 °C, and for 45 seconds at 72 °C for 40 times. The MMP-1 was kept for 30 seconds at 94 °C, for

**Table 4 – Primer sequence (in B16F10 cell)**

Gene	Primer	Sequence (5'→3')
MITF	Forward	AGC GTG TAT TTT CCC CAC AG
	Reverse	TAG CTC CTT AAT GCG GTC GT
TRP-1	Forward	ACT TCA CTC AAG CCA ACT GC
	Reverse	AGC TTC CCA TCA GAT GTC GT
TRP-2	Forward	GCT CCA AGT GGC TGT AGA CC
	Reverse	AAT GCA GTG GCT TGG AAA TC
Tyrosinase	Forward	GAC GGT CAC TGC ACA CTT TG
	Reverse	GCC ATG ACC AGG ATG AC
GAPDH	Sense	TGA AGG TCG GTG TGA ACG GAT TTG GC
	Anti-sense	CAT GTA GGC CAT GAG GTC CAC CAC
MMP-1	Forward	AGC GTG TGA CAG TAA GCT AA
	Reverse	GTT TTC CTC AGA AAG AGC AGC AT
$\beta$ -Actin	Forward	ATT GTT GCC ATC AAT GAC CC
	Reverse	AGT AGA GGC AGG GAT GAT



60 seconds at 56 °C, and for 60 seconds at 72 °C for 35 times. After synthesis by PCR, it was treated with electrophoresis for 40 minutes at 100 V in 0.002% ethidium bromide added with 1.5% agarose gel. The LAS 4000 instrument (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used to measure the band for quantitative analysis.

#### 2.9.5. Protein expression measurement by Western blot

B16F10 cells were seeded on a 100-mm culture dish and cultured for 24 hours. These were treated with  $\alpha$ -MSH (100 nM), TAM (5  $\mu$ g/mL, 25  $\mu$ g/mL, and 50  $\mu$ g/mL), and kojic acid (50 ppm) and cultured for 24–48 hours. Then the culture medium was removed. After washing twice with phosphate buffered saline, RIPA buffer (100 mL) added to the complete mini 1 tab was dissolved in 100  $\mu$ L. Next, it was centrifuged for 20 minutes at 4 °C, 12,000 rpm. The centrifuged supernatant was quantified by the Bradford assay, and 30  $\mu$ L protein was separated by electrophoresis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) using 10% gel. The semidry transfer cell instrument (Hoefer, Inc., San Francisco, CA, USA) was used to move the separated protein on the polyvinyl difluoride membrane. Then, it was cultured in the blocking buffer (5% skim milk in Tris-buffered saline with Tween) for 1 hour at room temperature. The primary antibody [protein kinase A (PKA),  $\beta$ -actin] was diluted and kept overnight at 4 °C. It was washed using Tris-buffered saline with Tween for three times in at 10-minute intervals. The secondary antibody (anti rabbit, anti goat) was diluted by 1:1000, and cultured for 2 hours at room temperature. Then, it was washed for three times. The LAS 4000 instrument (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used to measure the band for quantitative analysis.

#### 2.9.6. Statistical analysis

Every experiment was repeated three times to obtain the average and standard deviation values. SPSS version 10.0 (Chicago, IL, USA) was used to process the statistical results. For significant difference verification, significance was analyzed using Tukey's honest significant difference test at  $\alpha=0.05$  level after analysis of variance (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

### 2.10. Effect of TAM with PW on melanin generation in HRM-2 melanin-possessing hairless mice

#### 2.10.1. HRM-2 mice experimental group classification

To identify the effect of 2% TAM + DW and 2% TAM + PW when inducing melanin generation on HRM-2 mice, the experimental groups were classified as shown in Table 5.

#### 2.10.2. UVB irradiation and hyperpigmentation induction

The UVB lamp (15 W type, UV maximum wavelength 312 nm; UV intensity 100  $\mu$ W cm<sup>-2</sup>; Ieda Boeki Co., Tokyo, Japan) was used to irradiate UVB on the dorsal skins of 7-week-old HRM-2 mice to identify the effect of 2% TAM + DW and 2% TAM + PW on changes in skin thickness, elasticity, and pigmentation. The base ointment and each sample was made in the cream form (0.5% Sunblock cream, 2% TAM + DW cream, 2% TAM + PW cream). It was applied on the right dorsal skin of each HRM-2 mouse, and no treatment was made on the left dorsal skin. Each mouse was anesthetized and irradiated with UVB from a 30-cm distance for 5 minutes at a time. UVB was irradiated three times a week for 4 weeks. Based on studies on UVB using animal whitening experiments,<sup>25–27</sup> the experimental duration was configured for a total of 5 weeks.

#### 2.10.3. Visual pigmentation observation

To visually observe the dorsal skin in which pigmentation was induced by UVB irradiation, applied parts of the solvent control group and the sample group were observed for three times on the 1<sup>st</sup> week, 3<sup>rd</sup> week, and 5<sup>th</sup> week after the experiment was launched to compare the colored skin states of experimental animals for whitening effect. Mice were etherized, and a digital camera (D 70 model; Nikon, Tokyo, Japan) was used to take pictures of skin surface states.

#### 2.10.4. Software analysis on pigmented images

To analyze the images taken by the digital camera during the 1<sup>st</sup> week, 3<sup>rd</sup> week, and 5<sup>th</sup> week after the experiment start, the image analysis software (Bio-Rad) was used. To compare melanin deposition from the entire dorsal part, the untreated left dorsal part and the treated right dorsal part were separated. The melanin deposition level was derived from the difference between the pigmented areas of the left dorsal skin and the right dorsal skin.

#### 2.10.5. mRNA expression measurement by PCR

(1) Total RNA extraction and cDNA synthesis. The dorsal skin tissue was extracted from each HRM-2 mouse, added with RNazol<sup>B</sup> (500  $\mu$ L), and crushed with a homogenizer. Then 50  $\mu$ L chloroform (CHCl<sub>3</sub>) was added, and the mixture was blended for 15 seconds. It was left for 15 minutes on ice and centrifuged at 13,000 rpm. A supernatant of approximately 200  $\mu$ L was collected and mixed with 2-propanol (200  $\mu$ L), then shaken slowly and left for 15 minutes. It was centrifuged at 13,000 rpm again. Next, it was washed with 80% EtOH and dried by vacuum pump for 3 minutes; then, RNA was extracted. The extracted RNA was dissolved in the DEPC-treated DW (20  $\mu$ L). It was deactivated in the

**Table 5 – Classification of experimental groups (in HRM-2 mice)**

Group	Treatment	Treatment part
Normal	Base ointment application	Right
Negative control	UVB irradiation + Base ointment application	Right
Positive control	UVB irradiation + 0.5% Sunblock cream application	Right
Experimental 1	UVB irradiation + 2% TAM + DW cream application	Right Upper
Experimental 2	UVB irradiation + 2% TAM + PW cream application	Right Lower

DW, distilled water; PW, phreatic water; TAM, *Taraxacum mongolicum*.

**Table 6 – Primed Sequences (in HRM-2 mice)<sup>a</sup>**

Target gene	Primer	Sequences
MMP-9	Forward	5'-AATCTCTTCTAGAGACTGGGAAGGAG-3'
MMP-9	Reverse	5'-AGCTGATTGACTAAAGTAGCTGGA-3'
TRP-1	Forward	5'-GCTGCAGGAGCCTTCTTCTC-3'
TRP-1	Reverse	5'-AAGACGCTGCACTGCTGGTCT-3'
TRP-2	Forward	5'-GGATGACCGTGAGCAATGGCC-3'
TRP-2	Reverse	5'-CGGTTGTGACCAATGGGTGCC-3'
GAPDH-VIC	Probe	5'-CATGTTCCAGTATGACTCCACTCAGG-3'

<sup>a</sup> MMP, matrix metalloproteinase; TRP, tyrosinase related protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

heating block at 75 °C and used in the first-strand cDNA synthesis.

- (2) RT. The total RNA (3 µg) extracted from the dorsal skin tissue of the HRM-2 mouse was denaturated for 10 minutes at 75 °C. Then, 2.5 µL 10 mM dNTPs mix, 1 µL random sequence hexanucleotides (25 pmol/25 µL), 1 µL RNase inhibitor (20 U/µL) as the RNA inhibitor, 1 µL 100 mM DTT, and 4.5 µL 5 × RT buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl<sub>2</sub>) were added to it; again, 1 µL M-MLV RT (200 U/µL) was added to the mixture. A 20-µL volume was made with the DEPC-treated DW. This 20-µL reaction mixture solution was fully stirred and was centrifuged for 5 seconds at 2000 rpm. It was reacted for 60 minutes in the heating block at 37 °C to synthesize the first-strand cDNA. It was left at 95 °C for 5 minutes to deactivate the M-MLV RT. Finally, the synthesized cDNA was used for PCR.
- (3) RQ PCR. The Applied Biosystems 7500 Real-Time PCR system was used for RQ PCR, and the primer sequence used in the experiment is given in Table 6. The SYBR Green primer for MMP-9, TRP-1, TRP-2 mRNA gene expression was analyzed with SYBR Green PCR MasterMix (ABI), and taqman probe for GAPDH (pn,4352339E, VIC dye-labelled, ABI) were selected using Assays-on-Demand Gene Expression Products (ABI). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ABI) gene expression was used as an endogenous control. For RQ PCR conditions, predenaturation was conducted for 2 minutes at 50 °C and for 10 minutes at 94 °C, and 40 cycles of denaturation were conducted for 15 seconds at 95 °C and for 1 minute at 60 °C. GAPDH was used as the internal standard for the experimental group and the control group. The relative quantitative value of the target group was calculated for RQ PCR using the following equation:  $y = x(1 + e)^n$ , where y is the yield, x is the starting quantity, n is the number of cycles, and e denotes efficiency.

#### 2.10.6. Histological observation on skin

To observe the optical microscopic change in the skin tissue, the extracted tissue was fixed in a 100% neutral formalin solution for 12 hours and washed with running water. It was hydrated with 70%, 80%, 95%, and 100% ethanol, and made transparent with xylene. After the penetration process, it was embedded with paraffin. The microtome was used to make the 4-µm-thick microsection from the produced paraffin block. Xylene was used to deparaffinize the microsection,

which was then washed with salty water and tap water to dye it suitably for our purpose. To observe the overall change of the skin tissue, it was placed in the Harris hematoxylin solution for 5 minutes to dye its nucleus. Then, it was washed with running water. It was deposited for three times with 1% HCl-alcohol solution and was fully washed. A 1% ammonium solution was used to color it blue, and it was moved to 80% alcohol. Next, 95% alcohol and 100% alcohol were used to dehydrate it. After it cleared, it was enclosed with Canada balsam and then placed under the microscope for inspection.

#### 2.10.7. Statistical analysis

The unpaired Student t test statistics was used to process each experimental group result, and significance was determined at levels not greater than  $p < 0.05$  (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

## 3. Results

### 3.1. Effective HPLC component analysis on TAM

For behaviors of standard materials contained in TAM contents, patterns of the standard material luteolin (Fig. 1A) and TAM (Fig. 1B) were identified by matching the retention times through HPLC with diode array detection. The luteolin content of TAM was 0.69 mg/g (Fig. 1).

### 3.2. Effect of TAM on melanin generation in B16F10 mouse melanoma cell

#### 3.2.1. Cytotoxicity

By the result of cytotoxicity of TAM on the B16F10 cell, little cytotoxicity was exhibited from every concentration from 5 µg/mL to 1000 µg/mL (Fig. 2). Therefore, for the whitening related signal transfer factor measurement in B16F10 cell, concentrations of 5 µg/mL, 25 µg/mL, and 50 µg/mL were tested.

#### 3.2.2. Tyrosinase activation inhibition

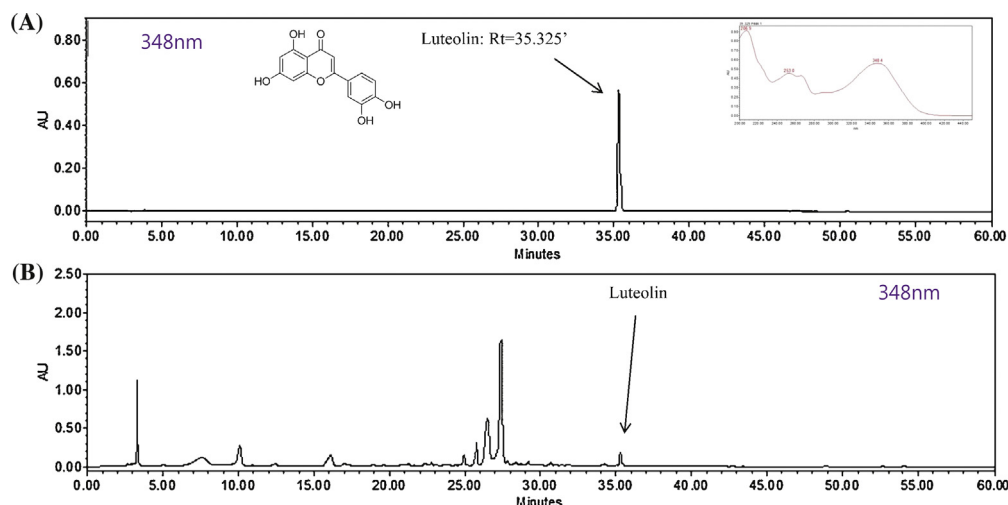
By the result of tyrosinase activation inhibition effect of TAM, 50 µg/mL significantly exhibited 7.3% activation inhibition ( $p < 0.05$ ), 100 µg/mL showed 7.7% activation inhibition ( $p < 0.01$ ), 500 µg/mL showed 29.9% activation inhibition ( $p < 0.01$ ), and 1000 µg/mL showed 46.5% activation inhibition ( $p < 0.001$ ; Fig. 3).

#### 3.2.3. TRP-1 mRNA gene expression

Whereas TRP-1 mRNA gene expression was increased in every concentration compared to the only a-MSH treated negative control group, it was decreased depending on the TAM concentration (Fig. 4).

#### 3.2.4. TRP-2 mRNA gene expression

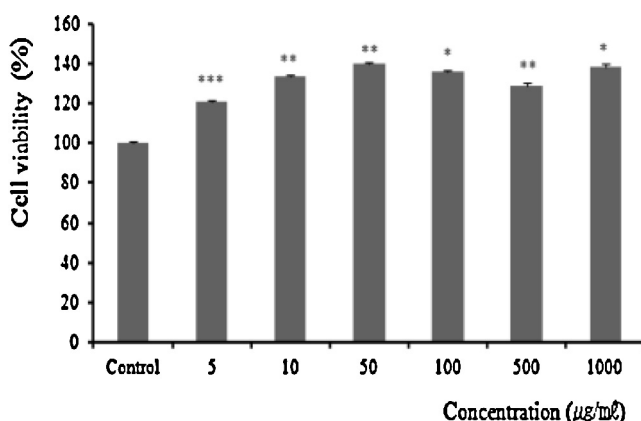
Whereas TRP-2 mRNA gene expression was increased in 5 µg/mL and 25 µg/mL concentrations compared to the only a-MSH treated negative control group, it was significantly decreased in 50 µg/mL concentration by 1.3% ( $p < 0.001$ ). In addition, it was decreased depending on the TAM concentration (Fig. 5).



**Fig. 1** – HPLC chromatogram of standard luteolin and TAM. Chromatograms of standard (A) luteolin and (B) TAM are recorded at 348 nm. Peaks of luteolin are shown. HPLC, high-performance liquid chromatography; TAM, *Taraxacum mongolicum*.

### 3.2.5. Tyrosinase mRNA gene expression

Whereas tyrosinase mRNA gene expression was increased in 5  $\mu\text{g/mL}$  concentration compared to the only a-MSH treated negative control group, it was significantly decreased in 25  $\mu\text{g/mL}$  concentration by 0.4% ( $p < 0.05$ ) and in 50  $\mu\text{g/mL}$  concentration by 1.3% ( $p < 0.05$ ). It was decreased in 5  $\mu\text{g/mL}$ , 25  $\mu\text{g/mL}$ , and 50  $\mu\text{g/mL}$  concentrations compared to kojic acid, the positive control group. In addition, it was decreased depending on the TAM concentration (Fig. 6).



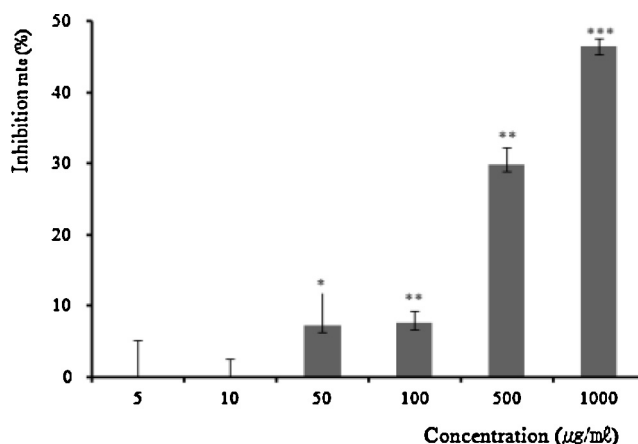
**Fig. 2** – Cytotoxicity effects of TAM in B16F10 cell. B16F10 cells were treated with various concentrations of TAM (5  $\mu\text{g/mL}$ , 10  $\mu\text{g/mL}$ , 50  $\mu\text{g/mL}$ , 100  $\mu\text{g/mL}$ , 500  $\mu\text{g/mL}$ , and 1000  $\mu\text{g/mL}$ ). Cell viabilities were measured by MTT assay. The results represent the mean  $\pm$  SD of three individual experiments. Statistically significant value was calculated by comparing with negative control group using Turkey's HSD test (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ). HSD, honest significant difference; MTT, 3-[4,5-dimethylthiazol]-2-yl]-2,5-diphenyl-tetrazoliumbromide; SD, standard deviation; TAM, *Taraxacum mongolicum*.

### 3.2.6. MITF mRNA gene expression

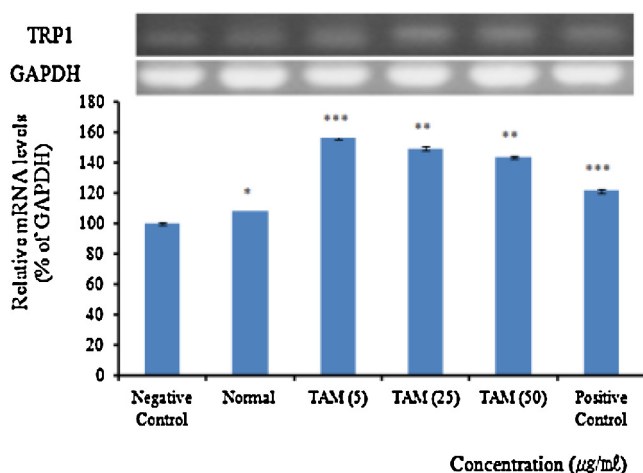
MITF mRNA gene expression was significantly decreased in 5  $\mu\text{g/mL}$  concentration by 12.8% ( $p < 0.05$ ) compared to the only a-MSH treated negative control group, in 25  $\mu\text{g/mL}$  concentration by 18.2% ( $p < 0.05$ ), and in 50  $\mu\text{g/mL}$  concentration by 25.6% ( $p < 0.01$ ). In addition, it was decreased depending on the TAM concentration (Fig. 7).

### 3.2.7. PKA protein expression

Whereas PKA protein expression was increased in 5  $\mu\text{g/mL}$  and 25  $\mu\text{g/mL}$  concentrations compared to the only a-MSH

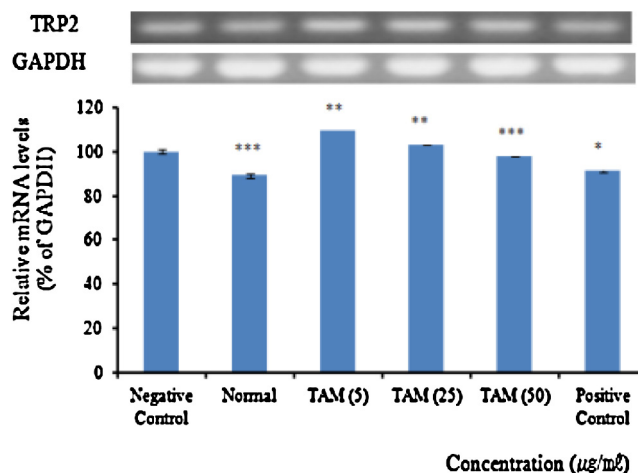


**Fig. 3** – Effect of TAM on tyrosinase activation inhibition in B16F10 cell. B16F10 cells were treated with various concentrations of TAM (5  $\mu\text{g/mL}$ , 10  $\mu\text{g/mL}$ , 50  $\mu\text{g/mL}$ , 100  $\mu\text{g/mL}$ , 500  $\mu\text{g/mL}$ , and 1000  $\mu\text{g/mL}$ ). Then the cells were tested for tyrosinase activation inhibition. The results represent the mean  $\pm$  SD of three individual experiments. Statistically significant value was calculated using Turkey's HSD test (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ). HSD, honest significant difference; SD, standard deviation; TAM, *Taraxacum mongolicum*.



**Fig. 4** – Effect of TAM on TRP-1 mRNA gene expression in B16F10 cell. B16F10 cells were seeded on 100-mm culture dish. After 24 hours, cells were treated with a-MSH, TAM (5 µg/mL, 25 µg/mL, and 50 µg/mL) or kojic acid (50 ppm) for 24 hours. TRP-1 mRNA gene expression was analyzed with RT PCR. The results represent the mean ± SD of three individual experiments. Statistically significant value was calculated by comparing with negative control group using Turkey's HSD test (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

a-MSH, a-melanocyte stimulating hormone; HSD, honest significant difference; Negative control, a-MSH (100 nM) treatment; Normal, nontreatment; Positive control, a-MSH (100 nM) + kojic acid (50 ppm) treatment; RT PCR, reverse transcription polymerase chain reaction; SD, standard deviation; TAM, *Taraxacum mongolicum*; TAM (5, 25, 50), a-MSH (100 nM) + TAM (5 µg/mL, 25 µg/mL, and 50 µg/mL) treatment.



**Fig. 5** – Effect of TAM on TRP-2 mRNA gene expression in B16F10 cell. B16F10 cells were seeded on 100-mm culture dish. After 24 hours, cells were treated with a-MSH, TAM (5 µg/mL, 25 µg/mL, and 50 µg/mL) or kojic acid (50 ppm) for 24 hours. TRP-2 mRNA gene expression was analyzed with RT PCR. The results represent the mean ± SD of three individual experiments. Statistically significant value was calculated by comparing with negative control group using Turkey's HSD test (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

a-MSH, a-melanocyte stimulating hormone; HSD, honest significant difference; Negative control, a-MSH (100 nM) treatment; Normal, nontreatment; Positive control, a-MSH (100 nM) + kojic acid (50 ppm) treatment; RT PCR, reverse transcription polymerase chain reaction; SD, standard deviation; TAM, *Taraxacum mongolicum*; TAM (5, 25, 50), a-MSH (100 nM) + TAM (5 µg/mL, 25 µg/mL, and 50 µg/mL) treatment.

treated negative control group, it was significantly decreased in 50 µg/mL concentration by 3.9% ( $p < 0.001$ ). In addition, it was decreased depending on the TAM concentration (Fig. 8).

### 3.2.8. ERK mRNA gene expression

ERK mRNA gene expression was increased in every concentration compared to the only a-MSH treated negative control group. It was increased in every concentration (5 µg/mL, 25 µg/mL, and 50 µg/mL) compared to kojic acid, the positive control group. There was no significant difference between TAM concentrations (Fig. 9).

## 3.3. Effect of TAM with PW on melanin generation in HRM-2 melanin-possessing hairless mice

### 3.3.1. Image analysis software

Melanin differences between the untreated left dorsal skin and the sample applied right dorsal skin for the 1<sup>st</sup> week, the 3<sup>rd</sup> week, and the 5<sup>th</sup> week of UVB irradiation are shown in Table 7.

For the 1<sup>st</sup> week of UVB irradiation, the positive control group, experimental Group 1, and experimental Group 2 were not significantly different from the negative control group (Figs. 10 and 11).

For the 3<sup>rd</sup> week of UVB irradiation, melanin difference between the untreated left dorsal skin and the sunblock cream applied right dorsal skin of the positive control group was significantly increased by 21.6 times compared to the control group ( $p < 0.001$ ), that of the untreated left upper dorsal skin and the TAM+DW cream applied right upper dorsal skin of the experimental Group 1 by 4.9 times ( $p < 0.01$ ), and that of the untreated left lower dorsal skin and the TAM+PW cream applied right lower dorsal skin of the experimental Group 2 by 9.4 times ( $p < 0.01$ ; Figs. 10 and 12).

For the 5<sup>th</sup> week of UVB irradiation, melanin difference between the untreated left dorsal skin and the sunblock cream applied right dorsal skin of the positive control group was significantly increased by 15.0 times compared to the control group ( $p < 0.001$ ), that of the untreated left upper dorsal skin and the TAM+DW cream applied right upper dorsal skin of the experimental Group 1 by 2.5 times, and that of the untreated left lower dorsal skin and the TAM+PW cream applied right lower dorsal skin of the experimental Group 2 by 4.7 times ( $p < 0.05$ ; Figs. 10 and 13).

### 3.3.2. RQ PCR analysis

(1) TRP-1 mRNA gene expression. The positive control group significantly inhibited TRP-1 mRNA gene expression by



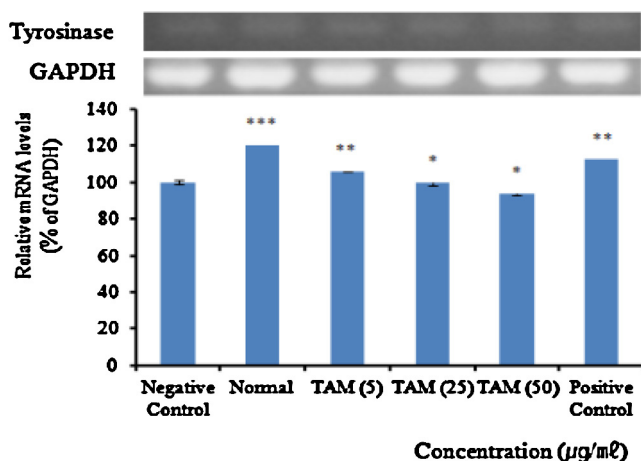
**Table 7 – Melanin difference between untreated part (left) and treated part (right)**

Group	1 <sup>st</sup> wk	3 <sup>rd</sup> wk	5 <sup>th</sup> wk
Normal	1.51 ± 0.16	0.63 ± 0.07	2.26 ± 1.40
Negative control	1.87 ± 1.09	1.27 ± 0.98	2.64 ± 2.29
Positive control	3.39 ± 1.00 (Fig. 12A)	27.35 ± 1.28 (Fig. 13A)	39.45 ± 1.61 (Fig. 14A)
Experimental 1	2.20 ± 1.07 (Fig. 12B)	6.19 ± 0.80 (Fig. 13B)	6.62 ± 2.59 (Fig. 14B)
Experimental 2	4.03 ± 1.67 (Fig. 12B)	11.81 ± 2.93 (Fig. 13B)	12.36 ± 3.66 (Fig. 14B)

DW, distilled water; Experimental 1, UVB irradiation + 2% TAM + DW cream application; Experimental 2, UVB irradiation + 2% TAM + PW cream application; Negative control, UVB irradiation + Base ointment application; Normal, base ointment application; Positive control, UVB irradiation + 0.5% Sunblock cream application; PW, phreatic water; TAM, *Taraxacum mongolicum*.

42.9% ( $p < 0.01$ ) compared to the negative control group, the experimental Group 1 by 19.2%, and the experimental Group 2 by 33.2% ( $p < 0.05$ ; Fig. 14).

- (2) TRP-2 mRNA gene expression. The positive control group significantly inhibited TRP-2 mRNA gene expression by 28.4% ( $p < 0.01$ ) compared to the negative control group, the experimental Group 1 by 1.5%, and the experimental Group 2 by 13.1% ( $p < 0.05$ ; Fig. 15).
- (3) MMP-9 mRNA gene expression. The positive control group significantly inhibited MMP-9 mRNA gene expression by 65.4% ( $p < 0.01$ ) compared to the negative control group, the experimental group 1 by 19.8%, and the experimental group 2 by 36.8% ( $p < 0.05$ ) (Fig. 16).



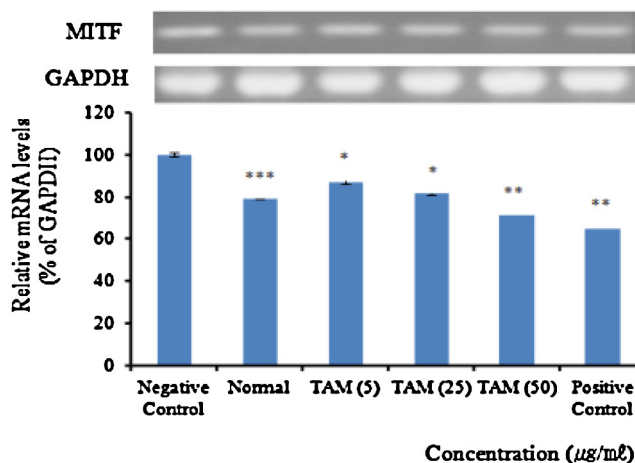
**Fig. 6 – Effect of TAM on tyrosinase mRNA gene expression in B16F10 cell.** B16F10 cells were seeded on 100-mm culture dish. After 24 hours, cells were treated with a-MSH, TAM (5 µg/mL, 25 µg/mL, and 50 µg/mL), or kojic acid (50 ppm) for 24 hours. Tyrosinase mRNA gene expression was analyzed with RT PCR. The results represent the mean ± SD of three individual experiments. Statistically significant value was calculated by comparing with negative control group using Turkey's HSD test (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

a-MSH, a-melanocyte stimulating hormone; HSD, honest significant difference; Negative control, a-MSH (100 nM) treatment; Normal, nontreatment; Positive control, a-MSH (100 nM) + kojic acid (50 ppm) treatment; RT PCR, reverse transcription polymerase chain reaction; SD, standard deviation; TAM, *Taraxacum mongolicum*; TAM (5, 25, 50), a-MSH (100 nM) + TAM (5 µg/mL, 25 µg/mL, and 50 µg/mL) treatment.

### 3.3.3. Histological analysis

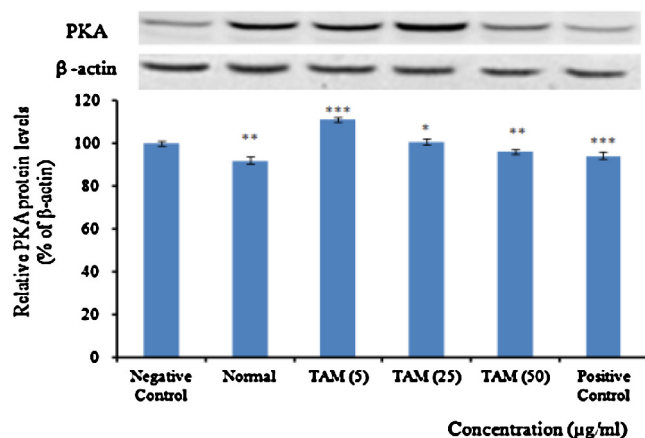
After the experiment, the dorsal skin of the HRM-2 mouse was extracted and dyed with hematoxylin and eosin. Based on the analysis result, compared to the normal group, the epidermis of the negative control group was excessively formed in thickness and expanded, and around it, hyperkeratinization, pigmentation, granum, and infiltration were significantly increased—indicating that the skin texture was damaged by UVB irradiation.

It was observed that, compared to the negative control group, the epidermis of the positive control group, experimental Group 1, and experimental Group 2 was narrowed,

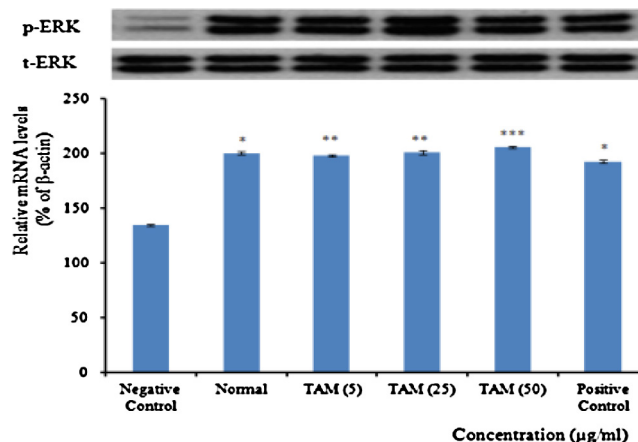


**Fig. 7 – Effect of TAM on MITF mRNA gene expression in B16F10 cell.** B16F10 cells were seeded on 100-mm culture dish. After 24 hours, cells were treated with a-MSH, TAM (5 µg/mL, 25 µg/mL, and 50 µg/mL) or kojic acid (50 ppm) for 24 hours. MITF mRNA gene expression was analyzed with RT PCR. The results represent the mean ± SD of three individual experiments. Statistically significant value was calculated by comparing with negative control group using Turkey's HSD test (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

a-MSH, a-melanocyte stimulating hormone; HSD, honest significant difference; MITF, microphthalmia-associated transcription factor; Negative control, a-MSH (100 nM) treatment; Normal, nontreatment; Positive control, a-MSH (100 nM) + kojic acid (50 ppm) treatment; RT PCR, reverse transcription polymerase chain reaction; SD, standard deviation; TAM, *Taraxacum mongolicum*; TAM (5, 25, 50), a-MSH (100 nM) + TAM (5 µg/mL, 25 µg/mL, and 50 µg/mL) treatment.



**Fig. 8** – Effect of TAM on PKA protein expression in B16F10 cell. B16F10 cells were seeded on 100-mm culture dish. After 24 hours, cells were treated with a-MSH, TAM (5 μg/mL, 25 μg/mL, and 50 μg/mL), or kojic acid (50 ppm) for 24 hours. PKA protein expression was analyzed with Western blot. The results represent the mean ± SD of three individual experiments. Statistically significant value was calculated by comparing with negative control group using Turkey's HSD test (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ). a-MSH, a-melanocyte stimulating hormone; HSD, honest significant difference; Negative control, a-MSH (100 nM) treatment; Normal, nontreatment; PKA, protein kinase A; Positive control, a-MSH (100 nM) + kojic acid (50 ppm) treatment; SD, standard deviation; TAM, *Taraxacum mongolicum*; TAM (5, 25, 50), a-MSH (100 nM) + TAM (5 μg/mL, 25 μg/mL, and 50 μg/mL) treatment.



**Fig. 9** – Effect of TAM on ERK mRNA gene expression in B16F10 cell. B16F10 cells were seeded on 100-mm culture dish. After 24 hours, cells were treated with a-MSH, TAM (5 μg/mL, 25 μg/mL, and 50 μg/mL) or kojic acid (50 ppm) for 24 hours. ERK mRNA gene expression was analyzed with RT PCR. The results represent the mean ± SD of three individual experiments. Statistically significant value was calculated by comparing with negative control group using Turkey's HSD test (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ). a-MSH, a-melanocyte stimulating hormone; HSD, honest significant difference; Negative control, a-MSH (100 nM) treatment; Normal, nontreatment; PKA, protein kinase A; Positive control, a-MSH (100 nM) + kojic acid (50 ppm) treatment; SD, standard deviation; TAM, *Taraxacum mongolicum*; TAM (5, 25, 50), a-MSH (100 nM) + TAM (5 μg/mL, 25 μg/mL, and 50 μg/mL) treatment.

and around it, cell deformation, keratinization, and infiltration were decreased, closely resembling what was observed in the normal group. In particular, it was observed that the epidermis of experimental Group 2 is narrowed, resembling more closely that of the normal group than that of experimental Group 1 (Fig. 17).

#### 4. Discussion

Skin color is mainly determined by melanin distributed in the epidermal layer, hemoglobin in the dermal layer, and carotene in the subcutaneous tissue. Among these factors, melanin plays the most important role in determining skin color.<sup>2</sup> The term melanin, which originates from the Greek word *melas*, means black.<sup>28</sup> It is a brownish black pigment and is largely distributed in the deep layer and the basal layer under the visible epidermal layer. The melanocyte distributed on the epidermis synthesizes melanin and plays an important role in skin pigmentation, and it is known that the generated melanin moves to the keratinocyte and performs a light-protection action that absorbs and blocks excessive UV rays.<sup>2-4</sup>

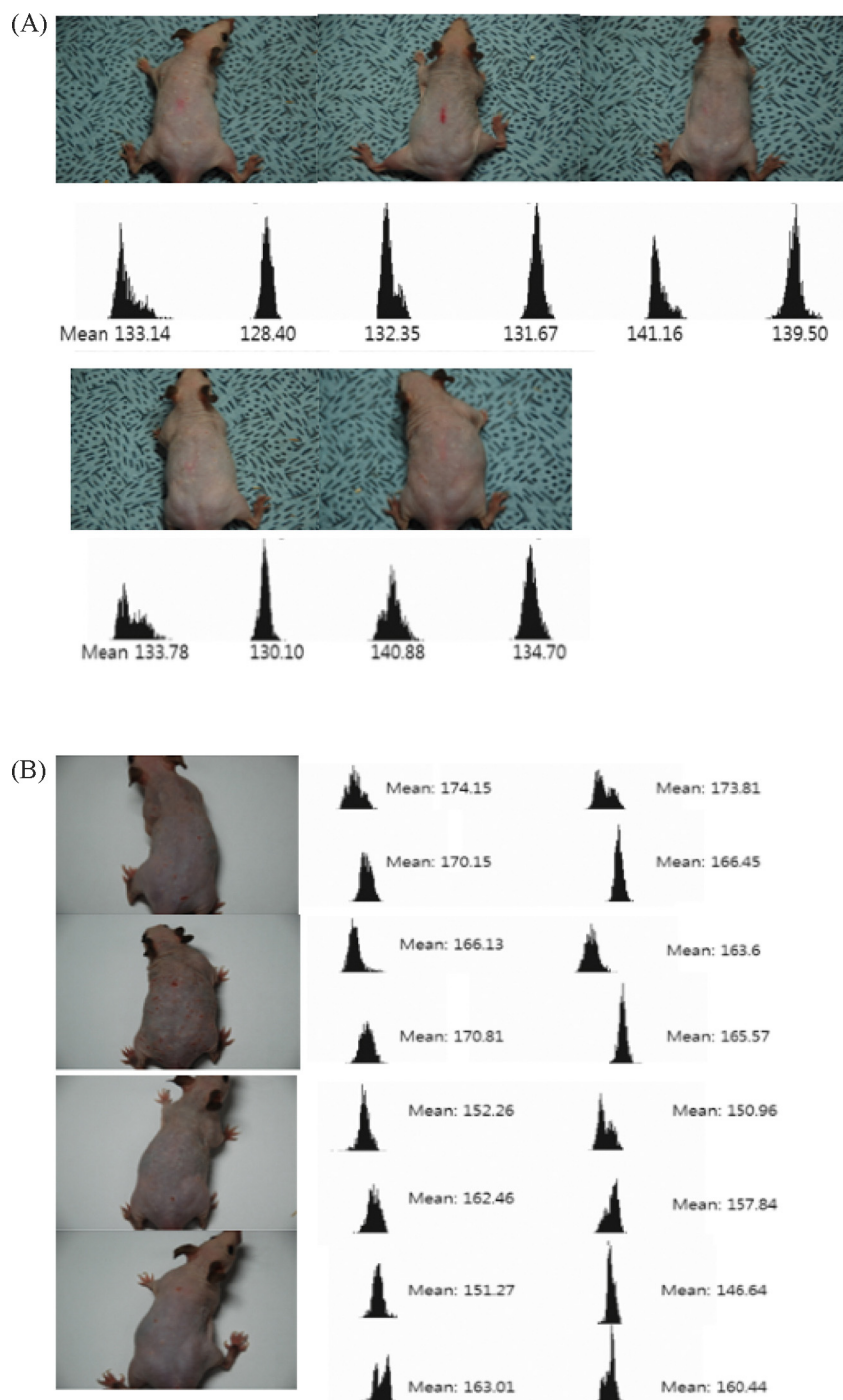
Melanin synthesis is started by the reaction in which tyrosine, one of the amino acids, is converted to DOPA quinone by tyrosinase. Whereas DOPA quinone acts as an important precursor that biosynthesizes pheomelanin and eumelanin by two different paths, eumelanin—which is brownish black—is

generally known as melanin.<sup>29</sup> Looking at the eumelanin formation process, DOPA quinone is converted to DOPA chrome, to 5- and 6-dihydroxyindole-2-carboxylic acids by TRP-2, and to indole-5 and 6-quinone-2-carboxylic acids by TRP-1. Through a series of these reactions, melanin is formed.<sup>30-32</sup>

The main intracellular signal transfer path of melanin is the cyclic monophosphate (cAMP)/PKA path and cAMP promotes MITF expression via the PKA and CREB1. MITF is an important transcription control factor in the melanin synthesis process, and it is known to promote transcription of tyrosinase, TRP-1 and TRP-2.<sup>33,34</sup> In addition, if the expression of ERK, which is the intracellular signal transfer protein and is known as the gene expression controlling factor, is inhibited, it is implied that melanin pigment generation is increased.<sup>35</sup>

In oriental medicine, <Jijinyodaeron ( )> of "Hwangjenegyeng-Somun ( )" <sup>36</sup> first mentioned skin hyperpigmentation as "If yangmyeong is in springwater, overflowing dryness win... seem to face in the dirt, it is no body shine and it has fever on foot outside. ( , , , )." <Myunganheukjeunghu ( )> of "Jebyungwonhuron ( )" <sup>37</sup> specifically describes its pathologic mechanism and types, stating that "Oma ( )" appears on the face if the pathogenic wind penetrates the skin and phlegm retention is accumulated in organs. Since then, it has been variously expressed as Ganjeung ( ), Ganjeom ( ), Jakran ( ), and Banganjeung





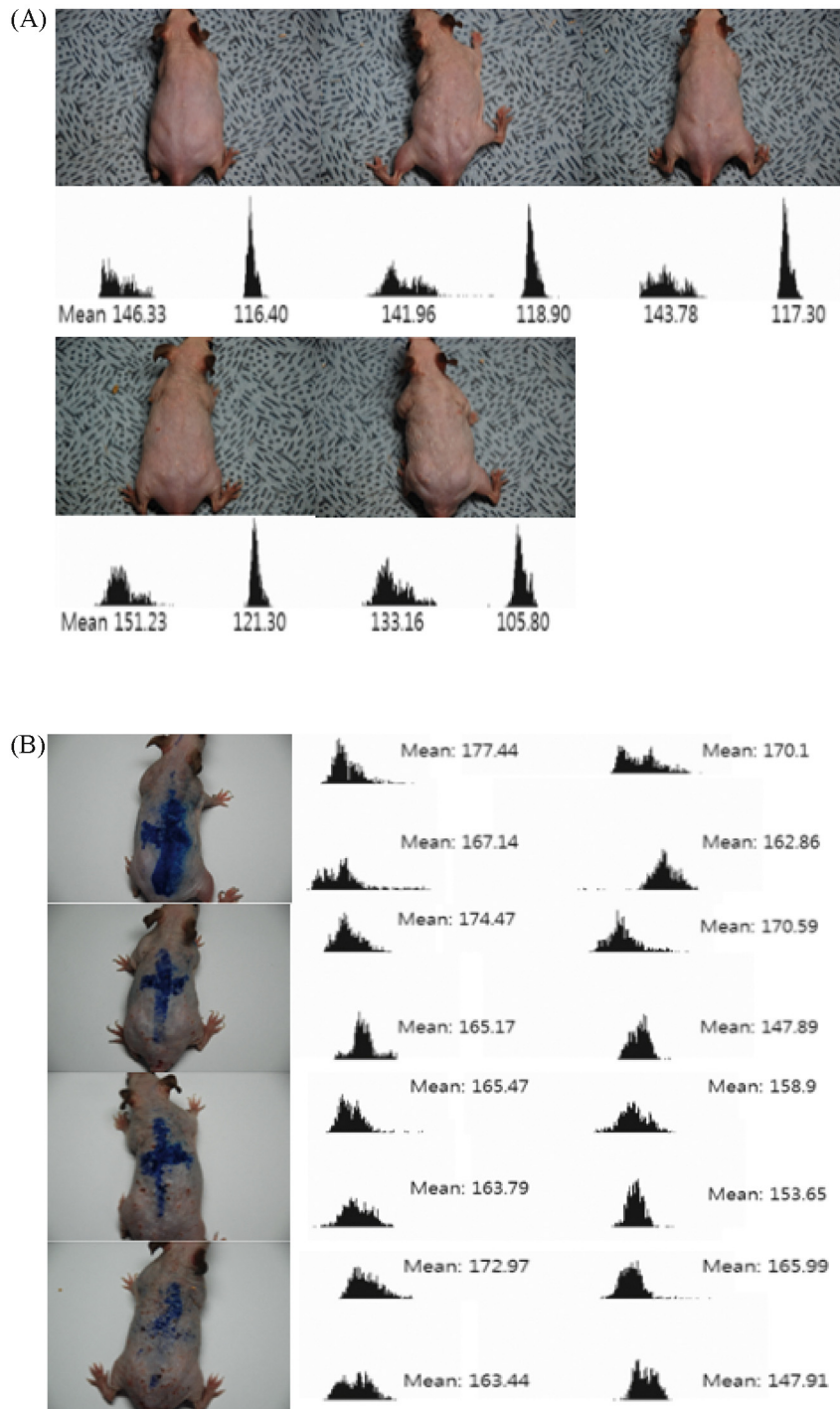
**Fig. 11 – Image analysis after 1 week of treatment. (A) Image analysis of treated part (right, 0.5% Sunblock Cream) after 1 week UVB irradiation compared to untreated part (left). Mean means peak area of a half dorsal skin calculated by image analysis software ( $n = 6$ ). (B) Image analysis of treated part (upper right: 2% TAM + DW; lower right: 2% TAM + PW) after 1 week UVB irradiation compared to untreated part (left). Mean means peak area of a quarter dorsal skin calculated using image analysis software ( $n = 6$ ).**

DW, distilled water; PW, phreatic water; TAM, *Taraxacum mongolicum*.

and atopic dermatitis. Although the mechanism for improving such skin disorders has not been completely discovered yet, it is known that the spa water effect multiplies the bathing effect and provides treatment effects on skin disorders.<sup>20</sup>

Looking at the efficacy of hot springs in more detail, it can be largely explained in terms of their chemical, physical, thermal, and immunological aspects. Its chemical effect is directly associated with its components—sulfur exerts an



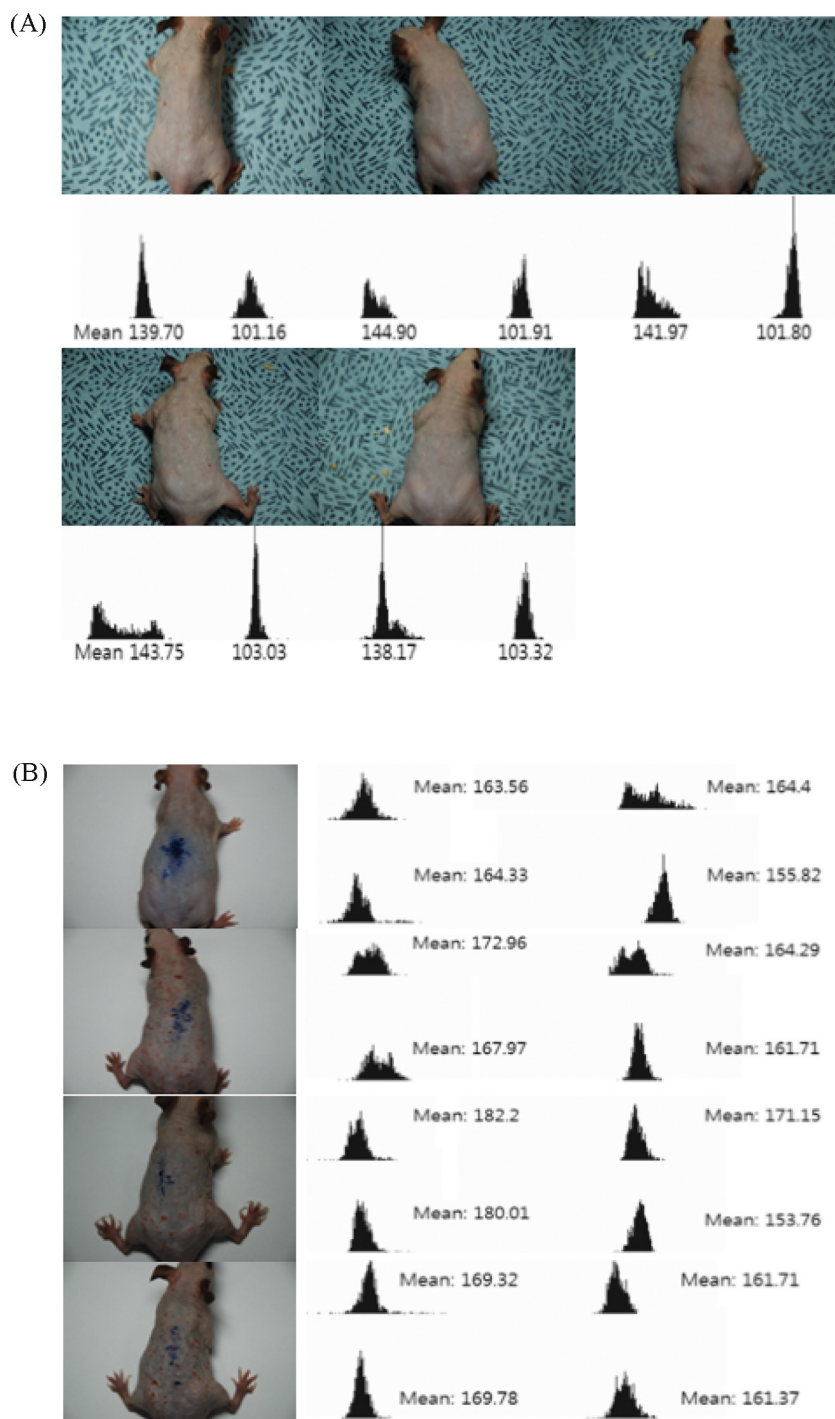


**Fig. 12 – Image analysis after 3 weeks of treatment. (A) Image analysis of treated part (right: 0.5% Sunblock Cream) after 3 weeks UVB irradiation compared to untreated part (left). Mean means peak area of a half dorsal skin calculated by image analysis software ( $n = 6$ ). (B) Image analysis of treated part (upper right: 2% TAM + DW; lower right: 2% TAM + PW) after 3 weeks UVB irradiation compared to untreated part (left). Mean means peak area of a quarter dorsal skin calculated using image analysis software ( $n = 6$ ).**

DW, distilled water; PW, phreatic water; TAM, *Taraxacum mongolicum*.

anti-inflammatory action; dekeratinization has antipruriginous action, antibacterial action, and antifungal action<sup>55</sup>; magnesium has psoriasis treatment action, as well as carcinostatic and vasodilation action<sup>56–58</sup>; and although selenium

is toxic if used in excessive amounts, in small, manageable doses, it promotes DNA synthesis and cell growth and it has antioxidant and anti-inflammatory properties as well as UVA and UVB protection action.<sup>59</sup> Aside from this, iron, lithium,

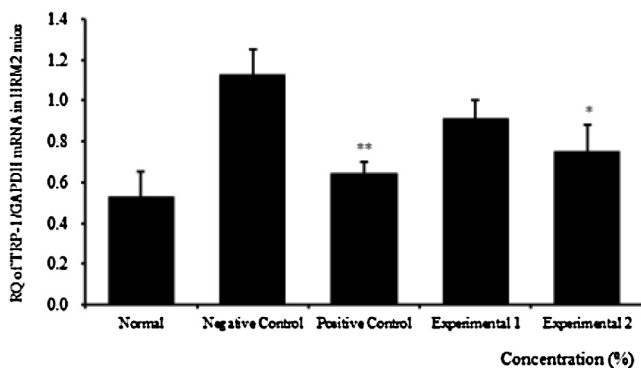


**Fig. 13 – Image analysis after 5 weeks of treatment. (A) Image analysis of treated part (right: 0.5% Sunblock Cream) after 5 weeks UVB irradiation compared to untreated part (left). Mean means peak area of a half dorsal skin calculated by image analysis software ( $n=6$ ). (B) Image analysis of treated part (upper right: 2% TAM + DW; lower right: 2% TAM + PW) after 5 weeks UVB irradiation compared to untreated part (left). Mean means peak area of a quarter dorsal skin calculated by image analysis software ( $n=6$ ).**

DW, distilled water; PW, phreatic water; TAM, *Taraxacum mongolicum*.

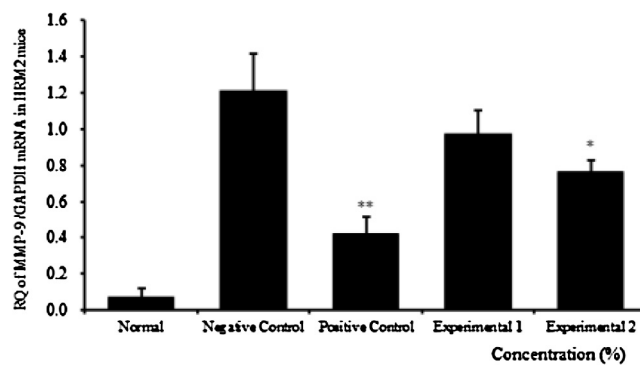
zinc, manganese, and strontium also have chemical actions. The thermal effect includes vasodilation, blood circulation promotion, blood pressure reduction, pain reduction, immune-inhibitory effect, and anti-inflammatory effect.<sup>60,61</sup>

The physical effect includes muscle tone reduction, joint mobility increase, and pain intensity reduction.<sup>62</sup> For the immunological effect, the sulfur water generates various cytokines and inhibits loss of cytokines.<sup>63</sup>



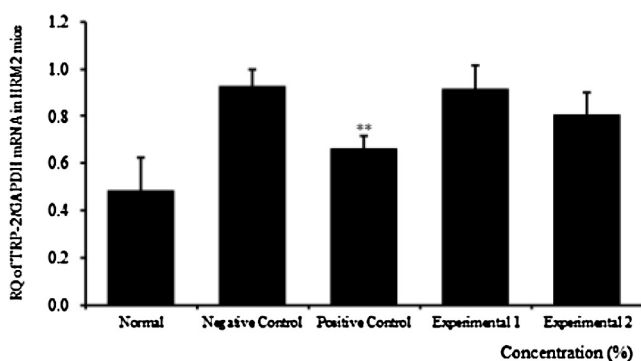
**Fig. 14** – Effect of TAM with PW on TRP-1 mRNA gene expression in dorsal skin of HRM-2 mice. HRM-2 mice were sacrificed, and TRP-1 mRNA gene expressions were checked in dorsal skin. TRP-1 mRNA gene expressions were analyzed with RQ PCR. The results represent the mean  $\pm$  SE ( $n = 6$ ). Statistically significant value was calculated by comparing with negative control group using Student t test (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

DW, distilled water; Experimental 1, UVB irradiation + 2% TAM + DW cream application; Experimental 2, UVB irradiation + 2% TAM + PW cream application; Negative control, UVB irradiation + Base ointment application; Normal, base ointment application; Positive control, UVB irradiation + 0.5% Sunblock cream application; PW, phreatic water; RQ PCR, real-time quantitative polymerase chain reaction; SE, standard error; TAM, *Taraxacum mongolicum*.



**Fig. 16** – Effect of TAM with PW on MMP-9 mRNA gene expression in dorsal skin of HRM-2 mice. HRM-2 mice were sacrificed, and MMP-9 mRNA gene expressions were checked in dorsal skin. MMP-9 mRNA gene expressions were analyzed with RQ PCR. The results represent the mean  $\pm$  SE ( $n = 6$ ). Statistically significant value was calculated by comparing with negative control group using Student t test (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

DW, distilled water; Experimental 1, UVB irradiation + 2% TAM + DW cream application; Experimental 2, UVB irradiation + 2% TAM + PW cream application; Negative Control, UVB irradiation + Base ointment application; Normal, Base ointment application; Positive Control, UVB irradiation + 0.5% Sunblock cream application; PW, phreatic water; RQ PCR, real-time quantitative polymerase chain reaction; SE, standard error; TAM, *Taraxacum mongolicum*.



**Fig. 15** – Effect of TAM with PW on TRP-2 mRNA gene expression in dorsal skin of HRM-2 mice. HRM-2 mice were sacrificed, and TRP-2 mRNA gene expressions were checked in dorsal skin. TRP-2 mRNA gene expressions were analyzed with RQ PCR. The results represent the mean  $\pm$  SE ( $n = 6$ ). Statistically significant value was calculated by comparing with negative control group using Student t test (\*\*  $p < 0.01$ ).

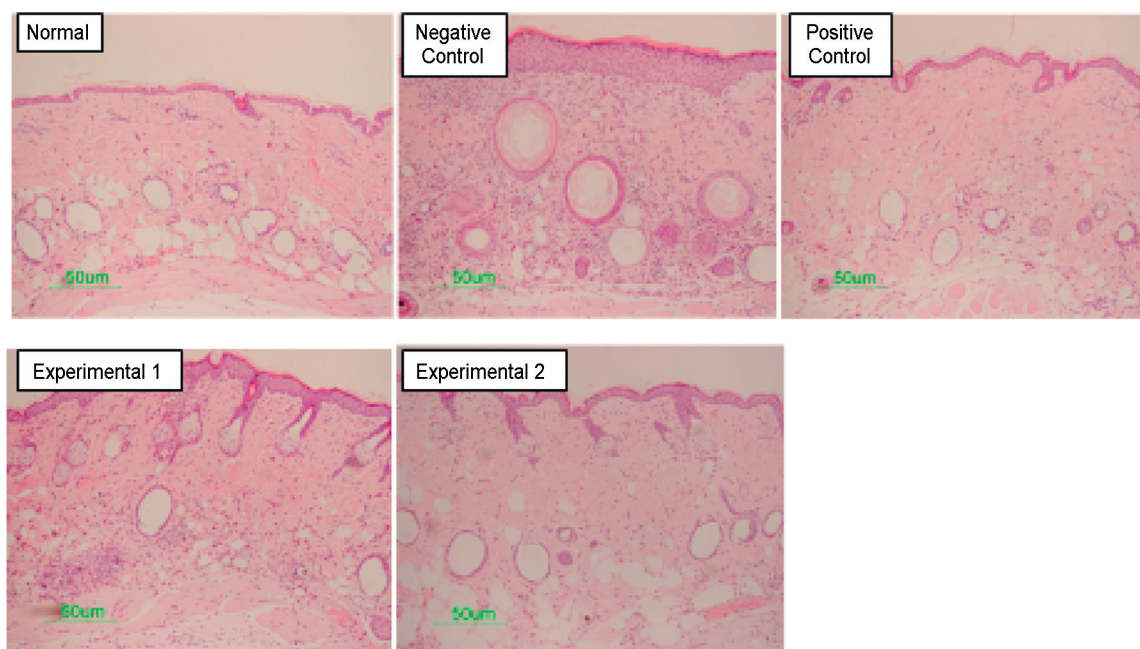
DW, distilled water; Experimental 1, UVB irradiation + 2% TAM + DW cream application; Experimental 2, UVB irradiation + 2% TAM + PW cream application; Negative control, UVB irradiation + Base ointment application; Normal, base ointment application; Positive control, UVB irradiation + 0.5% Sunblock cream application; PW, phreatic water; RQ PCR, real-time quantitative polymerase chain reaction; SE, standard error; TAM, *Taraxacum mongolicum*.

In oriental medicine, the principles of crenotherapy can be found in such statements as “Water is the source of all change ( ),” “The form is pure yin ( ) and the using is pure yang ( ) ( , ),” and “If there is no water, nutrients are removed ( ).” By nature PW is hot and a bit toxic, and spa bathing can remove scabies and skin disease.<sup>64</sup> In addition, <Supumron ( )> of “Donguibogam ( )”<sup>38</sup> divides water into 33 types and describes the nature and use of each type of water. Naturally, the nature and use of PW are also included.

In regard to recent studies on PW, there was a study that used PW from Suanbo Hot Springs, which is known as a simple spring of hot alkaline sodium-type silicon oxide, and found that it normalizes keratinocyte differentiation, restores the damaged skin barrier function, and reduces inflammatory cells from the skin by using atopic dermatitis induction NC/Nga mouse.<sup>65</sup> Another study used PW from Dogo Hot Springs and found that it effectively controls allergic inflammatory response and that it can be used for atopic skin disorder by using atopic dermatitis induction NC/Nga mouse.<sup>22</sup>

As such, PW can be used for various skin disorders, and many researchers have studied the variety of use and efficacy of PW. However, to date there have only been few studies on its whitening effect. Accordingly, this study was intended to figure out if using TAM together with PW from Dogo Hot Springs increases the inhibitory effect on melanin generation, as previous studies proved that TAM inhibits melanin





**Fig. 17 – Histological test in dorsal skin of HRM-2 mice. HRM-2 mice were sacrificed, and dorsal skin were processed for histology and stained with hematoxylin and eosin. It shows the thickening of the epidermis by bright microscope. DW, distilled water; Experimental 1, UVB irradiation + 2% TAM + DW cream application; Experimental 2, UVB irradiation + 2% TAM + PW cream application; Negative control, UVB irradiation + Base ointment application; Normal, base ointment application; Positive control, UVB irradiation + 0.5% Sunblock cream application; PW, phreatic water; TAM, *Taraxacum mongolicum*.**

generation and another previous study on organs analyzed the components of PW from Dogo Hot Springs.<sup>22</sup>

Paradise Spa Dogo, where this study was conducted, is located across Gigok-ri, Dogo-myeon, and Sinseong-ri, Seonjang-myeon in Asan-si, Chungcheongnam-do, Korea. It is a sulfurous spa classified as a light alkali spring and a light salty spring. Its water temperature is 25–32.5 °C.<sup>66</sup>

It was found that TAM with PW had a more excellent inhibitory effect on melanin generation in HRM-2 melanin-possessing hairless mice. It also showed an aging inhibitory effect as it identified that increased activation inhibited matrix metalloproteinase-9 (MMP-9),<sup>67</sup> which is the wrinkling material that destroys proteins in the skin (such as collagen) and progresses aging.

These results indicate that TAM with PW exert an inhibitory effect on melanin synthesis. Therefore, it is considered that TAM with PW can be used for treatment of skin hyperpigmentation such as melisma and freckle, and can be further used for development of whitening cosmetics with few skin side effects. In addition, because this study exhibited the aging inhibitory effect as well, it is considered that additional studies of TAM with PW on aging inhibition mechanism are needed.

However, because this study was performed on a limited number of experimental objects and conducted on animals only, it is considered that clinical studies on hyperpigmented individuals will be needed. In addition, because this study identified that TAM had an outstanding melanin generation inhibition and had an inhibitory effect when used along with PW, it is considered that further continuous and various

studies on the use of PW are needed in addition to more studies on TAM with PW on various categories.

### Conflicts of interest

All contributing authors declare no conflicts of interest.

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