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The Processed Radish Extract Melanogenesis in Humans and Induces Anti-Photoaging Effects in Ultraviolet B-Induced Hairless Mouse Model

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Abstract

The radish skin and radish greens are an edible part of the radish. But they are removed before eating the radish and used as a byproduct or an animal feed material because of their tough and rough texture.

Melanin is a pigment that gives colour to our skin. But increased production of melanin can turn into benign or malignant tumours. These days due to global warming, the amount of Ultra violet (UVB) rays has been extensively increased with sunlight. Due to this, a phenomenon called exogenous photo aging is widely observed for all skin colour and types. As a result of this phenomenon, a set of enzymes called matrix metalloproteinases (MMP's) that serves as degradation enzymes for extracellular matrix proteins mainly collagen is increased, causing depletion in collagen and resulting in early wrinkles formation. Therefore in our study we used the murine melanoma cell line B16/F10 to study the melanogenesis inhibition by Heated radish extract (HRE) in vitro and we used HRM-2 hair less mice exposed to artificial UVB for checking the efficacy of Heated radish extract in vivo. Furthermore, we prepared a 3% Heated radish extract (HRE) cream and checked its effects on human skin. Our results have clearly demonstrated that Heated radish extract (HRE) have potently suppressed the tyrosinase activity and melanin production in B16/F10 cells. It had also reduced the expression of components involved in melanin production pathway both transcriptionally and transitionally. In in vivo studies, HRE had potently suppressed the expression of MMP's and reduced the wrinkle formation and inhibited collagen degradation. Moreover, on human skin, ginseng cream increased the resilience, skin moisture and enhanced the skin tone. Therefore in light of these findings, we conclude that HRE is an excellent skin whitening and antiaging product.

Keywords*: Melanogenesis, antiaging, wrinkles, Heated radish extract, human trials*

1. INTRODUCTION

Melanin is a compound that gives colour to our skin and the variety of skin colours between the human races is attributed to the amount of melanin producing cells present in them [1-4]. Melanogenesis is the process that is responsible for the formation of melanin. In this process the α-Melanocyte Stimulating Hormone (α-MSH) binds with its receptor i.e. Melanocortin 1 receptor (MC1R) which causes the elevated levels of cAMP that in turn activates Microphathalamia associated factor (MITF) through various pathways like cAMP response element binding protein (CREB), Extracellular regulating kinase (ERK) and Protein kinase B (AKT) and

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thus causing its degradation. Due to this degradation, the rate limiting step in the process of melanogenesis i.e. tyrosinase enzyme (TYR), gets affected and is activated. Then TYR is responsible for the conversion of tyrosine protein to L-DOPA that forms melanin. Tyrosinase-related protein 1 (TRP1) and Tyrosinase-related protein (TRP2) are further downstream factors of TYR and MITF that are activated and are involved in formation of melanin. Therefore, during all this process, it's the TYR, that is most important in regulating the amount of melanin production in skin cells [5-7].

The radish is a vegetable of the family Cruciferae containing volatile sulfur-compounds that cause its unique spiciness. The spiciness peculiar to the radish is caused by the production of thiocyanate and isothiocyanate released enzymatically from the thioglucoside contained in the radish as a result of the enzyme glucosidase activity when the radish is cut to break cells. The radish contains a larger amount of free amino acids, sugars, calcium, phosphorus, etc. than other vegetables. The root of radishes contains sugar components like glucose and fructose and other ingredients, such as coumaric acid, caffeic aid, ferulic acid, phenylpyruvic acid, gentidin acid, hydroxyl benzoic acid, and a variety of amino acids. Particularly, it has the content of vitamin C amounting up to 20 to 25 mg and becomes an important source of vitamin C in winter. According to the ancient medicinal records, the root of radish, nabok, has the curative effects on phlegm, coughing, dysentery, etc. and eliminates food poisoning associated with fish, shellfish, and noodles. Diastase contained in the radish is used to promote digestion, neutralize the effects of food poisoning, and ease a hangover, and rapine is known as an antibiotic component against germs, fungus, parasites, etc.

Radish skin and radish greens are an edible part of the radish. But they are removed before eating the radish and used as a byproduct or an animal feed material because of their tough and rough texture. The research have conducted studies on a processing method capable of further increasing the physiological activity of radish, and as a result, have found that processed radish products including by-products may be effectively used to reduce skin whitening and anti-aging agent.

Up till now there has been many studies made on the effects of natural herb on melanin production and also on melasma. However, no study till now has reported the anti-melanogenic effects of heated radish extract (HRE) on melanin production and its skin whitening and anti-aging effects in particularly humans. Therefore, we made an attempt to investigate the tyrosinase inhibition and melanin production inhibition by HRE in vitro in B16/F10 melanoma cell line via the mechanistic study of pathway involved in this process. Our results have shown that HRE showed remarkable inhibition in tyrosinase activity and also showed decreased melanin content via the MITF degradation pathway. Moreover, our in vivo study on Hairless mouse (HRM-2) model of Ultra violet induced (UVB) photo aging and hyperpigmentation study revealed that application of Korean Heated radish extract (HRE 150 and 300mg/kg) substantially and remarkably reduced the production of melanin in HRM-2 mice. Furthermore, the 3% heated radish extract cream showed excellent anti-wrinkle and skin whitening qualities in humans. Therefore from our in vitro and in vivo study, we can conclude that HRE and its cream can guarantee its usage in the cosmetic industry as a skin whitening and anti-aging agent.

2. EXPERIMENT MATERIALS AND METHODS

2.1. Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM) [Daegu, Korea], foetal bovine serum (FBS) (WelGene Co., Korea), streptomycin and penicillin (Lonza,MD, USA), TRIZOL® reagent (Invitrogen, Carlsbad, CA, USA), oligodT (Bioneer oligo synthesis), MITF, TYR, TRP-1, TRP-2 and β-actin primers were obtained from Bioneer (Daejeon, Korea). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) were purchased from Sigma-Aldrich. Antibodies for MITF, TYR, TRP-1 and TRP-2 were obtained from Santa Cruz (Santa Cruz biotechonology, Inc, Texas, USA). Tyrosinase from mushroom and L-3,4-dihydroxyphenylalanine (L-DOPA) were purchased from Sigma (St. Louis, MO, USA). All other reagents were of local analytical grade.

2.2. Preparation of Heat-Treated Radish Extract (HRE)

Radish purchased from an agricultural and marine products market was washed, cut to a size of about 0.5 cm × 0.5 cm × 0.5 cm, and then freeze-dried. The freeze-dried sample was placed in the inner chamber of a

heat-treatment apparatus (Jisco, Seoul, Korea), which was designed and manufactured to be capable of resisting even a pressure of 10 kg/cm2 or higher. Water was placed in the outer chamber, and the sample was heat-treated at a temperature of 140 to 150°C for 6 hours. The apparatus could prevent the sample from coming into direct contact with water and also prevent the carbonization of the sample by direct heat transfer, from water contained in the outer chamber.

The heat-treated sample was cooled, and then crushed using a crusher, and a 10-fold volume (v/v) of distilled water was added, followed by extraction at 60°C for 2 hours. The extract was filtered, and then freeze-dried before use.

2.3. Cell Line

Murine melanoma cell line B16/F10, originating from American Type culture collection (ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 8% Fetal Bovine Serum (FBS) (WelGene Co, Daejeon) and 100 IU/mL penicillin and 100 µg/mL streptomycin sulfate (Lonza, MD, USA). The incubating conditions were humidified 5 % CO_2 at 37˚C.

2.4. Cell-free Tyrosinase inhibition assay

The assay was performed with slight modifications as previously described [6]. Briefly 10ul of HRE was put in 96 well in triplicates and mixed with 60uL of 50mmol/L phosphate buffer on ice (pH 6.8). Then 20ul of 0.9mg/mL L-DOPA was added to each well. Finally 10uL of mushroom tyrosinase was added in each well and the plate was incubated at 27ºC for 10 min. After incubation, the amount of dopachrome production was determined spectrophotometrically at 450nm by microplate reader (Versamax microplate reader, Molecular devices, LLC, CA, USA). Kojic acid in this experiment was taken as a positive control [16].

2.5. Melanin inhibition assay

The B16/F10 cells were seeded in the 6-well culture plate at a density of $2.5x10^3$ cells/well and then incubated for 5 days. After the cells reached the desired confluency, they were treated with KRG and then stimulated with α-MSH. The cells were then incubated again for 3 days and then harvested using 0.25% trypsin-EDTA solution and transferred to 1.5mL microcentrifuge tubes. The tubes were then centrifuged at 10,000rpm for 10 min and pellet was dissolved in 2mol/L NaOH for 15min at 60ºC. Later this dissolved mixture was transferred to 96 well plates and absorbance was measured at 450nm with a microplate reader (Versamax microplate reader, Molecular devices, LLC, CA, USA). The absorbance was compared to the synthetic standard melanin curve (Sigma).

2.6. Animal experiment and grouping

Six-week-old male HRM-2 melanin-possessing hairless mice were obtained from Central Lab Animal Inc. (Seoul, South Korea) and were housed in a controlled room (23±1°C, 55±5% humidity, 12-h light/dark cycle) with *ad libitum* access to water and feed. All animal care experimentations were strictly carried out according to the Institutional Animal Care and Use Committee of Daejeon University (Daejeon, Korea) (Permission number: DJUARB2017-033). After 1 week of acclimation period, mice were randomly divided into 5 groups with each group containing 5 animals. The first group was normal group with no treatment at all. Second group was control UVB treated group, third group was positive control group that received 0.01% sunblock with UVB. Fourth group received HRE 150mg/kg p.o. with UVB and fifth group received HRE 300mg/kg p.o. with UVB.

2.7. UVB irradiation and induction of photo aging

HRM-2 mice were irradiated at the dorsal skin with UVB lamp (15 W, maximum wave length 312 nm; UV intensity 100 µW cm -2, Ieda Boeki Co., Tokyo, Japan). To evaluate the effects of positive control (0.01% sunblock) and KRG on wrinkle formation and pigmentation, they were applied daily, 5-10 min before exposure of mice to UVB radiation. HRM-2 mice were irradiated with 100 mJ / cm^2 UVB radiation (1 minimal erythematic dose = 100 mJ / cm^2) daily for the first week and then UVB radiation was increased to 200 mJ / cm^2 from 2-5 weeks and mice were monitored 3 times in a week. Dietary intakes and body weight were taken

at regular intervals every week till 12 weeks.

2.8. Effect on melanin production in HRM-2 mice

For checking the melanin production as a result of exposure of mice to UVB, the dorsal portion of mice was divided into right and left sides. In all 4 groups except normal group, left side received UVB (control), sunblock, and HRE along with UVB for 5 weeks. The right side remained un-treated. UVB was given 3 times in a week for 5 weeks and pigmentation status of the skin was analyzed at 1 week, 3 weeks, and 5 weeks, respectively using the digital camera (D70 model, Nikon, Tokyo, Japan) after anesthetizing the mice with ether. Image analysis on the dorsal skin was analyzed using Image analysis software (Bio-Rad, USA) that harvested the photographs taken with the digital camera. The degree of melanin deposition was analyzed from the difference between the pigmented, UVB exposed and samples treated area on the left side with the un-treated and un-pigmented area on the right side.

2.9. Skin wrinkle measurement

The degree of skin aging induced by UVB was measured by observing wrinkle formation. To evaluate the formation of wrinkles, each HRM-2 mouse was anesthetized by intraperitoneal injection of chloral hydrate (body weight 0.1 ml / 7% CH / 25 g mouse) at 5th week. Exposure to UVB and samples treatment was same as described in the previous section. Skin wrinkles were measured using at 3rd, 4th and 5th weeks using DETAX System II (MIXPAC) and Double-Stick Disc (3M, Germany) after UVB irradiation. Double-Stick Disc (sprayed with DETAX System II) was attached to mouse skin and removed after 2-3 minutes. Disc wrinkles were evaluated according to the scoring system presented by [9]. According to this evaluation, grade 0 is defined as the absence of wrinkles, grade 1 as several shallow wrinkles, grade 2 as some wrinkles, and grade 3 as some deep wrinkles. For visual analysis of skin wrinkles, after disc removal, skin was cleaned with 70% ethanol and photographed with USB Digital Microscope (x400, CE FOROHS, China).

2.10. Enzyme-linked immunosorbent assay (ELISA)

In order to investigate the effect of UVB irradiation wrinkle-related genes i.e. matrix metalloproteinase (MMP-2), skin from all treated groups was harvested and proteins were analyzed using ELISA kit according to manufacturer's instructions, (MMP-2 ELISA kit) (R & D System, USA).

2.11. Histological observation of skin

The skin tissues extracted from each experimental group were fixed in 10% formalin solution for 48 hours, and then Hematoxylin and Eosin staining (H&E) was done according to [10] for epidermal thickness. For collagen visualization, Masson's Trichome (M-T) staining was performed according to established protocols [11].

2.12. RNA extraction and Real-Time PCR

Total RNA was extracted from the B16/F10 cells and from UVB irradiated mouse skin, after they were treated with HRE and stimulated with α-MSH and HRE by using Trizol® according to Manufacturer's instructions. RNA (2 μg) was annealed with Oligo dt (Bioneer Co, Daejeon) for 10 min at 70˚C and cooled for 5 min on ice, reverse transcribed using reverse transcriptase pre-mix (Bioneer Co, Daejeon) in 20 μL of reaction mixture and run for 90 min at 42.5˚C using thermal cycler. The reactions were terminated at 95˚C for 5 min to inactivate the reverse transcriptase. The reverse transcription polymerase chain reaction was performed using aliquots of cDNA obtained from RT reaction in a PCR premix (Bioneer Co, Daejeon). The PCR products were then electrophorised on 1% agarose gel stained with ethidium bromide and visualized using ImageQuant LAS 500 (GE health care life sciences, Seoul, South Korea). The intensity of band densities was normalized for corresponding GAPDH and primers sequence is given in Table 1. MMP-2, MMP-9 and IL-1β expression levels were analyzed with Real-time quantitative PCR using Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, USA).

Table 1. Oligonucleotide sequences of primers/probe used for qRT-PCR and Real-Time PCR.

Genes	Primer/probe	Oligonucleotide sequence (5'-3')
MITF	Forward	5'-CATCGCATAAAACCTGATGGC-3'
	Reverse	5'-GTCGTCACCCTGAAAATCCTAACT-3'
TYR	Forward	5'-TGGGTCATCTTGTCTTGCTG-3'
	Reverse	5'-TACCATCTGTTGTGGCTGGA-3'
TRP-1	Forward	5'-ACCCACACTGTGCCCATCTA-3'
	Reverse	5'-CGGAACCGCTCATTGCC-3'
TRP-2	Forward	5'-GTCCAATAGGTGCGTTTTCC-3'
	Reverse	5'-ACCCATTTGTCTCCCAATGA-3'
MMP-2	Forward	5'-CAGGGAATGAGTACTGGGTCTATT-3'
	Reverse	5'-ACTCCAGTTAAAGGCAGCATCTAC-3
MMP-9	Forward	5'-AATCTCTTCTAGAGACTGGGAAGGAG-3
	Reverse	5'-AGCTGATTGACTAAAGTAGCTGGA-3'
IL-1β	FAM	5'CTGTGTAATGAAAGACGGCACACCCACC-3'

2.13. Western blot analysis

B16/F10 cells were treated with HRE in the presence of α-MSH (10μM). Total proteins from cells and UVB irradiated mouse skin were extracted according to the instructions of PRO-PREP® lysis buffer (iNtRON Biotechonology Korea). Proteins were then measured using PROMEASURE assay kit (PRO-PREP, iNtRON Biotechonology, Korea). They were then separated by 10 % polyacrylamide gels through SDS-PAGE and transferred onto PVDF membranes (Millipore, Immobilion ®-P, Billerica MA, USA). Nonspecific binding on the PVDF membranes was minimized with a blocking buffer containing 5 % non-fat dry milk and 0.1 % Tween-20 in TBS. The membranes were then incubated with specific primary antibodies overnight at 4℃ followed by 1h incubation with horseradish peroxidase-conjugated anti-rabbit antibody (1: 3000 dilution). Bound antibodies were visualized using enhanced chemiluminescence (Supex, Daegu, Korea) and images were analysed using ImageJ software. β-actin was taken as internal control.

2.14. Statistical analysis

Data were presented as mean ±SEM. One way ANOVA, Dunnet's test and un-paired students T test were applied for the statistical evaluation of data or where specifically otherwise indicated. Statistical analyses with ***$p < 0.001$, **$p<0.05$ and *$p<0.01$ were considered significant.

3. RESULT AND DISCUSSION

3.1. Heated radish extract (HRE) inhibited tyrosinase activity in cell-free system and suppressed the melanin content

Tyrosinase (TYR) is an enzyme that is a rate limiting step in the process of melanogenesis [12, 13]. Therefore to check whether HRE possess the ability to suppress the production of this enzyme, we performed cell-free mushroom tyrosinase assay. As it is evident from fig. 1A, HRE had potently inhibited the TYR production. Moreover, we treated the melanoma cell line B16/F10 with HRE and stimulated with α-MSH for melanin secretion and found that it was efficiently suppressed by HRE as shown in Fig 1B. In both of these experiments we took kojic acid as our positive control, since it is a renowned whitening agent.

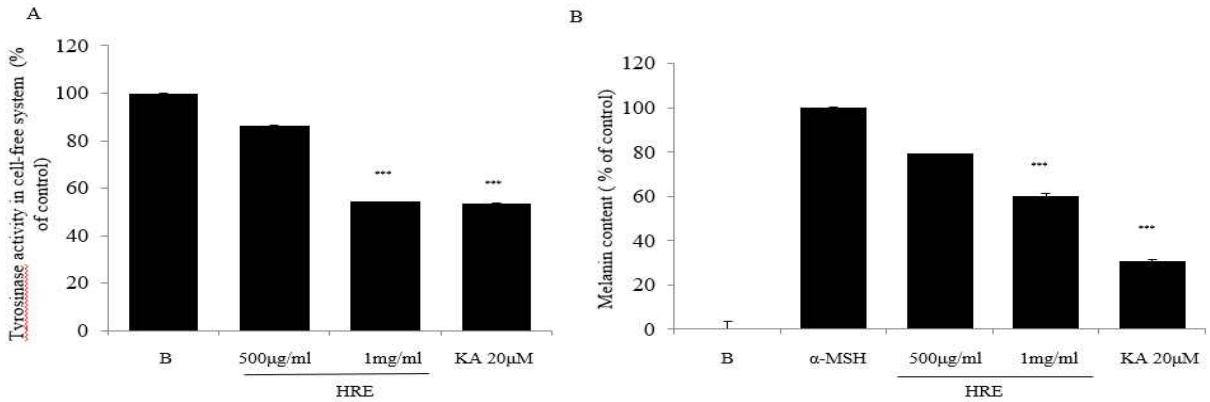

Figure 1. Inhibition of mushroom tyrosinase and production of melanin by HRE. (A) HRE inhibited the mushroom tyrosinase activity in a cell free system. (B) Melanin content in crude lysates was suppressed by HRE when B16/F10 cells were stimulated with α-MSH. Values in bar graph are mean ± SEM of at least 4 independent experiments. ***p<0.001 compared to untreated group.

3.2. Effects of Heated radish extract on the mRNA and protein expressions of MITF and tyrosinase related proteins

Above results elaborated the effects of HRE on tyrosinase and melanin contents. However, for the complete understanding of the mechanism that underlies for melanin inhibition by HRE, we checked the transcriptional and translational expression levels of the genes related to melanogenesis pathway. They are Tyrosinase related protein 1 and 2 (TRP-1 and TRP-2), the enzyme tyrosinase (TYR) and finally the Microphathalamia associated transcription factor (MITF). As can be seen in fig 2A-B, all the above mentioned four factors of the melanogenesis pathway were strongly inhibited by HRE. Henceforth we can justify HRE as good whitening agent at in vitro level study.

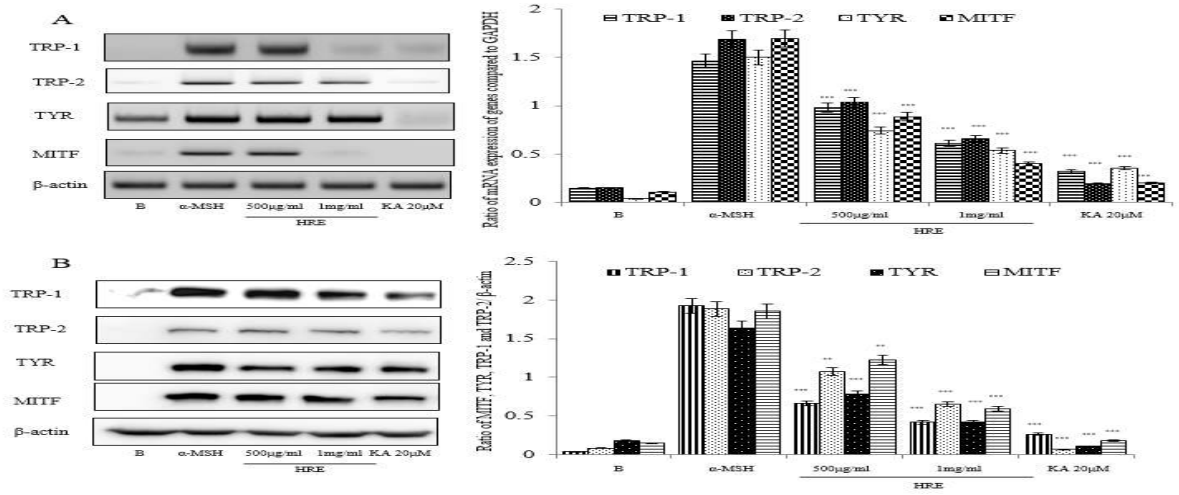

Figure 2. Depressed expression of MITF pathway genes by HRE. B16/F10 cells were seeded in 6 well plates and treated with indicated concentrations of HRE and then stimulated with α-MSH (10μM). Later RNA and proteins were extracted and MITF, TYR, TRP-1 and TRP-2 expression levels were checked by qRT-PCR (A) and also by western blotting (B). GAPDH was taken as internal control for checking transcriptional expression and β-actin was taken as translational control and all values were compared against them. ***p<0.001 and **p<0.05 were considered as statistically significant against α-MSH treated group only.

3.3. Effect of oral supplementation of HRE on body weight and food intake

Exposure to artificial UVB serves as a stress to animal skin resulting in loss of appetite, therefore we evaluated the dietary intake and dietary efficiency of HRM-2 mice exposed to UVB for 5 weeks and given sunblock and HRE orally. As can be seen in Fig 3A, there was no significant change in the body weights of normal, control UVB or the treatment groups. In addition food intake was also found to be significantly increased for positive control and HRE treated groups as shown in Fig. 3B. This proves that although UVB acted like stress inducer in mice, HRE treatment was effective in securing the mice from loss of appetite and weight.

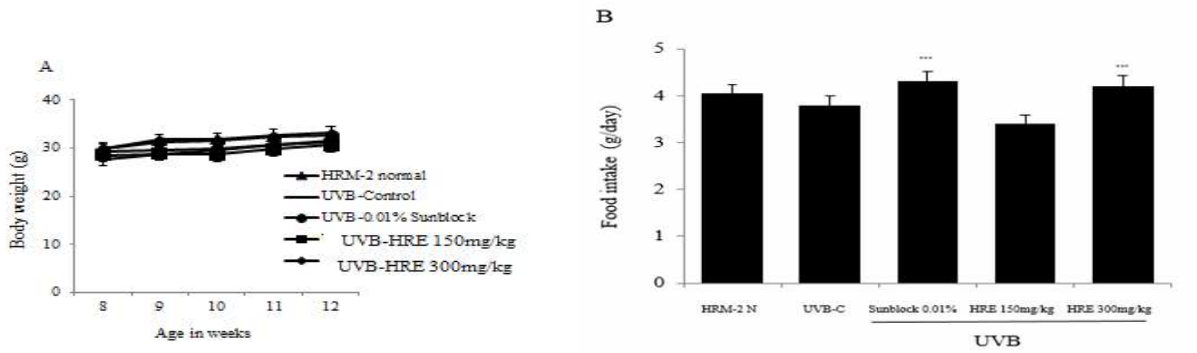

Figure 3. Effects of HRE on body weight and food intake. To measure body weight and food intake, HRM-2 mice were exposed to UVB for 5 weeks with the HRE orally and positive control treatment on skin. Food intake and body weight was taken daily during 5 weeks. (A) No significant change was observed in the control and treatment groups regarding weight change. (B) Daily food intake was significantly increased for Positive control, and HRE group. Values are expressed as means ± SEM from three - independent experiments. *** p<0.001 when compared with UVB control. HRM-2 N= normal mice, UVB-C= HRM-2 mice control (exposed to UVB).

3.4. Analysis of wrinkle-related genes expression in UVB irradiated skin damage induction model

In order to measure the effect of HRE on skin wrinkles formation, HRM-2 mice were irradiated with UVB to induce wrinkles. Then, after 5 weeks, skin tissues were isolated and wrinkle-related genes i.e. IL-1β, MMP-2 and MMP-9 was analyzed. As can be seen from fig. 4A-B, the proteins levels of MMP-2 gene as determined by ELISA and mRNA levels as determined by Real-Time PCR were significantly decreased in the positive control group and HRE groups when compared to control UVB group. Moreover, both the MMP-9 and IL-1β levels were also significantly decreased in HRE treated groups when compared to control UVB group through Real-Time PCR as shown in Fig. 4C-D.

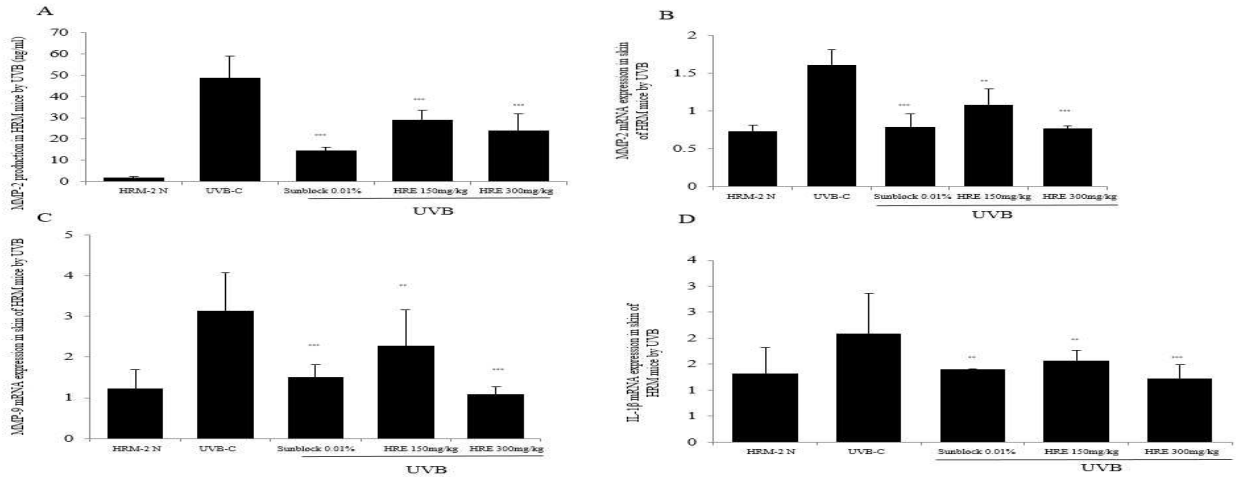

Figure 4. Analysis of wrinkle-related genes expression in UVB irradiated skin damage induction model. In order to measure the effect of HRE on the genes that are specifically related to wrinkle formation and photo

aging, HRM-2 mice were irradiated with UVB to induce wrinkles for 5 weeks along with positive control and HRE treatment. After 5 weeks, skin tissues were isolated and MMP-2, MMP-9 and IL-1β gene expression was analyzed. (A) MMP-2 production was significantly reduced by HRE as determined by ELISA. (B) MMP-2 mRNA expressions was significantly decreased by both HRE groups as determined by Real-Time PCR. (C) MMP-9 mRNA expressions was significantly decreased by HRE as determined by Real-Time PCR. (D) IL-1β expression was significantly decreased by HRE as determined by Real-Time PCR. Values in bar graphs are ± SEM from three -independent experiments. *** p<0.001 and **p<0.05 when compared with UVB control.

3.5. Effects of HRE on Melanogenesis in Skin Damage Induced by UVB Irradiation

In order to confirm the whitening efficacy of HRE, the difference in the formation of melanin at 1st , 3rd , and 5th weeks of UVB irradiation was examined by dividing the dorsal skin of HRM-2 mice into left(untreated) and right (UVB and samples treated sections). As can be seen from Fig. 5A, during the 1st week of exposure of mice to UVB with the treatment of sunblock and HRE, there was no significant decrease in production of melanin by both HRE doses. Then, in the 3rd week, there was a minor decrease in melanin production in positive control sunblock treated group and HRE groups when compared to control UVB group as can be seen in Fig. 5B. Then finally at 5th week as it is clearly evident from Fig 5C, the positive sunblock treated group and both HRE treated groups showed significant decrease in melanin production. These results clearly indicate that HRE can be effectively used as skin whitening agent.

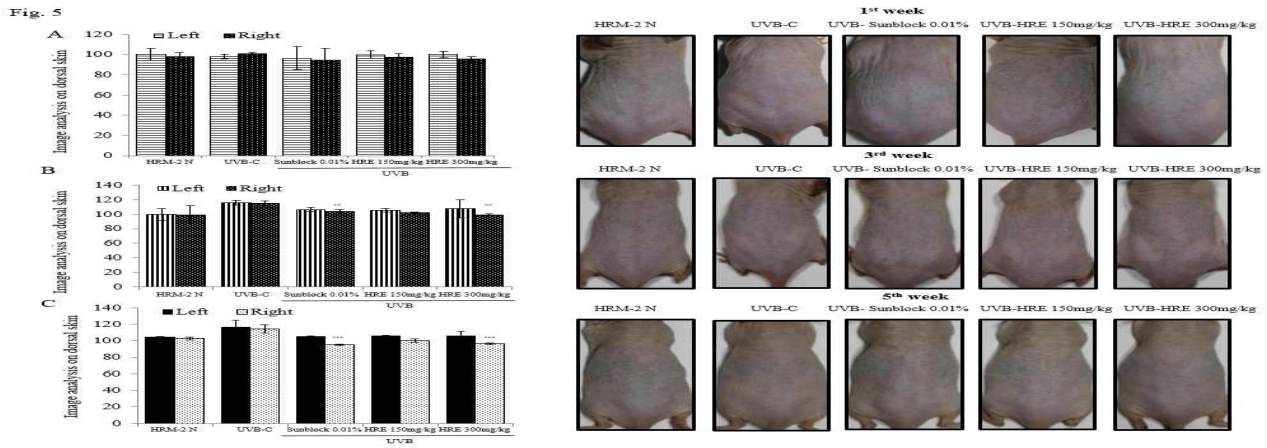

Figure 5. Effects of HRE on Melanogenesis in Skin Damage Induced Conditional Model by UVB Irradiation. In order to confirm the whitening efficacy of HRE, the difference in the formation of melanin at 1st , 3rd , and 5th weeks of UVB irradiation was examined by dividing the dorsal skin of HRM-2 mice into left (untreated) and right (treated) sides. Images were taken with digital camera as described in material and methods section. (A) No significant reduction was found in the melanin production in any groups in 1st week. (B) Minor reduction was found in the melanin production in HRE group in 3rd week. (C) Significant reduction was found in the melanin production in both HRE groups in 5th week .Values in bar graphs are ± SEM from three -independent experiments. *** p<0.001 and **p<0.05 when compared with UVB control. HRM-2 N= normal mice, UVB-C= HRM-2 mice control (exposed to UVB).

3.6. Analysis

To check the effects of HRE on formation of wrinkles and their depth, HRM-2 mice were irradiated with UVB to induce photo aging, and the skin wrinkle depth was measured using a 3D analyzer as given in materials and methods section for 3rd, 4th and 5th weeks. As can be clearly seen from fig. 6A-D, the positive sunblock treated group and both HRE treated groups showed significant decrease in wrinkle formation and depth.

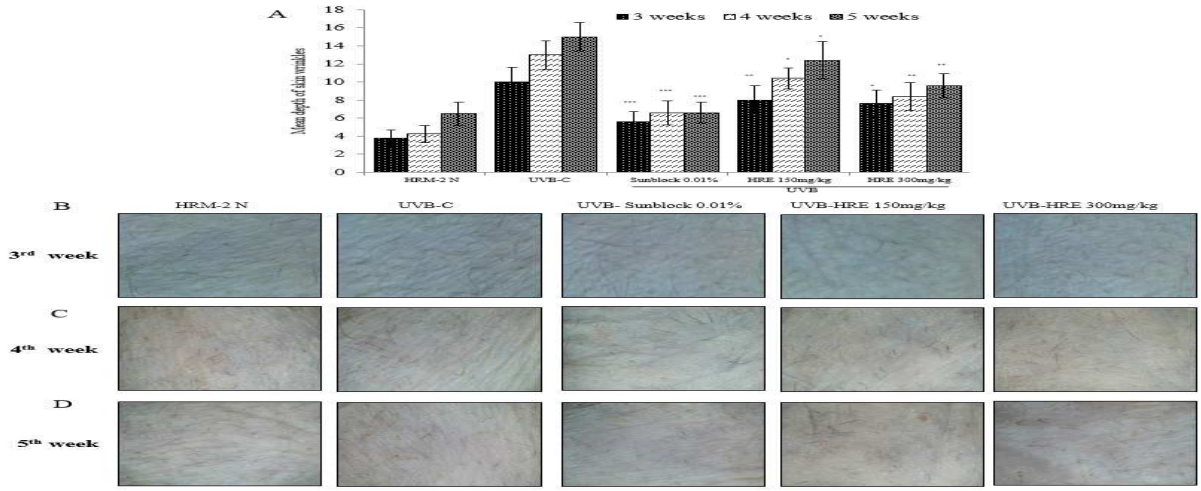

Figure 6. Fig.6. Effects of HRE on wrinkle depth and formation in HRM-2 mice.
In order to measure the effect of improving the wrinkle depth by HRE , HRM-2 mice were irradiated with UVB to induce photo aging, and then the skin wrinkle depth was measured and images were taken as given in the materials and methods section. (A) Significant reduction was found in the depth of wrinkles at 3rd, 4th and 5th weeks of HRE treatment. Values in bar graphs are ± SEM from three -independent experiments. *** p<0.001 **p<0.05 and *p<0.01 when compared with UVB control. (B) Wrinkles formation in 3rd week of HRE treatment. (C) Wrinkles formation in 4th week of HRE treatment. (D) Wrinkles formation in 5th week of HRE treatment. HRM-2 N= normal mice, UVB-C= HRM-2 mice control (exposed to UVB).

3.7. Decrease in epidermal thickness and increase in collagen by HRE

Ultraviolet radiations can cause increase in epidermal thickness causing the skin to become thicker and rough. Moreover continuous exposure to UVB can degrade the MMP's that causes a loss in collagen which is very essential to good skin health [14]. As shown in Fig. 7A, the epidermal thickness caused by UVB was potently reduced by the positive sunblock treated group and both HRE treated groups. Moreover it can be seen in Fig 7B that the epidermal thickness as visualized by H&E staining was also clearly decreased by the positive sunblock treated group and both HRE treated groups. The M-T staining was done to visualize the matrix components in the skin of HRM-2 mice. As shown in Fig 7C, the intensity of M-T stain is reduced in control UVB group as a result of degradation in collagen fibers but with the treatment of mice with sunblock and both HRE, the intensity of staining is dramatically increased when compared to UVB control group. These results clearly show that heated radish extract can be safely and efficaciously used for whitening and antiaging effects.

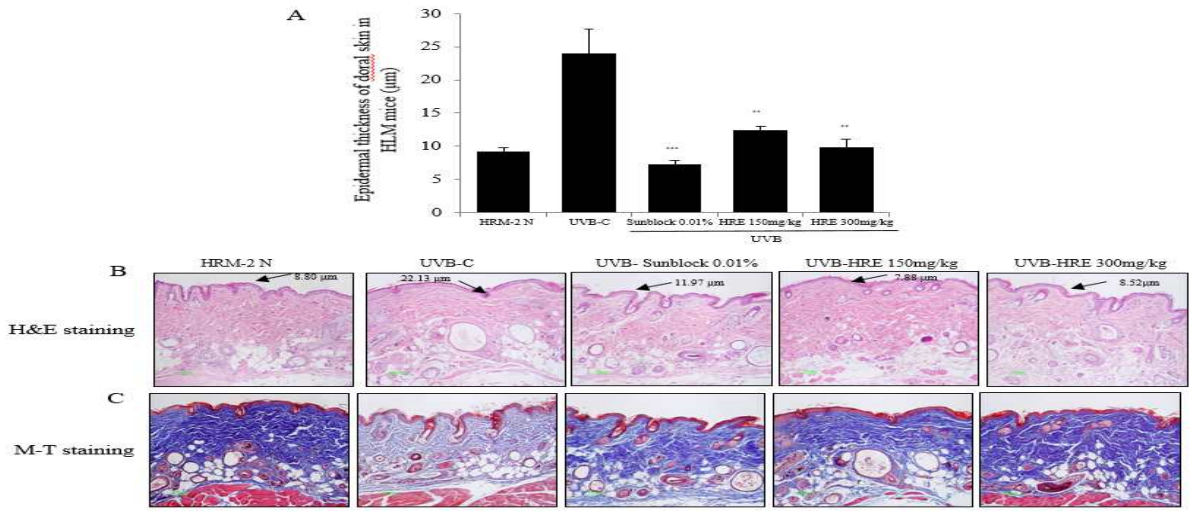

Figure 7. Effects of HRE on epithelial thickness and collagen fiber changes in HRM-2 mice UVB

irradiated mice. To evaluate the effects of HRE on epidermal thickness and amount of collagen fibers, HRM-2 mice were irradiated with UVB to induce photo aging for 5 weeks along with positive control and HRE administration and application. After termination of 5 weeks, skin tissues were stained with H & E to measure epithelial thickness using the microscope as given in materials and methods section. (A&B) Significant reduction was found in the epidermal thickness of HRE treated groups. Values in bar graphs are ± SEM from three -independent experiments. *** $p<0.001$ and ** $p<0.05$ when compared with UVB control. (C) The intensity of M-T staining was decreased in the UVB-control group compared to the normal group suggesting that collagen fiber degradation progressed and wrinkle formation accelerated. However, the amount of collagen fibers in HRE and positive control group were increased indicating that HRE reduced the amount of collagen degradation.

5. CONCLUSION

Melanocytes are the specialized melanin producing cells found in the basal layer of epidermis. These cells are involved in the production and transportation of melanin to the neighbouring keratinocytes and henceforth forming a uniform layer of skin pigmentation. Melanin is basically a pigment that serves two functions. The first and the most important one is to give colour to skin and the second one is to protect the skin from harmful Ultraviolet (UV) radiations by decreasing the amount of reactive oxygen species (ROS) [15, 16]. When the skin is exposed to UV, α-MSH hormone is activated that cause the production of melanin from melanocytes [17]. The key regulator this whole process of melanin production is the MITF that is the transcriptional factor for the regulation of tyrosinase enzyme (TYR) and Tyrosine related proteins (TRP-1 and TRP-2) [7, 18-20]. These all components are involved in the production of melanin and giving colour to skin.

Skin aging can be roughly classified into two types [21]. One is intrinsic or endogenous aging, which is an inevitable aging phenomenon with the increasing age of human. Clinical features of this type of aging are relatively mild, including fine lines, dry skin, and reduced elasticity [22]. The second is photo aging or exogenous aging, which refers to the aging phenomenon observed in the skin exposed to sunlight for a long time. In this case the element responsible is UVB via sunlight. This type of photo aging can be prevented by use of commercially available sunblock application before going into sun and also minimizing the exposure time under the sun. The clinical features of exogenous aging are deleterious like very rough, dry skin with reduced elasticity, deep wrinkles formation with severe skin sagging. It also makes skin very easily prone to pigmentation diseases like solar lentigo and so on [23, 24].

Moreover when the skin is excessively exposed to sunlight that contain UVB , the amount of extracellular matrix metalloproteinases (MMP's) increases that cause the degradation of matrix proteins and among them mainly collagen [25]. Since collagen is globally accepted element for skin elasticity and aging, its degradation causes early winkles formation and reduction in skin elasticity. After exposure to UVB, increased MMPs degrade collagen and other substrate proteins [26]. This is a sort of wound that the skin receives by the sun's rays, and our body makes efforts to heal wounds by synthesizing new collagen. However, since the process of wound healing is not always perfect, if the skin is continuously exposed to UVB, it becomes more aged clinically, including wrinkles.

Henceforth, to avoid the damage caused by UVB as a result of exposure to sunlight, many kind of herbal and pharmaceutical remedies are available like sunblock creams, lotions, sprays and so on. Even when going out in sun, it is prescribed to cover the body parts with caps and full clothes. However, in our research we have shown that the oral treatment and topical application of a herbal based HRE cream had effectively reduced the formation of wrinkles and decreased the dermal thickness as a result of UVB exposure. Moreover, they have also decreased the production of MMP'S and degradation of collagen.

Although melanocytes release a large number of soluble components, prostaglandin E2 prostaglandin F2α, adrenocorticotropic hormone (ACTH), and NO are regarded as well-known regulators of melanogenesis. [27-29] . But, the effect of cytokines on the process of melanogenesis is rather complicated. For example among the Inter leukines (IL) family, IL-1α/1β and granulocyte-macrophage colony-stimulating factor (GM-CSF) are involved in stimulation of melanogenesis, however, IL-6, TGF-β1, and TNF-α suppress melanin production. And in our results it has been shown that the levels of IL-1β were increased in the control UVB group while

with the treatment of HRE it was significantly decreased.

Henceforth, in a nutshell we conclude that HRE and HRE cream exhibited potent anti-melanogenic and whitening properties. Therefore it can be reckoned in future as a commercially available cosmetic agent.

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