Original Articles

Journal of Alternatives to Animal Experiments
14 (1), December, 2020 : 59~65

Mulberroside A 및 Oxyresveratrol의 병용 처리에 의한 미백 시너지 효과

박희진, 허윤정, 임경민*

이화여자대학교 약학대학

Synergistic Hypopigmentation Effects of Mulberroside A and Oxyresveratrol

Heui-Jin Park, Yoon-Jung Huh, Kyung-Min Lim*

College of Pharmacy, Ewha Womans University, Seoul, 120-750, Republic of Korea

ABSTRACT. Melasma is an acquired hyper-melanosis characterized by irregular light to dark brown macules and patches on sun-exposed areas of the skin. Topical treatments of melasma involve the use of hypo-pigmenting agents which modifies various stages of melanogenesis. The most common mode of action for hypo-pigmentation is the inhibition of the tyrosinase. Mulberroside A and oxyresveratrol, well known tyrosinase inhibitors, are found frequently together in natural products. The aim of the experiment is to find out the synergism in decreasing pigmentation by mulberroside A and oxyresveratrol. B16F10 murine melanoma cells were used for identification of melanin content and cell viability. In addition, inhibition against tyrosinase, a key enzyme for melanin synthesis, was determined. Single treatment of mulberroside A or oxyresveratrol exhibited dose-dependent inhibition of mushroom tyrosinase activity, which showed a reduction of extra melanin in B16F10 melanoma cells. Interestingly, while mulberroside A or oxyresveratrol rarely reduced melanin inside the melanocytes alone, their mixture could significantly attenuate the production of intracellular melanin, which might be manifested as synergistic hypopigmenting effects. Further studies are needed to understand the mechanism of synergism by mulberroside A and oxyresveratrol in the inhibition of melanin synthesis.

KEY WORDS: Mulberroside A, Oxyresveratrol, hypopigmentation, Melanin assay, Mushroom tyrosinase assay

Introduction

Melasma is frequently acquired symmetrical hyper-melanosis characterized by irregular light to dark brown macules and patches on sun-exposed areas of the skin especially cheeks, forehead, and upper lips (Passeron and Picardo, 2018). The major etiological factors include genetic influences, exposure to ultraviolet radiation, and sex hormones. It is common in oriental women, pregnant women, and exacerbates during summer season. The pathogenesis of melasma is not yet fully understood (Kim et al., 2007). One study demonstrated that melasma skin has more solar elastosis, suggesting that the dermal change may influence the development of melasma (Kang et al., 2002). Another study suggested that dermal inflammation induced by accumulation of UV irradiation is associated with the activation of fibroblasts which result in the up-regulation of stem cell factor in melasma dermal skin leading to melanogenesis (Kang et al., 2006). And a study has suggested that human melanocytes respond to angiogenic factor

Received: 9 December 2020
Revised: 22 December 2020
Accepted: 24 December 2020

* Corresponding author: Kyung-Min Lim
College of Pharmacy, Ewha Womans University,
Seoul, 120-750, Republic of Korea
Tel: 02-3277-3055; Fax: 02-3277-3760
E-mail: kmlim@ewha.ac.kr

because melanocyte expresses vascular endothelial growth factor (VEGF) receptor (Kim et al., 2007).

Topical treatments of melasma involve the use of hypo-pigmenting agents, such as hydroquinone, retinoids (tretinoin, isotretinoin, adapalene), azelaic acid, N-acetyl-4-S-cysteaminylphenol, kojic acid, topical steroids, glycolic acid, mequinol, arbutin, ascorbic acid or vitamin C, tranexamic acid, rucinol serum, and combination of these agents (Bandyopadhyay, 2009; Rendon et al., 2006). Less commonly used depigmenting agents include bearberry extract, paper mulberry plant extract, arbutin, licorice extract, melawhite, ascorbic acid, mercury, and indomethacin (Rendon et al., 2006). Topical medications modify various stages of melanogenesis. Especially the most common mode of action is inhibition of the enzyme, tyrosinase (Bandyopadhyay, 2009).

Melanogenesis is a complex pathway. The first step of melanin synthesis is the oxidation of L-tyrosine to L-3, 4-dihydroxyphenylalanine (L-DOPA), and then to dopaquinone, both of which are catalyzed by tyrosinase. The rest of pathway is a series of enzymatic and nonenzymatic chemical reactions to produce two types of melanin, eumelanin and pheomelanin (Nguyen and Fisher, 2019).

Mulberroside A is one of the major components of mulberry plant extract and has the moderate tyrosinase inhibitory activity (Erdogan Orhan and Tareq Hassan Khan, 2014). Oxyresveratrol is a deglucosylated form of mulberroside A, a stilbenoid found in the root of Morus alba, and has the strong tyrosinase inhibitory activity (Kim et al., 2002). Mulberroside A inhibits mushroom tyrosinase as a competitive inhibitor to L-tyrosine and L- DOPA, while oxyresveratrol inhibits enzyme as a noncompetitive and competitive inhibitor to L-tyrosine, or L- DOPA (Kim et al., 2012). With L-tyrosine as the tyrosinase substrate, mulberroside A and oxyresveratrol showed the inhibitory activity against mushroom tyrosinase with the half maximal inhibitory concentration (IC50) of 53.6 μM and 0.49 μM, respectively. Oxyresveratrol has 110-fold higher tyrosinase inhibitory activity than mulberroside A (Kim et al., 2010). Another study reported that number and positions of hydroxyl groups in hydroxystilbenes seem to play an important role in tyrosinase inhibitory activity, and poor inhibitory effect might be caused by the steric hindrance of bulky glucosyl moiety which does not allow it to reach the target site of the tyrosinase (Shin et al., 1998).

Oxyresveratrol has limitations to be used in cosmetic products because it is a strongly yellow powder. To overcome this, a previous study examined a mixture of oxyresveratrol and dioscin, which shows the tyrosinase inhibitory effect higher than combined activities of the respective substance (Liang et al., 2012).

Mulberroside A is white powder and has the tyrosinase inhibitory mechanism distinct from that of oxyresveratrol. As shown in the oxyresveratrol and dioscin study, a mixture of mulberroside A and oxyresveratrol, from the same natural product, might have the synergistic effects in inhibition of tyrosinase. The aim of the present study was to examine the synergism in decreasing pigmentation by mulberroside A and oxyresveratrol.

Materials and Methods

1. Materials

B16F10 murine melanoma cells, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from American Type Culture Collection (ATCC). Antibiotic-antimycotic solution (100 U/mL penicillin, 100 μg/mL streptomycin) were purchased from Hyclone Laboratories Inc. (Logan, UT, USA). Mushroom tyrosinase, α-melanocyte stimulating hormone (α-MSH), 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), L-tyrosine, L- DOPA, arbutin, oxyresveratrol and mulberroside A were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide (NaOH) were purchased from DAEJUNG (Gyeonggi-do, South Korea).

2. Melanin assay

Mouse B16F10 melanoma cells were cultured in DMEM medium containing 10% FBS and 1% penicillin/streptomycin. Each experiment was performed using the same passage cells. Six hundred μl of 2×10^4 cells were seeded on each well of a 48-well plate. After leaved for attachment to the plate for 24 h, the medium was removed. The cells were stimulated by α-MSH and co-treated with various concentrations of samples (mentioned in each figures) or arbutin (50 ppm) in culture medium containing 0.5% dimethyl sulfoxide (DMSO) for 48 h. α-MSH-untreated cells were used as negative controls, and arbutin-treated cells were used as positive controls. Extra melanin was quantified with an upper medium at absorbance of 405 nm using a microplate reader (Spectra max 190, Molecular Devices, Sunnyvale, CA, USA). To identify the intra melanin content, the extra medium in the same plate was removed and the cells were dissolved in 300 μl of 1 M NaOH at 60°C dry room for 1 h in the dark. Two hundred μl of solution was measured by absorbance at 405 nm. All measurements were performed in triplicate.

3. Cell viability assay

Cell viability was quantified using MTT assay by measuring mitochondrial activity as an indicator of cell viability and cytotoxicity (Kim et al., 2012). MTT, a yellow tetrazolium salt, is reduced by dehydrogenase in viable mitochondria to purple formazan crystals. After removal of treatment of samples, 250 μl of 0.5 mg/ml MTT was treated and incubated in 2-3 h at 37°C. MTT was removed and 300 μl of DMSO was refilled. After shaking 30 min at 350 rpm, 25°C condition, 150 μl of upper solution was measured by absorbance at 540 nm. The darker the color of the solution means the more cells are alive. Results are expressed as a percentage of the control cells.

4. Mushroom tyrosinase activity assay

For measuring mushroom tyrosinase activity using substrate as L-tyrosine, the reaction mixture consisted of 180 μl of 0.3 mg/ml L-tyrosine, 2 μl of sample dissolved in DMSO, 30 μl of 0.1 M phosphate buffer or 30 μl of mushroom tyrosinase (250 U). For L- DOPA, mixture consisted of 180 μl of 2 mg/ml L-DOPA, 2 μl of sample dissolved in DMSO, 30 μl of 0.1M phosphate buffer or 30 μl of mushroom tyrosinase (50U). The amount of dopachrome was measured by absorbance at 475 nm.

5. Statistics

Data are showed as mean ± SEM of three independent experiments. The statistical significance of differences between groups was estimated using a two-sided Student's t-test. A p value < 0.05 was considered significant.

Results

1. Effects on Melanogenesis

To decide the concentration range to examine synergism, the five concentrations of mulberroside A and oxyresveratrol were tested for melanin synthesis inhibition. Since the mushroom tyrosinase inhibitory activity of mulberroside A was about 100 times greater than that of oxyresveratrol in the previous study, the concentration ratio of the two substances was set as 1 to 100 (Kim et al., 2010). As a result, mulberroside A and oxyresveratrol could rarely inhibit melanin generated inside of melanocytes (Fig. 1A). In addition, remarkable reduction of melanin secreted outside of melanocytes was observed dose dependently (Fig. 1B). These inhibitory activities were stronger than arbutin, a well-known inhibitor of melanin synthesis.

Mulberroside A at concentrations of 0.1 ppm, 0.3 ppm, 1 ppm and oxyresveratrol at concentrations of 0.01 ppm, 0.03 ppm, 0.1 ppm were selected

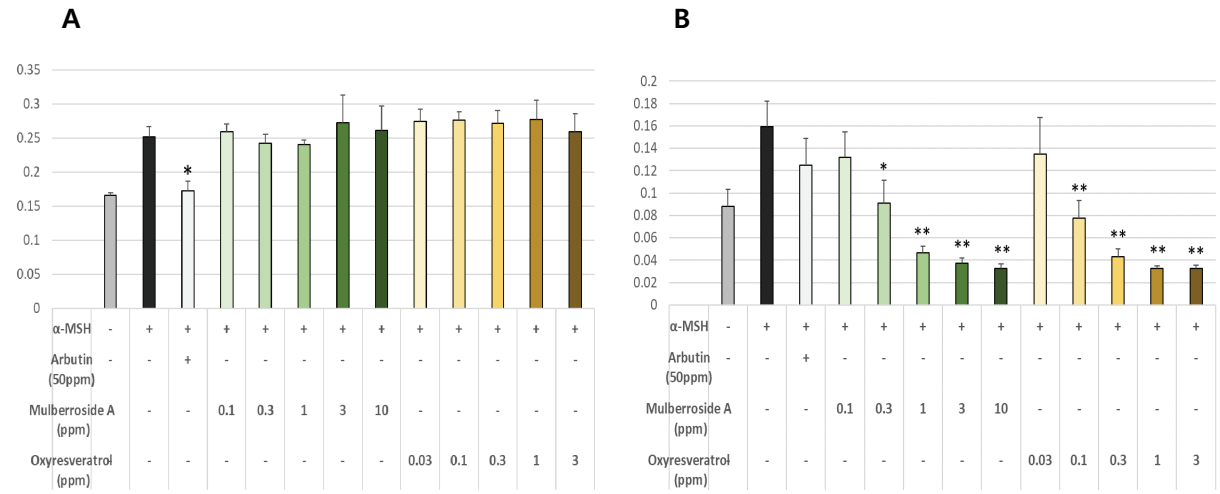

Fig. 1. Optical density (OD) of intra melanin (1A) and extra melanin (1B) at a single treatment of mulberroside A, oxyresveratrol and arbutin. The results are expressed as mean ± standard deviation (SD) (n=3). A p-value means that * < 0.05, ** < 0.01.

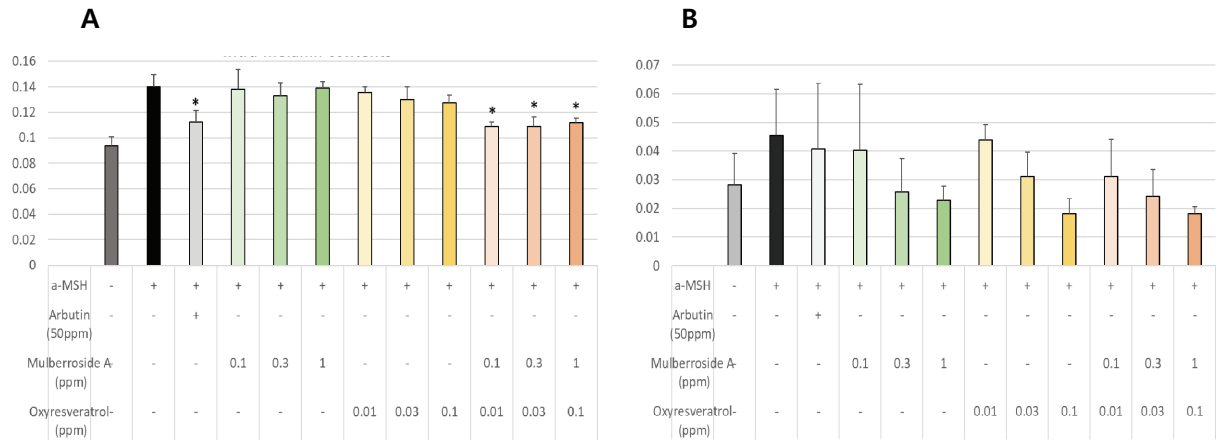

Fig. 2. Optical density (OD) of intra melanin (2A) and extra melanin (2B) at a mixed treatment of mulberroside A and oxyresveratrol and a single treatment of arbutin. The results are expressed as mean ± SD (n=3). A p-value means that * < 0.05.

for testing synergism based on the result of melanin inhibition and cell viability.

Interestingly, synergism was observed for the melanin synthesis inhibition inside of melanocyte by the mixture of mulberroside A and oxyresveratrol (Fig. 2A). Outside of melanocyte, however, synergism was rarely observed (Fig. 2B).

2. Effects on Cell viability

While the highest concentration of mulberroside A and all the concentrations of oxyresveratrol tested resulted in decreased cell viabilities, all of them were not toxic to the cells (Fig. 3).

In the synergism experiments, all of the tested samples were not toxic (Fig. 4). The most noteworthy was that the mixture of mulberroside A and oxyresveratrol resulted in increased cell viabilities as compared with same concentrations (0.03 ppm, 0.1 ppm) of oxyresveratrol.

3. Mushroom tyrosinase assay

The mushroom tyrosinase activity was measured with substrates, L-tyrosine and L- DOPA respectively with mulberroside A, oxyresveratrol

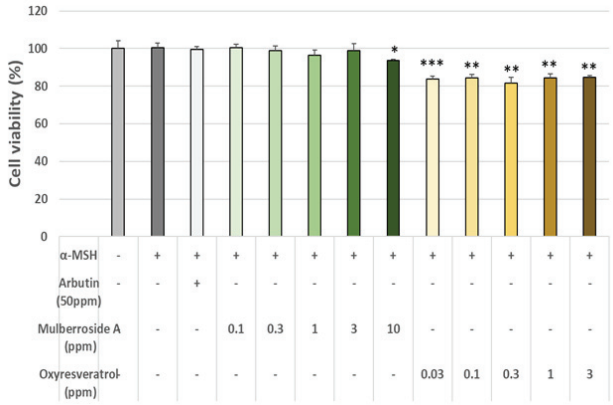

Fig. 3. Effect of a single treatment of mulberroside A, oxyresveratrol and arbutin on B16F10 cell viability, as assayed by MTT. The data are setting 100% to the control group which is untreated anything. The results are expressed as mean ± SD (n=3). A p-value means that * < 0.05, ** < 0.01, *** < 0.001.

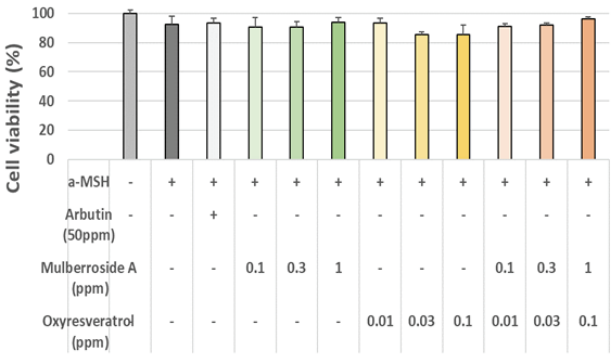

Fig. 4. Effect of a mixed treatment of mulberroside A and oxyresveratrol and a single treatment of arbutin on B16F10 cell viability, as assayed by MTT. The data are setting 100% to the control group which is untreated anything. The results are presented as mean ± SD (n=3).

and arbutin. Oxyresveratrol showed a strong inhibition against mushroom tyrosinase oxidase activity when using L-tyrosine as a substrate, while mulberroside A showed only weak inhibition (Fig. 5A). There was no synergism of mulberroside A and oxyresveratrol in tyrosinase inhibition with L-tyrosine (Fig. 5B).

On the result of the mushroom tyrosinase activity inhibition for L- DOPA, all materials showed only weak inhibition (< 50%) (Fig. 6A). There was no synergism by the mixture of mulberroside A and oxyresveratrol. Rather, the mixture showed lower inhibition activity than mulberroside A or oxyresveratrol alone (Fig. 6B).

Discussion

Mulberroside A and oxyresveratrol strongly decreased the amount of melanin secreted out of the melanocyte. Both substances might respectively inhibit the pathway associated with the melanosome transfer from melanocytes to the keratinocytes, which results in the pigmentation of skin and hair (Wasmeier et al., 2008). During melanosome transfer, melanocyte extends multiple dendrites which allow the transport of mature melanosomes to the keratinocyte. In this pathway, the complex of Rab27a, its effector melanophilin and myosin 5A (MYO5A) that is expressed in the melanocyte promotes the association of the melanosome and actin in the dendrite of the melanocyte (Serre et al., 2018). Proteinase-activated receptor 2 (PAR-2), the keratinocyte cell-surface receptors, and the keratinocyte growth factor receptor (KGFR) are also associated with the melanosome transfer (Wasmeier et al., 2008). It would be interesting to examine whether the mulberroside A and oxyresveratrol may affect this pathway.

Inhibition of melanin synthesis inside of the melanocytes was slightly influenced by mulberroside A or oxyresveratrol. On the other hand, a mixture of two materials had synergistic effect on the inhibition of the intra melanin synthesis (Fig. 7B). Based on the results of mushroom tyrosinase activity assay, this synergism appears not from the inhibition of the tyrosinase. There might be many hypotheses to explain the synergism. Maturation process of melanosome can be affected by the mixture of mulberroside A and oxyresveratrol. Also, the microphthalmia associated transcription factor (MITF), a master regulator of the tyrosinase expression, might be affected (Wasmeier et al., 2008). Further studies are needed to understand the mechanism of synergism in the hypopigmentation by mulberroside A and oxyresveratrol.

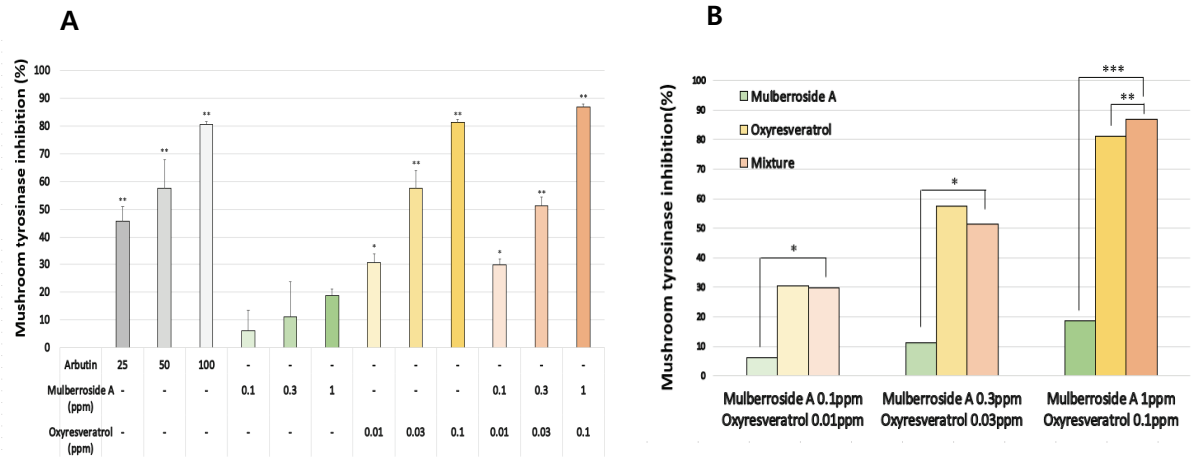

Fig. 5. Inhibition of mushroom tyrosinase when using L-tyrosine as a substrate by mulberroside A, oxyresveratrol and arbutin at three different concentrations after treatment of samples in 60 min. The results are presented as mean ± SD (n=3). A p-value means that * < 0.05, ** < 0.01, *** < 0.001.

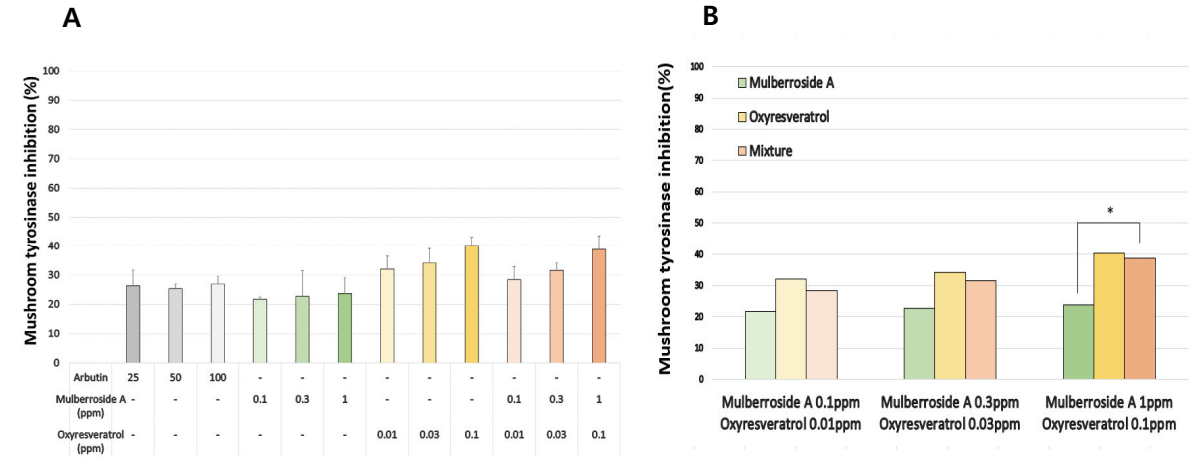

Fig. 6. Inhibition of mushroom tyrosinase when using L-DOPA as a substrate by mulberroside A, oxyresveratrol and arbutin at three different concentrations after treatment of samples in 15 min. The results are presented as mean ± SD (n=3). A p-value means that * < 0.05.

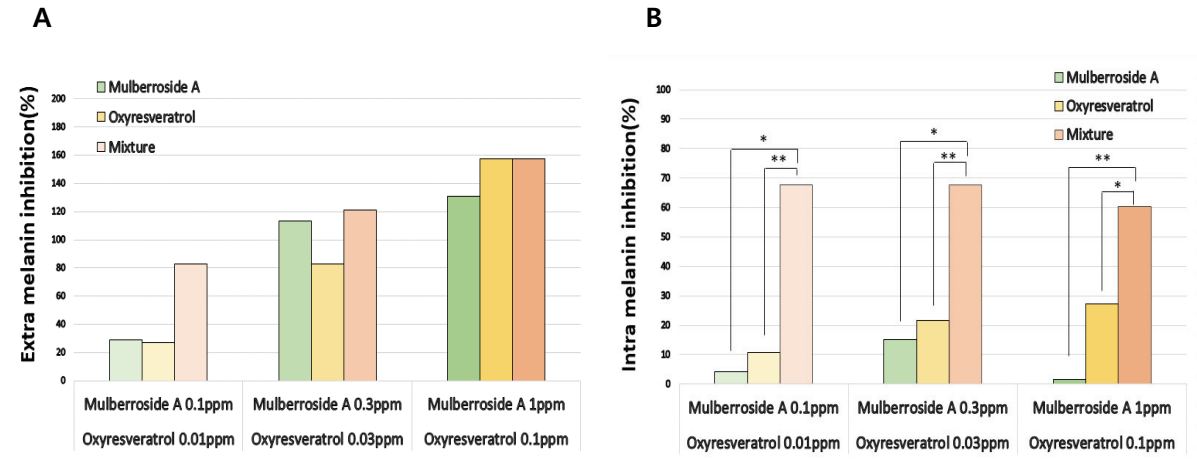

Fig. 7. Inhibition rate of extra melanin (A) and intra melanin (B) at 405nm. The results are expressed as mean ± SD (n=3). A p-value means that * < 0.05.

References

Bandyopadhyay, D. (2009). Topical treatment of melasma. Indian Journal of Dermatology *54*, 303.

Erdogan Orhan, I., and Tareq Hassan Khan, M. (2014). Flavonoid derivatives as potent tyrosinase inhibitors–a survey of recent findings between 2008-2013. Current Topics in Medicinal Chemistry *14*, 1486-1493.

Kang, H., Hwang, J., Lee, J., Ahn, J., Kim, J.Y., Lee, E.S., and Kang, W. (2006). The dermal stem cell factor and c -kit are overexpressed in melasma. British Journal of Dermatology *154*, 1094-1099.

Kang, W., Yoon, K., Lee, E.S., Kim, J., Lee, K., Yim, H., Sohn, S., and Im, S. (2002). Melasma: histopathological characteristics in 56 Korean patients. British Journal of Dermatology *146*, 228-237.

Kim, E.H., Kim, Y.C., Lee, E.-S., and Kang, H.Y. (2007). The vascular characteristics of melasma. Journal of Dermatological Science *46*, 111-116.

Kim, J.-K., Kim, M., Cho, S.-G., Kim, M.-K., Kim, S.W., and Lim, Y.-H. (2010). Biotransformation of mulberroside A from Morus alba results in enhancement of tyrosinase inhibition. Journal of Industrial Microbiology & Biotechnology *37*, 631-637.

Kim, J.-K., Park, K.-T., Lee, H.-S., Kim, M., and Lim, Y.-H. (2012). Evaluation of the inhibition of mushroom tyrosinase and cellular tyrosinase activities of oxyresveratrol: comparison with mulberroside A. Journal of Enzyme Inhibition and Medicinal Chemistry *27*, 495-503.

Kim, Y.M., Yun, J., Lee, C.-K., Lee, H., Min, K.R., and Kim, Y. (2002). Oxyresveratrol and hydroxystilbene compounds inhibitory effect on tyrosinase and mechanism of action. Journal of Biological Chemistry *277*, 16340- 16344.

Liang, C., Lim, J.-H., Kim, S.-H., and Kim, D.-S. (2012). Dioscin: a synergistic tyrosinase inhibitor from the roots of Smilax china. Food Chemistry *134*, 1146-1148.

Nguyen, N.T., and Fisher, D.E. (2019). MITF and UV responses in skin: From pigmentation to addiction. Pigment Cell & Melanoma Research *32*, 224-236.

Passeron, T., and Picardo, M. (2018). Melasma, a photoaging disorder. Pigment Cell & Melanoma Research *31*, 461-465.

Rendon, M., Berneburg, M., Arellano, I., and Picardo, M. (2006). Treatment of melasma. Journal of the American Academy of Dermatology *54*, S272-S281.

Serre, C., Busuttil, V., and Botto, J.M. (2018). Intrinsic and extrinsic regulation of human skin melanogenesis and pigmentation. International Journal of Cosmetic Science *40*, 328-347.

Shin, N.-H., Ryu, S.Y., Choi, E.J., Kang, S.-H., Chang, I.-M., Min, K.R., and Kim, Y. (1998). Oxyresveratrol as the potent inhibitor on dopa oxidase activity of mushroom tyrosinase. Biochemical and Biophysical Research Communications *243*, 801-803.

Wasmeier, C., Hume, A.N., Bolasco, G., and Seabra, M.C. (2008). Melanosomes at a glance. Journal of Cell Science *121*, 3995-3999.