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Short Communication

# The Anti-Melanogenic Effects of *Petalonia binghamiae* Extracts in α-Melanocyte Stimulating Hormone-Induced B16/F10 Murine Melanoma Cells

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***Petalonia binghamiae* extracts (PBE) suppressed melanin synthesis in a dose-dependent manner in α-melanocyte stimulating hormone (α-MSH)-treated B16/F10 murine melanoma cells. Specifically, the cell tyrosinase activity and melanin content were inhibited by 72% and 48%, respectively, in response to treatment with 100 μg/mL of PBE. The results of western blot analysis suggest that PBE induced the inhibition of tyrosinase and TRP-1 protein expression through suppression of α-MSH induced p38 and ERK activation.**

**Key words:** *depigmentation, melanin,* Petalonia binghamiae*, tyrosinase*

Skin color is determined based on the content of melanin that melanocytes synthesize in the epidermal-dermal junction. Once the physiological function of skin has deteriorated in response to aging or extreme overproduction of melanin, various types of hyper-pigmentation such as solar lentigo are caused by pigmenting melanin in the skin surface [Hill *et al*., 1997]. Exposure of the skin to ultraviolet rays (UVR) causes keratinocyte-secreted factors such as α-melanocyte stimulating hormone (α-MSH), endothelin-1 (ET-1) and nitric oxide (NO) to stimulate melanin biogenesis within melanocytes [Imokawa *et al*., 1997]. One of the factors that regulate melanocytes and skin pigmentation is the locally produced melanocortin peptide, α-MSH. Upon binding to the melanocortin-1 receptor (MC1R), α-MSH activates adenylate cyclase, which then causes an increase in the production of intracellular cyclic adenosine monophosphate (cAMP). Increased levels of cAMP result in activation of tyrosinase *via* protein kinase A (PKA), which is the rate limiting enzyme in the melanogenic pathway [Thody and Graham, 1998]. Additionally, factors stimulated by melanogenesis in response to UVR such as basic fibroblast growth factor (bFGF) and stem cell factor (SCF) are also secreted in keratinocytes [Gilchrest *et al*., 1996].

The synthesis of melanin occurs in the melanosome, which is a specialized intracellular membrane-coated organelle that originates from the endoplasmic reticulum. During development of the melanosome, it acquires tyrosinase, tyrosinase-related protein-1 (TRP-1) and tyrosinase-related protein-2 (TRP-2). Tyrosinase is a key enzyme involved in melanogenesis that catalyzes the conversion of L-tyrosine to dopaquinone, which is required for synthesis of both eumelanin and pheomelanin. Tyrosinase has several enzyme activities of tyrosine hydroxylase, dihydroxyphenylalanine (DOPA) oxidase and 5,6-dihydroxyindole (DHI) oxidase. Although TRP-1 has 5,6-dihydroxyindole carboxylic acid (DHICA) oxidase activity, its functions are not clear. It has been suggested that TRP-1 stabilizes tyrosinase [Hearing and Tsukamoto, 1991]. In addition, the function of TRP-2 is DOPAchrome tautomerase. Thus, TRP-2 regulates a switch that controls the proportion of carboxylated subunits in the melanin biopolymer [Tsukamoto *et al*.,

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**Abbreviations:** ERK, extracellular signal-regulated kinase; α-MSH, α-melanocyte stimulating hormone; MTT, 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; PBE, *Petalonia binghamiae* extracts; TRP, tyrosinase-related protein

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1992]. It has been reported that melanogenic activators such as α-MSH mediate opposite effects in tyrosinase and TRP-2 in B16/F10 murine melanoma cells [Martinez-Liarte *et al.*, 1992].

The depigmenting process can be accomplished by attenuating the transcription and activity of Tyrosinase, TRP-1, TRP-2 and/or peroxidase. Tyrosinase and TRP-1 are key enzymes involved in the biosynthesis of eumelanin. Most whitening agents act specifically to attenuate the function of tyrosinase by several mechanisms. For example, $C_2$-ceramide and tretinoin suppress the transcription of tyrosinase, while hydroquinone and arbutin are competitive inhibitors of tyrosinase, and linoleic acid and α-linolenic acid induce the degradation of tyrosinase [Briganti *et al.*, 2003]. Additionally, the extracts of many plants and algae have recently been reported to inhibit melanogenesis [Yoon and Kim, 2007; Kang *et al*., 2008a].

*Petalonia binghamiae* is a marine algae that has been found to have anti-oxidant and anti-diabetic properties [Kuda *et al*., 2006; Kang *et al*., 2008b]. Additionally, galactosyldiacyl-glycerol and fucoxanthin-related compounds produced by *P. binghamiae* have been reported to inhibit mammalian DNA polymerase α and to suppress adipocyte differentiation in 3T3-L1 cells, respectively [Mizushina *et al*., 2001; Mori *et al*., 2004]. Therefore, this study was conducted to determine if *P. binghamiae* would be useful as a skin whitening agent. *P. binghamiae* was collected on the coast of Jeju Island, South Korea. The entire body (dry weight, 100 g) of the samples was then extracted in 2 L of water at 100°C for 2 h with stirring. The *P. binghamiae* extracts (PBE) were concentrated on a rotary evaporator under reduced pressure and freeze-dried to a powder. PBE was dissolved in 50% ethanol at a concentration of 100 mg/mL and used for an *in vitro* assay. To validate the anti-melanogenic effects of PBE, the cell-free tyrosinase activity and cellular tyrosinase activity were evaluated and western blot analysis of B16/F10 murine melanoma cells was conducted.

The tyrosinase activity was determined based on the amount of DOPA chrome produced in response to the use of various substrates, including L-tyrosine and L-DOPA. To accomplish this, B16/F10 murine melanoma cells were seeded into 6 well plates at a density of $1\times10^5$ cells per well. The cells were then treated with or without α-MSH and the test extracts at 37°C for 3 days. Next, the cells were washed with 1×phosphate-buffered saline (PBS) and then collected using 1×trypsin-ethylene-diaminetetraacetic acid (EDTA), after which they were lysed with 0.2 mM and 1% Triton-X 100 in 67 mM sodium phosphate buffer (pH 6.8). The samples were then sonicated, and 80 μL of the sample, 40 μL of 25 mM L-DOPA, 5 μL of 1.5 mM L-tyrosine and 120 μL of 67 mM sodium phosphate buffer (pH 6.8) were then mixed with the sonicated samples and incubated at 37°C for at least 2 h. The absorbance was then measured at 475 nm, after which the corresponding total protein was used to normalize the absorbance.

B16/F10 murine melanoma cells were seeded into 6 well plates at a density of $1\times10^5$ cells per well. The cells were then treated with or without the test extracts at 37°C for 3 days. Next, the cells were washed with 1×PBS and then collected in 1×trypsin-EDTA, after which they were lysed with 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 1% Triton-X 100 in 67 mM sodium phosphate buffer (pH 6.8). The samples were then sonicated and centrifuged at 15,000 rpm for 15 min at 4°C. To extract the melanin from the pellets, 1 N sodium hydroxide (NaOH) was added to the pellets, which were subsequently incubated at 70°C for 4 h. The absorbance was then measured at 405 nm and the corresponding total protein was used to normalize the absorbance. The cell viability was determined by an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cell viability assay. Briefly, cells were seeded into 96 well plates at a density of $1\times10^4$ cells per well in the presence or absence of α-MSH and/or PBE. After three days of α-MSH and/or PBE treatment, the mitochondrial enzyme activity, which is an indirect measure of the number of viable respiring cells, was determined using MTT reagent. Finally, the absorbance was measured at 540 nm and the effect of α-MSH and/or PBE on cell viability was evaluated as the relative absorbance when compared with that of control cultures.

Cells were collected and lysed in 1×RIPA buffer (10×RIPA lysis buffer (Upstate, Boston, MA), 0.1 mM PMSF, 0.1 M $Na_3VO_4$, 0.5 M NaF, 5 mg/mL aportinin, and 5 mg/mL leupeptin). Thirty micrograms of protein per lane were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently blotted onto nitrocellulose membranes. The nitrocellulose membranes were then blocked with 5% dried milk in Tris-buffered saline containing 0.05% Tween 20. Next, the blots were incubated with primary antibodies at a dilution of 1:1,000 and then further incubated with horseradish peroxidase-conjugated secondary antibody. The bound antibodies were then detected using an enhanced chemiluminescence kit (Amersham Cat. No. RPN2106V2). Phosphorylated ERK (E-4, sc-7873), tyrosinase (M-19, sc-7834), TRP-1 (M-19, sc-10448) and TRP-2 (H-150, sc-25544) were purchased from Santa Cruz Biotech (Santa Cruz, CA), phosphorylated p38 (Thr180/Tyr182) and p38 were obtained from Cell Signaling Technology (Beverly, MA)

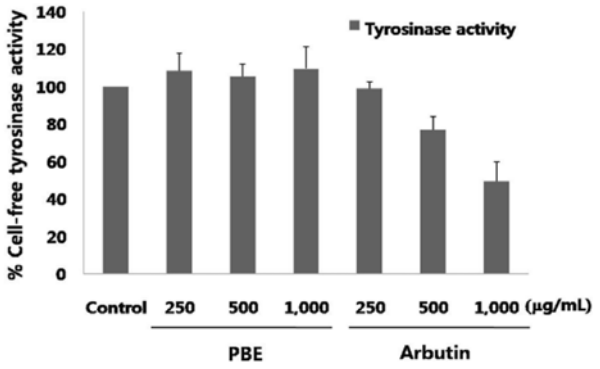


**Fig. 1. Effects of *Petalonia binghamiae* extracts (PBE) on cell-free tyrosinase activity in total protein extracted from B16/F10 melanoma cells.** Each bar represents the mean±SD of three independent experiments.

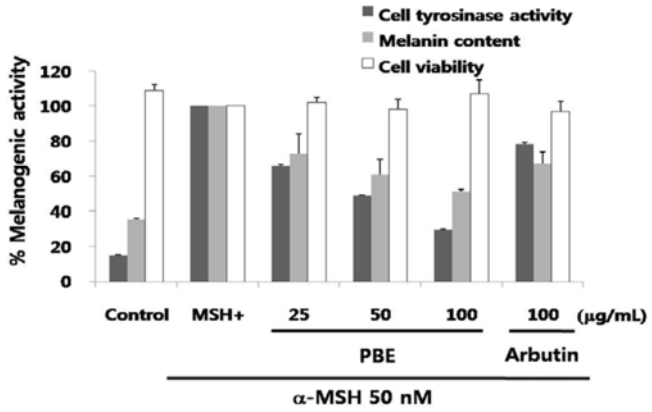


**Fig. 2. Effects of PBE on melanin biosynthesis and cell viability in α-MSH stimulated B16/F10 melanoma cells.** Each bar represents the mean±SD of three independent experiments.

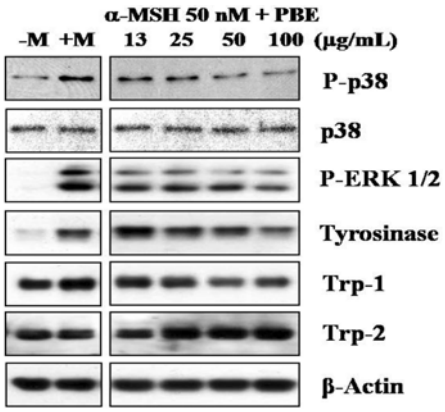


**Fig. 3. Effects of PBE on the expression of protein components of melanogenic signaling pathways in α-MSH stimulated B16/F10 melanoma cells.** Equal protein loading was confirmed using anti-β-actin antibody. -M represents a negative control cultured for 72 hours in the absence of α-MSH, +M represents a positive control cultured for 72 hours in the presence of 50 nM α-MSH.

and β-actin was obtained from Sigma Chemical Co. (St. Louis, MO).

Many drugs used to inhibit melanin synthesis have phenol structures and act as competitive inhibitors of tyrosinase [Briganti *et al.*, 2003]; therefore, PBE was evaluated to determine if it had an inhibitory effect on the cell-free tyrosinase activity. Briefly, tyrosinase was released from the melanosomes of untreated B16/F10 melanoma cells after treatment with sonication buffer. The lysates were then centrifuged and the supernatants containing soluble tyrosinase were incubated with or without PBE at the indicated concentrations for 2 h in reaction buffer. The tyrosinase activity was then determined by measuring the amount of L-DOPA chrome produced as compared to a control. Arbutin was used as a reference material.

The results showed that PBE had no inhibitory effect on the cell-free tyrosinase activity in B16/F10 melanoma cells. Indeed, even at high concentrations (1,000 μg/mL), PBE did not directly inhibit mammalian tyrosinase extracted from B16/F10 melanoma cells, whereas arbutin directly inhibited the activity in a concentration-dependent manner (Fig. 1).

PBE was also evaluated to determine if it had an indirect effect on cellular tyrosinase activity in α-MSH-stimulated B16/F10 melanoma cells. Cells were incubated with or without α-MSH and/or PBE at the indicated concentrations for 72 h. At 36 h, α-MSH and/or PBE were retreated at the indicated concentrations. Arbutin was used as a reference material. PBE showed a greater inhibitory effect on tyrosinase activity than arbutin, and these effects occurred in a dose-dependent manner, with up to 72% of the activity being inhibited in response to treatment with 100 μg/mL PBE (Fig. 2, black bars). Furthermore, the decrease in cellular tyrosinase activity showed a similar pattern when its ability to inhibit melanin synthesis was evaluated, with up to 48% of the activity being inhibited in response to treatment with PBE at 100 μg/mL (Fig. 2, gray bars). However, exposure to 100 μg/mL of PBE for 72 h did not affect the viability of α-MSH induced B16/F10 melanoma cells (Fig. 2, empty bars). Taken together, these findings demonstrate that PBE inhibited melanin synthesis by suppressing the cellular tyrosinase activity indirectly in α-MSH-stimulated B16/F10 melanoma cells.

Cells were treated with or without α-MSH and/or PBE for 72 h and then harvested. The total protein was then extracted and subjected to western blot analysis. Western blot analysis revealed that PBE suppressed the melanogenic enzymes, tyrosinase and Trp-1, by inhibiting α-MSH-stimulated ERK and p38 MAP kinase activation. The

results of previous studies have suggested that the effects of α-MSH are associated with melanogenesis via sustained ERK activation in B16/F10 murine melanoma cells [Goding, 2006]. Additionally, UVA irradiation-induced melanogenesis has been shown to be associated with the activation of ERK in melanocytes [Yanase *et al*., 2001]. Furthermore, UV-induced activation of MC1R promoters is mediated by p38 stress-activated kinase signaling [Corre *et al*., 2004]. Taken together, these results suggest that PBE may be used as a skin whitening agent, and that it functions via inhibition of the melanogenic signaling pathway through p38 and ERK activation in cells that are in an α-MSH induced state.

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## References

Briganti S, Camera E, and Picardo M (2003) Chemical and instrumental approaches to treat hyperpigmentation. *Pigment Cell Res* **16**, 101-110.

Corre S, Primot A, Sviderskaya E, Bennett DC, Vaulont S, Goding CR, and Galibert MD (2004) UV-induced expression of key component of the tanning process, the POMC and MC1R genes, is dependent on the p-38-activated upstream stimulating factor-1 (USF-1). *J Biol Chem* **279**, 51226-51233.

Gilchrest BA, Park HY, Eller MS, and Yaar M (1996) Mechanisms of ultraviolet light-induced pigmentation. *Photochem Photobiol* **63**, 1-10.

Goding CR (2006) Mitf from neural crest to melanoma: signal transduction and transcription in the melanocyte lineage. *Genes Dev* **14**, 1712-1728.

Hearing VJ and Tsukamoto K (1991) Enzymatic control of pigmentation in mammals. *FASEB J* **5**, 2902-2909.

Hill HZ, Li W, Xin P, and Michell DL (1997) Melanin: a two edged sword? *Pigment Cell Res* **10**, 158-161.

Imokawa G, Kobayashi T, Miyagishi M, Higashi K, and Yada Y (1997) The role of endrothelin-1 in epidermal hyperpigmentation and signaling mechanisms of mitogenesis and melanogenesis. *Pigment Cell Res* **10**, 218-228.

Kang JR, Lee MK, and Kang SM (2008a) Anti-oxidant property and tyrosinase inhibition activity of various extracts from plants in Compositae plants. *J Korean Soc Appl Biol Chem* **51**, 321-328.

Kang SI, Jin YJ, Ko HC, Choi SY, Hwang JH, Whang I, Kim MH, Shin HS, Jeong HB, and Kim SJ (2008b) *Petalonia* improves glucose homeostasis in streptozotocin-induced diabetic mice. *Biochem Biophys Res Commun* **373**, 265-269.

Kobayashi T, Imokawa G, Bennett DC, and Hearing VJ (1998) Tyrosinase stabilization by Tyrp1 (the brown locus protein). *J Biol Chem* **273**, 31801-31805.

Kuda T, Hishi T, and Maekawa S (2006) Anti-oxidant properties of dried product of 'habanori', an edible brown alga, *Petalonia binhamiae* (J. Agaradh) Vinogrdova. *Food Chem* **98**, 545-550.

Martinez-Liarte JH, Solano F, Garcia-Borron JC, Jara JR, and Lozano JA (1992) α-MSH and other melanogenic activators mediate opposite effects on tyrosinase and dopachrome tautomerase in B16/F10 mouse melanoma cells. *J Invest Dermatol* **99**, 435-439.

Mizushina Y, Sugiyama Y, Yoshida H, Hanashima S, Yamzaki T, Kamisuki S, Ohta K, Takemura M, Yamaguchi T, Matsukage A, Yoshida S, Saneyoshi M, Sugawara F, and Sakagauchi K (2001) Galactosyldiacylglycerol, a mammalian DNA polymerase α-specific inhibitor from a sea alga, *Petalonia binhamiae*. *Biol Pharm Bull* **24**, 982-987.

Mori K, Ooi T, Hiraoka M, Oka N, Hamada H, Tamuara M, and Kusumi T (2004) Fucoxanthin and its metabolites in edible brown algae cultivated in deep seawater. *Mar Drugs* **2**, 63-72.

Thody AJ and Graham A (1998) Does alpha-MSH have a role in regulating skin pigmentation in humans? *Pigment Cell Res* **11**, 265-274.

Tsukamoto K, Jackson IJ, Urabe K, Montague PM, and Hearing VJ (1992) A second tyrosinase-related protein, TRP-2, is a melanogenic enzyme termed DOPAchrome tautomerase. *EMBO J* **11**, 519-526.

Yanase H, Ando H, Horikawa M, Watanabe M, Mori T, and Matsuda N (2001) Possible involvement of ERK 1/2 in UVA-induced melanogenesis in cultured normal human epidermal melanocytes. *Pigment Cell Res* **14**, 103-109.

Yoon HS and Kim JK (2007) The inhibitory effect of Acanthopeltis japonica on melanogenesis. *J Soc Cosmet Sci Korea* **33**, 87-92.