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남극 지의류 Ramalina terebrata로부터 분리된 라말린의 미백효과

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Inhibition of Melanogenesis by Ramalin from the Antarctic Lichen *Ramalina terebrata*

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요 약: 남극 지의류인 *Ramalina terebrata*에서 분리 정제된 라말린(γ-glutamyl-N'-(2-hydroxyphenyl)hydrazide)은 이전 연구에서 강력한 항산화능을 확인하였다. 본 연구에서는 라말린의 추가적인 효능을 확인하기 위하여, 비암세포 세포주인 멜란에이 세포를 이용하여 라말린의 멜라닌 합성에 대한 효과를 확인하였다. 라말린은 세포 독성이 없는 농도에서, 멜란에이 세포에서 멜라닌 합성을 농도 의존적으로 감소시켰으며, 이러한 효과는 널리 사용되고 있는 미백제인 알부틴보다 우수하였다. 라말린은 무세포 타이로시네이즈의 활성을 직접 저해했을 뿐만 아니라, 세포 내의 타이로시네이즈의 활성도 저해하는 효과를 보였다. 라말린의 이러한 멜라닌 합성 저해의 기전 연구를 위하여, 멜라닌 합성에 중요한 단백질인 타이로시네이즈, TRP-1, TRP-2의 mRNA와 단백질 발현을 조사한 결과, mRNA양에는 영향을 주지 않고, 단백질의 발현은 감소되는 것으로 나타났다. 또한, 0.2 % 라말린을 포함한 제형을 사람피부에 도포하였을 때, 3주 후에 피부 밝기가 개선됨을 확인하였다. 이상의 결과를 통해서, 라말린은 타이로시네이즈의 직접적인 저해뿐만 아니라 멜라닌 합성과 관련된 단백질의 발현을 저해함으로써 미백효과를 나타낸다고 할 수 있으며, 이러한 효과를 인체시험을 통해 확인하였다. 따라서, 라말린은 멜라닌 합성을 저해하는 미백 소재로 활용될 수 있을 것으로 판단된다.

Abstract: Ramalin (γ-glutamyl-N'-(2-hydroxyphenyl)hydrazide) isolated from the Antarctic lichen *Ramalina terebrata* has been shown to have strong antioxidant activities in the previous study. To investigate additional activities of ramalin, we studied the effects of ramalin on melanogenesis in melan-a cells, a non-tumorigenic melanocyte cell line. At a non-cytotoxic concentration, ramalin dramatically decreased melanin synthesis in melan-a cells in a dose-dependent manner, which was more potent than arbutin, a well-known tyrosinase inhibitor. Ramalin inhibited cell-free tyrosinase activity directly and intracellular tyrosinase activity as well. Its inhibitory mechanisms on melanin production were further assessed, and we found that ramalin significantly decreased the protein levels of melanogenic enzymes such as tyrosinase, tyrosinase-related protein 1 (TRP-1), and tyrosinase-related protein 2 (TRP-2). However, the mRNA levels of these enzymes were not altered. In a clinical study, application of 0.2 % ramalin on human skin significantly improved the degree of skin brightness after 3 weeks. In conclusion, ramalin has strong anti-melanogenic activity that is exerted both by the direct inhibition of tyrosinase activity and by down-regulation of melanogenic proteins. Furthermore, ramalin showed skin brightening effect in a clinical study. Collectively, these results suggest that ramalin may be a useful inhibitor for melanogenesis in skin.

Keywords: *ramalina terebrata, ramalin, melanogenesis, tyrosinase, melan-a cell*

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1. Introduction

Melanin is a biopigment, synthesized in the specialized cellular organelles called melanosomes in melanocytes residing within the basal layer of epidermis. Melanin production in human skin is an important defense mechanism against ultraviolet (UV) radiation and one of major determinants of skin color [1,2]. Melanin is synthesized by coordinated actions of various melanogenic enzymes such as tyrosinase, tyrosinase-related protein 1 (TRP-1), and tyrosinase-related protein 2 (TRP-2) [3-5]. Tyrosinase is a key enzyme that catalyzes two initial rate-limiting reactions in melanogenesis, the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to dopaquinone. TRP-2, which functions as dopachrome tautomerase, catalyzes the rearrangement of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA), and TRP-1 oxidizes DHICA to a carboxylated indole-quinone. The melanosomes enclosing melanin polymers are transferred to keratinocytes, providing protection from harmful UV radiation and eventually disappear with desquamation of the skin [6]. Although melanin plays this protective role, excess melanin synthesis can lead to abnormal hyperpigmentations such as freckles, melasma, and senile lentigo. Therefore, active agents for the improvement of hyperpigmentation have been sought for clinical and cosmetic usage and many inhibitors of melanogenesis such as hydroquinone, arbutin, kojic acid, and ascorbic acid have been developed so far [7,8].

Therefore, we investigated the additional effects of ramalin on melanogenesis *in vitro* and *in vivo* in this study. In the previous study, ramalin (γ-glutamyl-N'-(2-hydroxyphenyl)hydrazide) from the Antarctic lichen *Ramalina terebrata* showed the strong antibacterial activity against *Bacillus subtilis* [9], and the more potent antioxidant capacity than the commercial antioxidants [10]. Since melanin is produced through series of oxidation reactions, most antioxidants are thought to inhibit melanin production [11]. Therefore, we investigated the additional effects of ramalin on melanogenesis *in vitro* and *in vivo* in this study.

2. Materials and Methods

2.1. Preparation of Ramalin

Ramalina terebrata Hook and Taylor (*Ramalinaceae*) was collected from Barton Peninsula (S62°13.3', W58° 47.0') on King George Island, Antarctica and identified as described previously [12]. Extraction and isolation of ramalin was performed by a previously described method [9]. Briefly, a freeze-dried and ground lichen sample was extracted in mixture of methanol and water. The resulting crude extract was dissolved in the distilled water and sequentially partitioned with n-hexane and $CHCl_3$ to yield n-hexane, $CHCl_3$, and water soluble fractions. Of these fractions, the water soluble extract was then subjected to automated mild pressure liquid chromatography and a stepwise gradient solvent system of methanol in water. The fraction eluted at 0 % methanol was subjected to semi preparative reverse phase HPLC. The gradient solvent system was 10 ~ 30 % acetonitrile in water and ramalin was eluted [10].

2.2. Materials

All tissue culture medium and components were purchased from Invitrogen (NY, USA). Arbutin, L-tyrosine, L-Dopa, phorbol-12-myristate-13-acetate (PMA), Triton X-100, sodium phosphate, and β-mercaptoethanol were from Sigma Chemical Co. (MO, USA). Protease inhibitor cocktail was from Boehringer-Mannheim (IN, USA), and L-[U-^{14}C] tyrosine and [3,5-^{3}H] tyrosine were from Amersham (NJ, USA).

2.3. Cells and Culture

Melan-a cells were a kind of gift from Dr. Bennette D.C. (University of London, London, UK). Melan-a cells are a non-tumorigenic melanocyte cell line originally derived from C57BL mice [13]. Melan-a cells were cultured in RPMI 1640 supplemented with 5 % heat-inactivated fetal bovine serum, 100 μM β-mercaptoethanol, 100 mM penicillin, 50 U/mL streptomycin, and 200 nM PMA, at 37 ℃ in a humidified atmosphere containing 10 % CO_2.

2.4. Cell Treatment

Melan-a cells were seeded and allowed to attach for 24 h. Then, triplicate cultures were fed with fresh medium containing various concentrations of ramalin. After 48 h, the medium was replaced with the same, fresh test medium. After a further 48 h, cells were harvested with 0.5 mL of 0.25 % trypsin/EDTA. After dislodging the cells with occasional agitation, 2 mL of medium was immediately added to inactivate the trypsin, and 100 mL aliquots were seeded into 96-well plates for alamarblue assay, as described below. The remaining cell suspensions were centrifuged for 5 min at 12,000 rpm, washed with PBS, and then solubilized in 200 μL of extraction buffer (1 % Nonidet P-40, 0.01 % SDS, 0.1 M Tris-HCl, pH 7.2, and protease inhibitor cocktail). Extracts were solubilized at 4 ℃ for at least 1 h and then assays were conducted for each sample in triplicate [14].

2.5. Cell Viability Assay

The alamarblue assay was used to determine cell viability. After treating as described above, 100 μL aliquots of harvested cells were plated. Cells were allowed to attach overnight at 37 ℃. 10 μL of alamarblue solution (Promega, USA) was added to each well and incubated at 37 ℃ for 4 h. After incubation, the fluorescence was monitored at 530 nm excitation and 590 nm emission wavelengths in Victor 2 Fluorometer (MS, USA).

2.6. Melanin Content Assay

After treating and extracting as described above, the resulting pellets were lysed with 1 N NaOH and transferred to 96-well plates in triplicate. The relative melanin content was determined by absorbance at 405 nm in a PowerWave X340 ELISA reader (Bio-tech Instruments, USA).

2.7. Measurement of Cell-free Tyrosinase activity

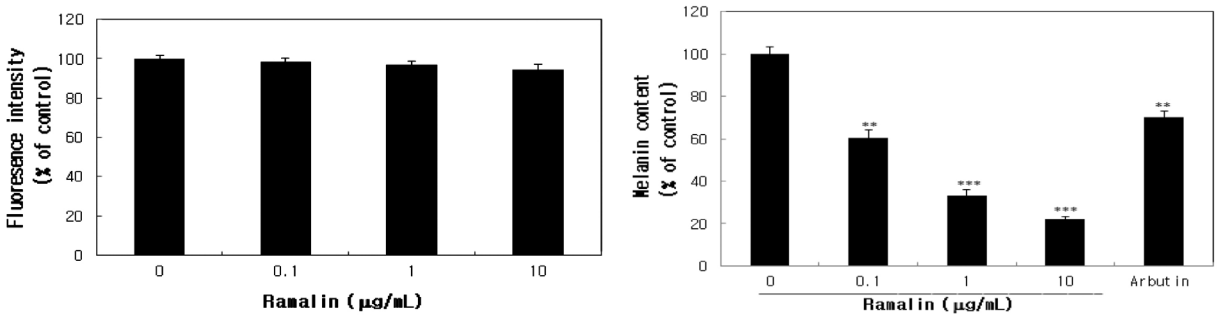
To prepare solubilized tyrosinase, 5×10^6 cells were removed from stock culture flasks and centrifuged at 1,200 rpm for 10 min, and the cell pellet was sonicated in 0.1 M sodium phosphate buffer (pH 6.8) containing 1 % Triton X-100 and 0.1 mM PMSF. After 1 h incubation at 4 ℃, the resulting extract was centrifuged at 12,000 rpm for 20 min at 4 ℃. 50 μL of the resulting supernatant was added to 1 mL of a reaction mixture containing 0.1 mM L-tyrosine, 2 mCi [3,5-^{3}H] tyrosine, 0.1 mM L-DOPA, and various concentrations of ramalin. After 3 h at 37 ℃, the reaction was terminated by addition of 1 mL of activated charcoal. Samples were centrifuged at 12,000 rpm for 10 min, the supernatant was removed and mixed with scintillation cocktail, and the radioactivity was determined using the scintillation system (LS 6500, Beckman, USA).

2.8. Measurement of Intracellular Tyrosinase Activity

Intracellular tyrosinase assay was performed in 96-well plates by adding 30 μL of cell extracts prepared as described above, 10 μL of L-[U-^{14}C] tyrosine, and 10 μL of 0.25 μM L-Dopa cofactor in 1 M sodium phosphate buffer, pH 7.2. Reactions were incubated for 4 h at 37 ℃ and the reaction was stopped by cooling. The contents of each well were transferred to the prelabeled 3 mm filter disks (Whatman, UK) and air-dried. The disks were washed with 0.1 N HCl containing excess cold L-tyrosine, twice with 95 % ethanol, and once with acetone. Each disk was air-dried and its radioactivity was determined using the scintillation system (LS 6500, Beckman, USA).

2.9. Western Blotting

Protein extracts were prepared described as above. Protein concentrations were determined with a BCA assay kit (Pierce, USA). Equal amounts of each protein extract (10 μg per lane) were resolved using 8 % SDS polyacrylamide gel (Koma Biotech, Korea) and transblotted onto nitrocellulose membranes (Amersham, USA), and the membranes were blocked with 5 % nonfat milk in TBST buffer. After blocking, the membranes were incubated with specific antibodies (1 : 1,000 dilution), αPEP7, αPEP1, and αPEP8, which recognize the COOH-terminal of tyrosinase, TRP-1, and TRP-2, respectively. αPEP7, αPEP1, and αPEP8 were a kind of gift from Dr. Vincent J. Hearing (NCI, NIH, USA). The membranes were then incubated with HRP-conjugated anti-rabbit IgG at a dilution of 1 :



(A) (B)

Figure 1. Effects of ramalin on viability and melanin synthesis in melan-a cells. Melan-a cells were treated with ramalin (0.1 to 10 μg/mL) for 4 d and then harvested. (A) Cell viability was evaluated through alarmablue assay. (B) The harvested cells were pelleted and their melanin contents were assayed. Data were expressed as a percentage of control, and each bar represents mean ± S.D. of three separate experiments. *Significantly different from the control (**$p < 0.01$, ***$p < 0.001$). Arbutin (200 μg/mL) was used as a positive control.

2,000. Immunoreactive bands were detected with an ECL kit (Amersham, USA).

2.10. Measurements of Skin Brightness in Human Skin

To evaluate the effect of ramalin in human, essence formulations containing 0.2 % ramalin as an active ingredient were made. Twenty two healthy female volunteers, average age of 45.1 (range 37-52), were involved in this study. The study was approved by the local ethics committee. Oral and written information were given to all participating subjects, and an informed consent agreement was obtained from each subjects. Skin brightness was measured on the site under the eye at week 0, week 3 and week 6 by a Chromameter (CR 300, Minolta, Japan). Five measurements on the same site were done and the average of three 'L' values, excluding maximum and minimum values, were used. L is the parameter for brightening of skin in Chromameter [15].

2.11. Statistical Aalysis

The results of each group are expressed as mean ± standard deviation (S. D.). In *in vitro* experiment, data were analyzed using one-way ANOVA with *Dunnett's post hoc* test between control and sample-treated groups. In *in vivo* experiment, the statistical difference was evaluated using student's *t*-test. A p value of < 0.05 was considered to represent a statistically significant difference.

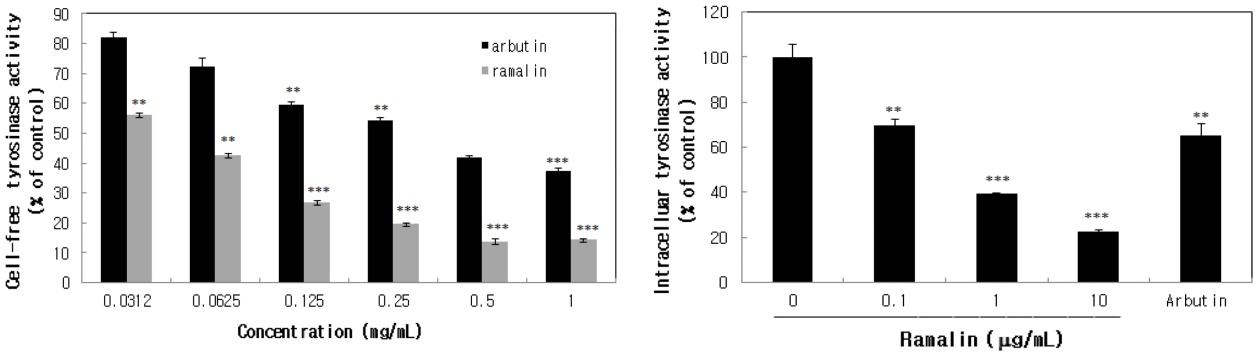
3. Results

3.1. Effects of Ramalin on Melanin Synthesis

When selecting skin-lightening compounds for cosmetic formulations, one of the important check points is that they should have minimal effects on melanocyte viability. Thus, cells were treated with ramalin for 4 d and viability of the cells was evaluated with alarmablue assay. Ramalin showed little inhibitory effects on cell viability at the tested concentrations (0.1, 1, and 10 μg/mL) (Figure 1A). To evaluate the effect of ramalin on melanin production, melan-a cells were treated at the non-cytotoxic concentrations for 4 d. Arbutin was used as a positive control. As shown in Figure 1B, the melanin content of cells that were treated with ramalin was decreased in a dose-dependent manner. At a concentration of 10 μg/mL, ramalin decreased melanin content by almost 80 % compared to the control. This suggests that the inhibitory effects of ramalin were not attributable to cytotoxicity.

3.2. Effects of Ramalin on Cell-free Tyrosinase Activity

Tyrosinase, a rate-limiting enzyme of melanogenesis, is known to closely correlate with melanin content of melanocytes [16] and provides a more sensitive indicator of changes in melanin synthesis than determi



(A) (B)

Figure 2. Effects of ramalin on cell-free tyrosinase activity and intracellular tyrosinase activity in melan-a cells. (A) For the assay of cell-free tyrosinase activity, tyrosinase was released from the untreated melan-a cells. The tyrosinase were incubated without or with ramalin and arbutin (0.03125 to 1 μg/mL) for 3 h in reaction buffer containing [3,5-^{3}H] tyrosine. Cell-free tyrosinase activity was determined by measuring the amount of tritiated water produced during this time. (B) For the assay of intracellular tyrosinase activity, melan-a cells were treated with ramalin (0.1 to 10 μg/mL) for 4 d and then harvested. Tyrosinase was extracted from the treated cells and the tyrosinase activity was assessed by counting L-[U-^{14}C] tyrosine incorporation into nascent insoluble melanin. Arbutin (200 μg/mL) was used as a positive control. Data were expressed as a percentage of control, and each bar represents mean ± S.D. of three separate experiments. *Significantly different from the control (**$p < 0.01$, ***$p < 0.001$). Arbutin was used as a positive control.

nation of total melanin levels. Therefore it has been an extensive screening target for the development of depigmentation agents for decades. To investigate whether the reduced melanin content is caused by the direct inhibition of tyrosinase activity, a cell-free tyrosinase assay with solubilized tyrosinase from untreated cell extracts was performed. Ramalin inhibited the cell-free tyrosinase in a dose-dependent manner. Compared with the untreated control, ramalin reduced *in vitro* tyrosinase activity by almost 90 % at 1 mg/mL, suggesting that ramalin is a direct inhibitor of tyrosinase catalytic activity (Figure 2A). Arbutin was used as a comparative control.

3.3. Effects of Ramalin on Intracelluar Tyrosinase Activity in Melan-a Cells

Reduction of tyrosinase activity in the melanocytes could be achieved either by the direct inhibition of tyrosinase itself or by the repression of tyrosinase expression. To investigate the effect of ramalin on the intracellular tyrosinase activity, the activity of tyrosinase purified from ramalin-treated melan-a cells was measured by counting L-[U-^{14}C] tyrosine incorporation into nascent insoluble melanin. Compared with the untreated control, ramalin significantly reduced the tyrosinase activity in a dose-dependent manner and almost 80 % of tyrosinase activity was reduced at 10 μg/mL (Figure 2B). This result suggests that inhibition of intracellular tyrosinase activity by ramalin also contributed to the significant reduction of melanin content.

3.4. Effects of Ramalin on mRNA and Protein Expression of Melanogenic Enzymes

Decrease in the melanin content and the intracellular tyrosinase activity by ramalin suggests that the regulation might be exerted at the level of gene and protein expression. In order to confirm this possibility, changes in the mRNA levels and protein levels of three important melanogenic enzymes (tyrosinase, TRP-1, and TRP-2) was measured after 4 d treatment using real-time quantitative PCR and Western blotting, respectively. Real-time quantitative PCR showed that ramalin did not significantly affect mRNA level of the melanogenic enzymes (data not shown). However, Western blot analysis revealed that ramalin decreased

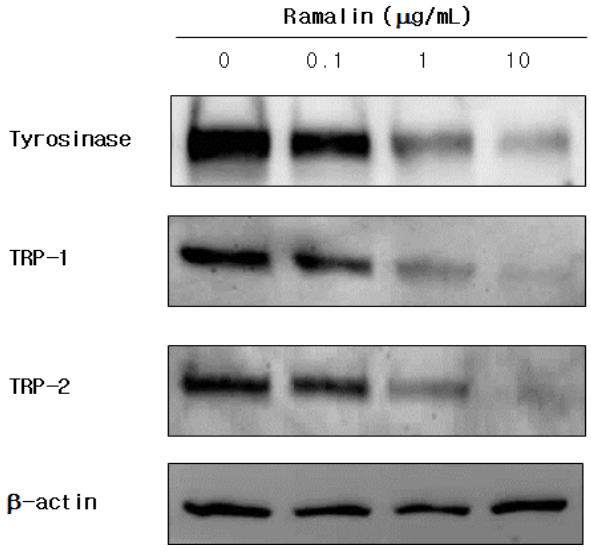


Figure 3. Melan-a cells were treated with ramalin (0.1 to 10 μg/mL) for 4 d and then harvested. Total protein was extracted and subjected to Western blotting. Specific detection of tyrosinase, TRP-1, and TRP-2 was performed using αPEP7 (anti-tyrosinase), αPEP1 (anti-TRP-1), and αPEP8 (anti-TRP-2), respectively. The loading control was assesed using β-actin.

the protein expression levels of tyrosinase, TRP-1 and TRP-2 in a dose-dependent manner (Figure 3). These results suggest that ramalin may inhibit melanogenesis through post-transcriptional modifications of melanogenic enzyme, which resulted in down-regulation of their protein expression, while the level of their mRNA transcription was not affected.

3.5. Effect of Ramalin on Skin Brightness *in vivo*

To investigate the effect of ramalin on skin brightness in human skin, L values were measured using a Chromameter before application and at week 3 and week 6 after topical application of 0.2 % ramalin. The measured values by chromameter were expressed as L, a, and b coordinate system. In this coordinate system, L values designate skin brightness, while a and b values point to skin chromaticity. Therefore, only L values were used to compare the extent of skin brightness in this study. As shown in Figure 4, L value was increased by 0.51 % at week 3 and 1.37 % at week 6 respectively. All volunteers participated in the study

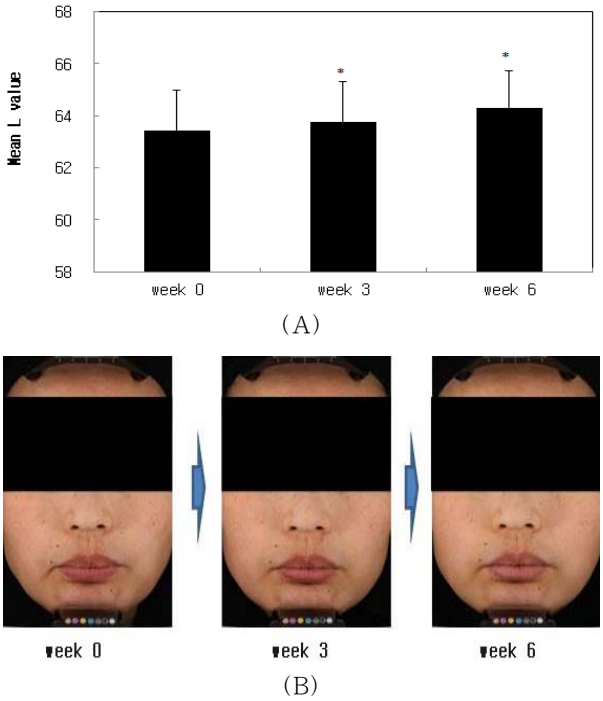


Figure 4. Improvement of skin brightness after ramalin treatment. (A) After topical treatment of 0.2 % ramalin, L values were significantly increased by 0.51 % and 1.37 % at week 3 and week 6, respectively compared to week 0. Results are described as mean ± SD of separate measurements. *$p < 0.05$ by a student *t*-test. (B) Clinical photographs were taken at week 0, week 3 and week 6 using a JANUS skin analyzer.

were subjectively satisfied with brightness effect of ramalin treatment (data was not shown). Furthermore, no side effect from the topical application of 0.2 % ramalin was revealed for 6 weeks. Therefore, these results suggest that topical applications of ramalin can improve skin brightness without any side effect *in vivo*.

4. Discussion

In this study, the activity of ramalin in the melanogenesis was investigated and found that ramalin reduced the melanin content in melan-a cells (Figure 1B) at non-cytotoxic concentration (Figure 1A). To investigate mechanisms of ramalin on the inhibition of melanin synthesis, the inhibitory effect of ramalin on cell-free tyrosinase activity was examined. IC_{50} value of

ramalin (0.046 mg/mL) was more potent than that of arbutin (0.37 mg/mL), confirming ramalin to be a potent inhibitor of melanogenesis (Figure 2A). In addition to this direct inhibition of tyrosinase activity, ramalin also dramatically decreased the intracellular tyrosinase activity in melan-a cells (Figure 2B). To investigate the further mechanism of ramalin, the effects of ramalin on mRNA and protein expression of three important melanogenic enzymes such as tyrosinase, TRP-1 and TRP-2 was examined. Ramalin treatment for 4 d did not decrease the mRNA expression level of tyrosinase, TRP-1 and TRP-2. However, protein expression levels of these melanogenic enzymes were reduced in a dose-dependent manner (Figure 3). Despite their potent inhibitory actions on melanogenesis *in vitro*, some compounds lose their activity in the finished cosmetic products due to the interaction and the interference with other ingredients. In order to verify the actual effect of ramalin in human volunteers, twenty two subjects were included in this *in vivo* study. After 3 weeks and 6 weeks of application, the degrees of skin brightness were significantly increased compared to that of the before treatment.

Ramalin (γ-glutamyl-N'-(2-hydroxyphenyl)hydrazide) was isolated from the methanol-water extract of the Antarctic lichen *Ramalina terebrata* by several chromatographic methods [10]. Lichens are the symbiotic association of a fungus (mycobiant) and an alga (photobiant) and/or cyanobacteria. In lichens, the fungus forms a thallus or lichenized stroma that contains characteristic secondary metabolites [17] and these lichen metabolites have been reported with various biological activities such as cytotoxic, fungicidal, antimicrobial, antioxidant, and anti-inflammatory [18]. Ramalin was a strong antioxidant when measured *in vitro* in both the DPPH assay and the superoxide anion scavenging assay [10]. Since melanin is produced through series of oxidation reactions, most antioxidants are thought to inhibit melanin production *via* tyrosinase inhibition. For example, ascorbic acid and its analogs have anti-oxidation function and are potent inhibitors of melanin synthesis [11].

Depigmentation agents can be classified on the basis of interfering mechanisms such as regulating 1) the transcriptional and post-transcriptional control of melanogenic enzymes, 2) glycosylation and maturation of melanogenic enzymes, 3) the uptake and distribution of melanosomes in keratinocytes, and 4) melanin degradation and turnover of pigmented keratinocytes [7]. Depigmentation agents, which are able to regulate melanin synthesis without affecting the expression of melanogenic genes, are likely to exert a post-transcriptional control of melanogenic enzymes. For example, linoleic acid (C18 : 2) decreased melanogenesis by proteolytic degradation of tyrosinase without the alteration in melanogenic enzyme mRNA level. Therefore, further molecular details of the mechanism of melanogenesis inhibition by ramalin need to be determined in the future.

In conclusion, we have shown that ramalin acts through dual mechanisms to reduce melanin synthesis; first by the direct inhibition of cell-free tyrosinase activity *via* an anti-oxidant effect, and second, by the decreased expression of melanogenic enzymes. Also in human clinical test, ramalin showed skin brightening effect in three weeks. Therefore, ramalin may be a useful inhibitor of melanogenesis and may have beneficial effects in the treatment of hyperpigmentation.

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