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Original Research

Poria cocos Wolf extracts represses pigmentation *in vitro* and *in vivo*

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Abstract: In skin, melanocytes determine skin color using melanogenesis, which induces protective mechanism to oxidative stress and UV damage. However, when melanin is excessive produced by the various stimulus, the accumulated melanin induces hyperpigmentation disease such as melasma, freckles, Melanism ware induced. Therefore, it is implicated to finding potential agents for whitening to be used in cosmetic products. In our present study, we show that *Poria cocos* Wolf extracts decreased melanin synthesis in B16F10. And then this inhibition of melanogenesis was provoked by regulation of tyrosinase activity and tyrosinase and MITF expression. Moreover, *Poria cocos* Wolf extracts contained cream improved skin tone using increase of bright value. Overall, these results provide evidence to potential agent for whitening to be used in cosmetic products.

Key words: *Melanogenesis*; *Tyrosinase*; *MITF*; *Poria cocos Wolf*; *B16F10*; *Whitening.*

Introduction

Skin is outermost layer separating between environment and human body (1). It is composed of the epidermis and dermis (2). In epidermis, melanocytes determine skin color using production of melanin, which production mechanism is melanogenesis (2, 3). Melanogenesis is one of the skin protective effects, since melanin decreases oxidative stress and UV damage (4). Excessive melanogenesis causes hyperpigmentation disease such as melasma, freckles, Melanism (5, 6). Melanogenesis is occurred by variety of stimuli, such as UV, hormone, and Etc. (7-9). In UV-mediated melanogenesis, melanocytes synthesize melanin by α-MSH produced by keratinocytes (10-12). And then the synthesized melanin transfers to keratinocytes using melnosome. Oxidative enzyme, such as a tyrosinase, TRP1 and TRP2, produced melanin from L-tyrosine (13-14).

Mushroom has long been used as food materials to contains various nutrients, peculiar fragrance and flavor (15). Additionally, mushroom is used as medical drug by various physiological roles (16,17). *Poria cocos* Wolf, a one of Polyporaceae, has been used as an oriental medicine in East Asia and is parasitic on rotten root of pine-tree (18). Extracts of *Poria cocos* Wolf are evaluated beneficial efficacy such as diuretic, calm, a ventricular contraction mineralization, anti-cancer, anti-inflammation and antiemetic (19-22). Especially, polysaccharides, such as pachyman, carboxymethyl pachyman and (1,3)-(1,6)-β-D-glucan, which are separated in *Poria cocos* Wolf is revealed as anti-cancer agents (23,24). In addition, triterpenes contained in *Poria cocos* Wolf reduce inflammation of skin (18).

In this study, we show that *Poria cocos* Wolf extracts regulation melanogenesis and expression melanin synthesis related enzymes in B16F10, a mouse melanoma cell line. Additionally, *Poria cocos* Wolf extracts contained cream reduced pigmentation in human skin.

Materials and Methods

Preparation of *Poria cocos* Wolf extracts

Poria cocos Wolf extracts was extracted with 70% ethyl alcohol using ultrasonication for 1 h. The extract was sequentially filtrated using filter paper (Whatman/GE Healthcare Life Sciences, PA, USA) and 0.45 μm membrane filter (Whatman/GE Healthcare Life Sciences). The solvent was removed by rotary evaporator (EYELA, Tokyo, Japan) and Freeze dryer (Ilshinbiobase, Dongducheon-si, Korea). The dried extracts were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) and stored at −20°C until use.

Cell culture

B16F10 were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and grown using DMEM (Life Technologies Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Life Technologies Gibco) and 1% penicillin-streptomycin (Life Technologies Gibco) in a humidified atmosphere containing 5% CO_2 at 37°C.

Cell viability assays

B16F10 were seeded into the wells of the plate at densities of 2×10^3 cells. After 24 h, cells were treated with 0-100 μg/ml *Poria cocos* Wolf extracts for 48 h. Cell viability was determined using WST-1 assay EZ-Cytox Cell Viability Assay kit; ITSBio, Seoul, Korea). The absorbance of each well was measured at 450 nm

which measured redacted WST-1 and 650 nm which measured reference using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Melanin content assays

B16F10 were seeded into the wells of the plate at densities of 2×10^5 cells. After 24 h, cells were treated with 50 nM α-MSH and 100 μg/ml *Poria cocos* Wolf extracts for 48 h. After α-MSH and *Poria cocos* Wolf extracts treatment, cells harvested, and these pallet is taken picture using digital camera (Coolpix P330; Nikon, Tokyo, Japan). The pallets were lysed with 1 N NaOH at 94°C for 20 min. The melanin content was determined by optical density at 415 nm and normalized to the amount of total protein. Results are presented as the relative percentage of melanin content.

Mushroom tyrosinase activity assays

3,4 Dihydroxy-L-phehylalanine (L-DOPA; Sigma-Aldrich) and mushroom tyrosinase (Sigma-Aldrich) were incubated with 0- 100 μg/ml *Poria cocos* Wolf extracts at 37°C for 20 min. We measured dopachrome metabolized from L-DOPA by mushroom tyrosinase at 475 nm using a microplate reader.

Cellular tyrosinase activity assays

B16F10 were seeded into the wells of the plate at densities of 2×10^5 cells. After 24 h, cells were treated with 50 nM α-MSH and 100 μg/ml *Poria cocos* Wolf extracts for 48 h. Cells were harvested and lysed in cell lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100). Lysates were incubated for 20 min at 37°C with L-DOPA, the tyrosinase substrate, in 0.1 M sodium phosphate buffer (pH 7.0). The absorbance of each sample was measured at 475 nm using a microplate reader.

Western blotting analyses

B16F10 were seeded into the wells of the plate at densities of 2×10^5 cells. After 24 h, cells were treated with 100 μg/ml *Poria cocos* Wolf extracts for 48 h. Cells were harvested and lysed using 1% SDS lysis buffer (1% SDS, 20 mM Tris-Cl (pH 7.4), 2 mM EDTA). Cell lysates were separated with protein size using SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and detected with the following primary antibodies: anti-β-actin, anti-MITF, anti-tyrosinase. Horseradish peroxidase-conjugated horse anti-mouse, goat anti-rabbit, and bovine anti-goat were used as secondary antibodies. anti-β-actin, anti-MITF, anti-tyrosinase, horseradish peroxidase-conjugated horse anti-mouse, goat anti-rabbit, and bovine anti-goat were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). And anti-β-actin were purchased from Sigma-Aldrich. Each protein was visualized using an enhanced chemiluminescence HRP substrate (Thermo Fisher Scientific, Waltham, MA, USA) on bimolecular imager (FUSION-FX7 SPECTRA; Vilber Lourmat, Eberhardzell, Germany). Western blot data are representative of at least three independent experiments and are quantified using image analysis software (FUSION CAP advanced; Vilber Lourmat).

Quantitative real-time PCR

B16F10 were seeded into the wells of the plate at densities of 2×10^5 cells. After 24 h, cells were treated with 100 μg/ml *Poria cocos* Wolf extracts for 48 h. Total RNA was isolated from cells using the TRIzol reagent (Life Technologies Invitrogen, Grand Island, NY, USA) and cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen). Each cDNA was mixed with EvaGreen qPCR master mix (Solis BioDyne, Tartu, Estonia). And then PCR reaction was performed by StepOne™ Plus (Applied Biosystems, Life Technologies, Forster city, CA, USA). Quantitative real-time PCR data are quantified using $2^{\Delta\Delta CT}$ method, which were normalized β-actin Ct values (1). Values represent the mean ± standard deviation (SD) of triplicate experiments. The following primer sequences were used for PCR: Mouse *β-actin* forward primer: 5'-CGACAGGATGCAGAAGGAG-3', Mouse *β-actin* reverse primer: 5'-ACATCTGCTGGAAGGTGGA-3', Mouse *Mitf* forward primer: 5'-GGAACAGCAACGAGCTAAGG -3', Mouse *Mitf* reverse primer: 5'-TGATGATCCGATTCACCAGA -3', Mouse *Tyr* forward primer: 5'-GGCAGATTGTCTGTAGCCGA-3', Mouse *Tyr* reverse primer: 5'-CCTTGGGGTTCTGGATTTGT-3'.

Subjects for clinical evaluation

The study protocols were approved by the Institutional Review Board of Korea Institute of Dermatological Sciences (Seoul, Korea). All subjects were informed about the objective of the study and provided informed consent and agreed to use products for skin care during the study. Forty women, aged 20–30 years, were selected for a randomized and double-blind clinical trial. The subjects were selected based on age, with signs of skin aging, and were neither pregnant nor nursing. Early study exit was due to itching, erythema, or hindrance to evaluation by excessive drinking or smoking.

Application process of cream and evaluation

The cream provided to the all subjects contained 2% (wt %) *Poria cocos* Wolf extracts and control cream which was prepared using the same volume of water in place of *Poria cocos* Wolf extracts. Subjects were applied with control cream and *Poria cocos* Wolf extracts contained cream by 2 g to each left and right cheeks in every morning and night for 4 weeks. All conditions were the same except for the test material used on right and left cheeks. The study progressed for 4 weeks. Biometric parameters were measured three times: before application, and 2 weeks and 4 weeks after application. Subjects and investigators were blinded to the test and control treatments. During this study, the investigator asked subjects about their condition and performed a visual evaluation of skin disorders like erythema, itching, scale, edema, and tingling and burning sensations at every visit. In addition, at every visit, all subjects washed with the cleanser provided and remained quietly in a room with constant temperature (22±1 °C) and humidity (45±5%) so that all subjects would be evaluated under the same conditions before measurements.

Evaluation of skin tone improvement

Skin tone improvement was measured using the Spectrophotometer (Spectrophotometer CR 2600D,

Konica Minolta, Inc., Japan). The right and left cheek was measured 3 consecutive times, and the average value was determined. The measurements were conducted before application and after 2 and 4 weeks of the test material application. The L* which indicates brightness of 3 measurement values were determined as a measure of skin tone.

Statistical analysis.

In vitro tests, all results are presented as the mean percentage ± standard deviation (SD) of three independent experiments. And statistical significance was determined by Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference. In clinical efficacy tests, statistical analyses were conducted using graph pad Prism (La Jolla, CA, USA). One-way ANOVA were performed among application times. And $p<0.01$ was considered to indicate a statistically significant difference.

Results

Poria cocos Wolf extracts decreased melanin content in B16F10 cells

To determine depigmentation effects of *Poria cocos* Wolf extracts, we first choose experimental concentration of *Poria cocos* Wolf extracts using cell viability in B16F10 cells. As shown in Fig. 1, cell viability was not decreased under 100 μg/ml *Poria cocos* Wolf extracts. Thus, we determined experimental concentration of *Poria cocos* Wolf extracts at 100 μg/ml. Interestingly, by 100 μg/ml *Poria cocos* Wolf extracts, α-MSH-induced melanin contents was significantly decreased (Fig. 2A). In addition, we show that the cell pellets were black in α-MSH-treated B16F10 cells, but returned to white upon the addition of 100 μg/ml *Poria cocos* Wolf extracts (Fig. 2B).

Poria cocos Wolf extracts decreased α-MSH-induced cellular tyrosinase activity in B16F10 cells

Mushroom tyrosinase activity assay was performed for determining whether *Poria cocos* Wolf extracts directly decreased tyrosinase activity. As shown in Fig. 3A, *Poria cocos* Wolf extracts did not directly decrease tyrosinase activity. However, as shown Fig. 2B, *Poria cocos* Wolf extracts were treated in α-MSH –treated B16F10, which significantly decreased cellular tyrosinase activity. These results suggested that *Poria cocos* Wolf extracts repressed melanogenesis by cellular signal pathway, such as regulating repression of tyrosinase expression, degradation of tyrosinase and post-translational modification of tyrosinase (25, 26). Thus, we show whether *Poria cocos* Wolf extracts altered amount of tyrosinase expression.

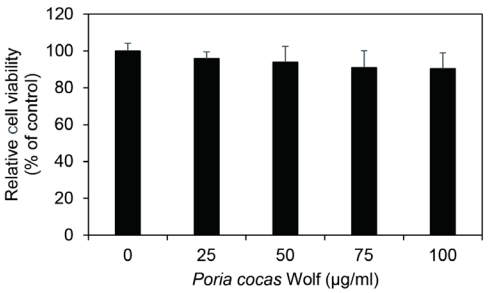

Figure 1. Effect of *Poria cocas* Wolf extracts on B16F10 cell viability. *Poria cocas* Wolf extracts causes no obvious cytotoxicity in B16F10 cells. Cells were seeded in 96-well culture plates and treated with the indicated doses of *Poria cocas* Wolf extracts (0, 25, 50, 75, and 100 μg/ml). After a 48 h incubation, a WST-1-based cytotoxicity assay was performed. Each bar represents the mean ± SD from three independent experiments.

Poria cocos Wolf extracts decreased tyrosinase and MITF in B16F10 cells

Because *Poria cocos* Wolf extracts decreased melanogenesis activity, we examined that *Poria cocos* Wolf extracts altered protein level of tyrosinase in B16F10 cells. In addition, mRNA expression tyrosinase is also decreased by *Poria cocos* Wolf extracts (Fig. 4B). These results suggested that *Poria cocos* Wolf extracts regulate melanogenesis through repression of tyrosinase expression. In α-MSH-mediated melanogenesis, expression of tyrosinase is typically promoted by MITF in various experiment models including B16F10 cells (27, 28). As shown in Fig. 4A and B, MITF protein and mRNA were decreased by *Poria cocos* Wolf extracts in α-MSH-treated B16F10 cells.

Poria cocos Wolf extracts decreased color value in human skin

We were revealed that *Poria cocos* Wolf extracts decreased melanogenesis using MITF-tyrosinase pathway. Thus, to determine whether *Poria cocos* Wolf extracts induced depigmentation in human skin, we performed clinical evaluation of skin tone measured by Spectrophotometer. As shown in Fig. 5, *Poria cocos* Wolf extracts was significantly increased L*value in application time dependent manners ($F=23.72$, p value$=1.73e^{-5}$). However, control cream slightly increased L*value but did not statistically increased ($F=7.19$, p value$=0.068$).

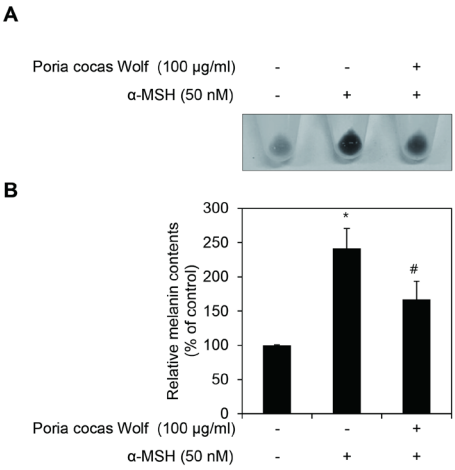

Figure 2. Effect of *Poria cocas* Wolf extracts on α-MSH-mediated melanin synthesis in B16F10 cells. Melanin synthesis was inhibited by *Poria cocas* Wolf extracts in α-MSH-stimulated B16F10 cells. Cells were treated with 50 nM α-MSH in absence and presence of 100 μg/ml *Poria cocas* Wolf extracts. (A) Pallet is taken picture using digital camera. (B) And melanin content was determined by optical density at 415 nm. Each bar represents the mean ± SD from three independent experiments. *$P < 0.05$ compared with controls. #$P < 0.05$ compared with α-MSH-treated B16F10 cells.

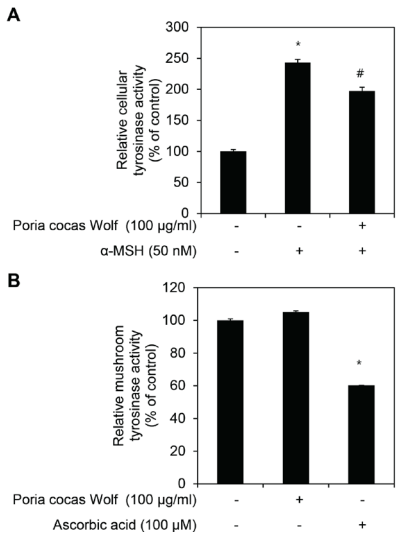

Figure 3. Effect of *Poria cocas* Wolf extracts on tyrosinase activity. (A) *Poria cocas* Wolf extracts decreased cellular tyrosinase activity. Cells were seeded in 60 mm culture plates and treated with the indicated doses of *Poria cocas* Wolf extracts (100 μg/ml) and 50 nM α-MSH. After a 48 h incubation, tyrosinase activity of cell lysate was determined by synthesized melanin conversed from L-DOPA. (B) *Poria cocas* Wolf extracts directly causes no obvious tyrosinase activity in B16F10 cells. Mushroom tyrosinase was incubated with *Poria cocas* Wolf extracts. And melanin content was determined by optical density at 415 nm. Each bar represents the mean ± SD from three independent experiments. *P < 0.05 compared with controls. #P < 0.05 compared with α-MSH-treated B16F10 cells.

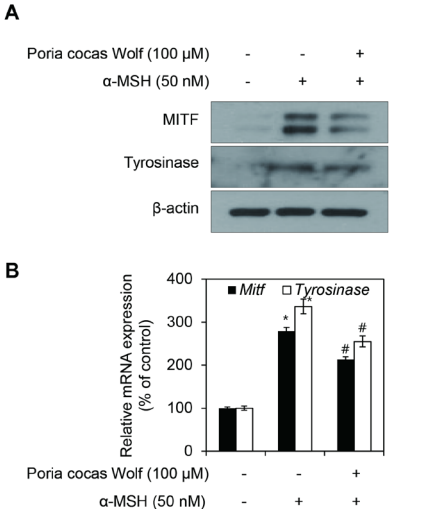

Figure 4. Effect of *Poria cocas* Wolf extracts on tyrosinase and MITF expression in B16F10 cells. *Poria cocas* Wolf extracts decreased MITF and Tyrosinase mRNA and protein in B16F10 cells. (A) Western analysis of MITF and Tyrosinase expression in 50 nM α-MSH and *Poria cocas* Wolf extracts-treated B16F10 cells. After 48 h of incubation, the levels of MITF and Tyrosinase proteins were evaluated by western blotting. β-actin was used as an internal control. (B) Analysis of relative MITF and Tyrosinase mRNA expression in *Poria cocas* Wolf extracts -treated B16F10 cells. The level of MITF and Tyrosinase mRNA was determined by qRT-PCR. Values represent the mean ± SD of three independent experiments. *P < 0.05 compared with controls. #P < 0.05 compared with α-MSH-treated B16F10 cells.

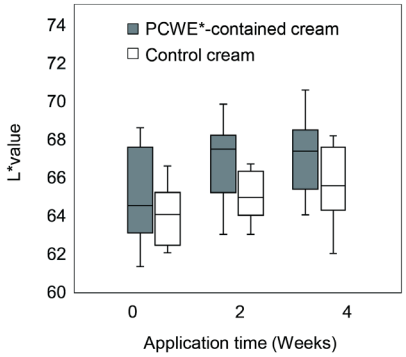

Figure 5. Effect of *Poria cocas* Wolf extracts on skin tone. *Poria cocas* Wolf extracts increased bright value in skin. Measurements were taken three times, namely before application and after 2 and 4 weeks of application. To evaluate skin tone, L* value was measured by spectrophotometer and data were statically analyzed using one-way ANOVA. Gray bar was *Poria cocas* Wolf extracts contained cream applied group. And white bar was control cream applied group. *PCWE is *Poria cocas* Wolf extracts.

Discussion

In present study, we first measured whether *Poria cocos* Wolf extracts induced cytotoxicity in B16F10 cells. Under 100 μg/ml *Poria cocos* Wolf extracts was not affected on cell viability in B16F10 (Fig. 1). Melanogenesis was initiated by various extrinsic and intrinsic stimuli in melanocytes (10, 29, 30). However, since these stimuli are mainly induced by paracrine or autocrine on surrounding cells such as keratinocytes, dermal fibroblasts (17). Especially, intercellular signal transmission between keratinocytes and melanocytes is implicated in UV-mediated pigmentation in skin (11). When UV radiation is exposed to skin, skin produced melanin through two steps (11). In first step, UV induces intercellular signal transmission between keratinocytes and melanocytes (11, 31). The intercellular signal is transmitted by α-MSH produced in UV-exposed keratinocytes (11). And then α-MSH stimulates melanocyte by melanocortin receptor (11, 31). Thus, in our study, we show that *Poria cocos* Wolf extracts repressed α-MSH-mediated melanogenesis in B16F10 cells. As shown in Fig. 2, 100 μg/ml *Poria cocos* Wolf extracts significantly decreased melanin contents in α-MSH exposed B16F10 cells. In second step, melanocortin receptor activated by α-MSH increases tyrosinase amount and activity using PKA/MITF pathway (25, 26, 28). Thus, regulation of tyrosinase activity and amount is implicated to find novel whitening agent (32). In our study, we show that activity and mRNA and protein amount of tyrosinase were also decreased by 100 μg/ml *Poria cocos* Wolf extracts (Fig. 3-4). In addition, we revealed that MITF expression was decreased by 100 μg/ml *Poria cocos* Wolf extracts (Fig. 4). These results suggest that *Poria cocos* Wolf extracts can be a potential agent for whitening to be used in cosmetic products. We have to prove this hypothesis, we performed clinical efficacy test against alteration of skin bright value. As shown Fig. 5, *Poria cocos* Wolf extracts significantly increased bright value in human skin.

Overall, the results suggested *Poria cocos* Wolf extracts decreased melanin synthesis in B16F10. And then this inhibition of melanogenesis improved skin tone using increase of bright value. These results provide evidence to potential agent for whitening to be used in cosmetic products.

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Interest conflict
The researcher claims no conflicts of interest

Author's contribution
HyunKyung Lee and Hwa Jun Cha designed the experiments and analysed the data. HyunKyung Lee performed the experiments. Hwa Jun Cha wrote the paper.

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