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금선련 조직 배양체 추출물의 멜라닌 합성 및 지방축적 억제 효과

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Inhibitory Effect of Jewel Orchid (*Anoectochilus Formosanus*) Plantlet Extract against Melanogenesis and Lipid Droplet Accumulation

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요 약: 일반적으로 보석란으로 알려진 금선련은 대만에서 폐나 간의 질병 및 발열이나 두통 치료를 위한 전통식물약제로 사용되어 왔다. 본 연구에서는 생물반응장치를 이용하여 조직배양된 금선련 식물체에 대하여 화장품 성분으로써 응용 가치를 평가하였다. 이미 몇몇 보고 된 논문에서 금선련은 항암활성, 면역 활성, 간 보호 활성 및 지질대사의 약리학적 활성 등에 대한 연구가 되고 있지만 화장품 성분으로 효능들에 대한 연구는 알려져 있지 않다. 따라서 본 연구에서는 생물반응장치를 이용하여 조직배양된 금선련 추출물에 대하여 미백 및 항비만 관련한 효능 효과를 평가하였다. 실험결과 조직배양된 금선련 추출물은 tyrosinase 활성 및 멜라닌 합성 억제 효과뿐만 아니라 지방 전구 세포의 지방세포로의 분화를 억제시킴으로써 세포 내 지질 축적을 억제하였다. 이러한 결과들은 피부보호를 위한 화장품 성분으로서 응용 가능성을 제공 할 수 있을 것으로 사료된다.

Abstract: *Anoectochilus formosanus*, commonly known as "Jewel Orchids", which has been used in traditional folk medicines for feber, pain, and diseases of the lung and liver in Taiwan. We artificially cultured *Anoectochilus formosanus* plantlet by using the bioreactor culture system for this study from *Anoectochilus formosanus*. Previously, several studies have been reported on pharmacological activities of lipid-metabolism, hepatoprotective activity, anti-tumor activity and immuno-stimulating effects but other efficacy were not well known as a cosmetic ingredient for skin care. In this study, we investigated the effect of melanogenesis in B16 mouse melanoma cells and lipid droplet accumulation in 3T3-L1 preadipocytes about *Anoectochilus formosanus* plantlet extract. We report that *Anoectochilus formosanus* plantlet extract inhibits the cytoplasmic lipid droplet accumulation through adipogenic differentiation of preadipocytes as well as inhibition of tyorsinase activity and melanogenesis. As a result, our findings indicate that *Anoectochilus formosanus* plantlet extract may be the potential natural ingredient for whitening and slimming cosmetic products.

Keywords: *Anoectochilus formosanus, melanogenesis, adipogenesis, plantlet, tissue-culture*

1. Introduction

The genus *Anoectochilus* (*Orchidaceae*) grows in deep shade on moist forest floors with a rich layer of humus and consists of approximately 40 species[1]. Several species have been used as traditional folk medicines. Among the genus *Anoectochilus*, *Anoectochilus for-*

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mosanus Hayata is a native perennial and terrestrial orchid plant, which is grown in Taiwan (China) and Okinawa (Japan)[2]. In general, *Anoectochilus formosanus* Hayata is well known as "Jewel Orchids" because its leaves have network of colorful venation. *Anoectochilus formosanus* is mainly used to treat hypertension, diabetes, heart and bright's disease and other diseases as well as in traditional folk medicine against lung and liver disease in China, Taiwan and many other countries[3,4]. *Anoectochilus formosanus* is conventionally propagated by seed. However, the germination rate is very low. *Anoectochilus formosanus* is also an expensive Chinese folk medicinal plant. It has great value in herbal medicines because of its pharmacological activities of lipid-metabolism, hepatoprotective activity, anti-tumor activity and immuno-stimulating effects, anti-inflammation and antioxidant activities[5,6]. Melanin is a biological pigment found in skin, which mainly determines human skin color by the content of the pigment melanin. In addition, melanin plays an important role in protecting human skin from the harmful effects of UV radiation from the sun. Melanin is produced in unique organelles melanocytes in the epidermis of skin[7,8]. Melanogenesis is initiated with the conversion of L-tyrosine through L-dopa to dopaquinone by tyrosinase[9,10]. Although melanin plays an important role as a photoprotective function of sun-induced skin injury, its abnormal accumulation results in serious skin problems. Adipocyte differentiation is influenced by a large number of growth factors. Insulin, IGF-1, fibroblast growth factor, platelet-derived growth factor and epidermal growth factor promote preadipocyte proliferation but only insulin or IGF-1 can induce adipocyte differentiation. Preadipocyte differentiation is characterized by changes in cell morphology, hormone sensitivity and gene expression in anti-obesity study. The cells accumulate lipid droplets[11,12]. In the present study, we investigated the effect of melanogenesis and anti-obesity whether *Anoectochilus formosanus* plantlet cultured by using the bioreactor culture system has a merit as a cosmetic ingredient.

2. Materials and Methods

2.1. Reagents and Cell Culture

Antibodies against tyrosinase and ß-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Insulin, dexamethasone, isobutylmethylxanthine (IBMX) and Oil Red O were purchased from sigma-aldrich (St. Louis, MO, USA). Other commercially available reagents and solvents were used as received. 3T3-L1 preadipocytes procured from American Type Culture Collection and B16F10 mouse melanoma cell line procured Korean Cell Line Bank were used in this study. Preadipocyte line 3T3-L1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, WelGENE, Korea) supplemented with 10 % heat-inactivated calf serum (CS, Gibco BRL, USA), 100 U/mL penicillin (Gibco BRL, USA) and 100 μg/mL streptomycin (Gibco BRL, USA) at 37 ℃ in a humidified atmosphere containing 5 % CO_2. B16 mouse melanoma cells was maintained in Dulbecco's modified Eagle's medium (DMEM, WelGENE, Korea) supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Gibco BRL, USA), 100 U/mL penicillin (Gibco BRL, USA) and 100 μg/mL streptomycin (Gibco BRL, USA) at 37 ℃ in a humidified atmosphere containing 5 % CO_2.

2.2. Preparation of *Anoectochilus Formosanus* Plantlet Extract

Anoectochilus formosanus plantlet was obtained from Research Center for The Development of Advanced Horticultural Technology, Chungbuk National University. **Extraction** : After dried, *Anoectochilus formosanus* plantlet was immersed in 70 % ethanol solution and 1,3-butyleneglycol (1,3-BG) for sufficient time and mixed enough for 48 h in 45 ℃ using agitator. Then, the solution was filtered through a filter paper, followed by the removal of ethanol from the filtrate at 50 ~ 60 ℃ to obtain a *Anoectochilus formosanus* plantlet extract.

2.3. Measurement of Melanin Content in B16 Mouse Melanoma Cells

Measurement of melanin content was performed using a modification of the method reported by Funasaka

[13]. B16 mouse melanoma cells were cultured in DMEM supplemented with 10 % FBS in humidified incubator at 37 ℃ under 5 % CO_2 in 6 well plate at density of 2×10^4 cells/well. After cells were attached, medium was replaced with DMEM containing 10 % FBS, 0.2 uM α-MSH, 2 mM theophylline and samples addition. After 4 days, trypsin was added and suspended cells were collected by centrifugation. Then cell pellets were dried and dissolved in 1 N NaOH. Melanin synthesis inhibition rates were measured 490 nm using ELISA reader.

2.4. Assay of Tyrosinase Activity in a Cell Culture-free System

A mushroom tyrosinase was dissolved in a phosphate buffer (pH 6.5) to prepare a tyrosinase enzyme solution. L-tyrosine was dissolved in a phosphate buffer (pH 6.5) to the concentration of 1.5 mM to prepare a substrate solution. Tyrosinase enzyme solution was added to the test solution. After incubation at 37 ℃ for 10 min, substrate solution was added there to. Absorbance was measured using an ELISA reader at 490 nm.

2.5. Pre-adipocyte (3T3-L1) Differentiation

To induce differentiation, pre-adipocytes were grown in 24-well plates until 2 d post-confluence. Confluent cells were differentiated by incubation with MID hormone mixture (10 μg/mL insulin, 0.5 μM dexamethasone, and 1 mM IBMX) in 10 % fetal bovine serum (FBS) / DMEM for 36 h and maintained in post-differentiation medium containing 10 μg/mL insulin for more 6 d.

2.6. Oil Red O Staining Assay

The degree of anti-adipogenic effects, with and without *Anoectochilus formosanus* plantlet, was evaluated by observation of the lipid accumulation with Oil Red O staining. At 6 d after the initiation, cells were washed twice with PBS and then fixed for 20 min with 3.7 % formaldehyde. Cells were washed twice in PBS and the lipidic content of the cells is evaluated by the red oil coloration method. Briefly, cells were stained for 10 min using 0.5 % oil red in isopropanol/water at room temperature. Then the stain was solubilized in pure isopropanol, and the absorbance was measured at 540 nm on a spectrophotometer.

2.7. Cell cytotoxicity by MTT Assay

Cells (1×10^5 cells/well) were seeded in 10 % BCS/DMEM medium and incubated in 5 % CO_2 incubator at 37 ℃ after treatment with *Anoectochilus formosanus* plantlet extract for indicate times. Measurement of mitochondrial activity to form purple formazan by MTT was used to assess the cytotoxicity of cell following extract treatment : MTT (0.5 mg/mL), one tenth of the original culture volume, was added to each culture and incubated for 3 h at 37 ℃ in 5 % CO_2. The purple formazan formed by viable cells was dissolved by the addition of DMSO and absorbance at the dual ranges of 540 nm and 630 nm was measured by using spectrophotometer.

2.8. Western Blot Analysis

Cells were treated with various dose and lysed in lysis buffer as described previously[14]. After differentiation, cells were lysed in lysis buffer. The lysates were clarified by centrifugation at 12,000 × g for 15 min at 4 ℃ and protein content was measured by 12.5 % SDS-PAGE and blotted to nitrocellulose membrane (0.2 mm, Amersham, Arlington Heights, IL). The membrane was blocked with 5 % non-fat skim milk in TBS-T and incubated with the primary and secondary antibodies. Immunoblots were visualized by enhanced chemiluminescence (Amersham, UK), according to the manufacturer's protocol.

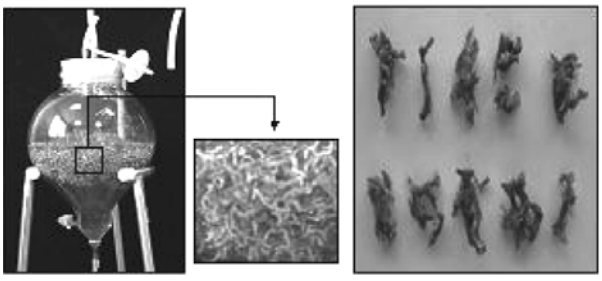
2.9. Statistical Analysis

Data are presentated as mean ± SD. Comparisons between groups were used to the paired Student's t-test. Asterisk (*, $p < 0.05$) was considered to bestatistically significant.

3. Results

3.1. *Anoectochilus Formosanus* Plantlet Cultured by the Bioreactor Culture System

Anoectochilus formosanus is precious in the Taiwanese



(A) (B)

Figure 1. The *Anoectochilus formosanus* plantlet cultured by the bioreactor culture system. Figure 1A is tissue-cultured *Anoectochilus formosanus* plantlet culturing by the bioreactor culture system using liquid medium and Figure 1B is a picture of a dried *Anoectochilus formosanus* plantlet.

market but seriously is reduced its populations. Tissue culture protocols have been developed for preservation of this valuable plant. Figure 1(A) is tissue-cultured *Anoectochilus formosanus* plantlet cultured by the bioreactor culture system using liquid medium. Figure 1(B) is the picture of a dried *Anoectochilus formosanus* plantlet cultured by the bioreactor culture system. We extract the dried *Anoectochilus formosanus* plantlet and evaluate the efficacy as cosmetic ingredients.

3.2. Inhibitory Effect of *Anoectochilus Formosanus* Plantlet on Mushroom Tyrosinase Activity in a Cell Culture Free System

To investigate the whitening effect of *Anoectochilus formosanus* plantlet extract, we first analyzed inhibition rate of mushroon tyrosinase activity in a cell-culture free system. As shown in Figure 2, *Anoectochilus formosanus* plantlet extract inhibited the tyrosinase activity in a concentration-dependent manner. The tyrosinase activity was reduced to 39 % at 0.1 % and to 28 % at 0.05 %, as compared with the non-treated control.

3.3. Inhibitory Effect of *Anoectochilus Formosanus* Plantlet on Melanogenesis and Tyrosinase Protein Expression in a Cell Based System

We investigated whether *Anoectochilus formosanus* plantlet extract inhibits melanin synthesis and tyrosinase expression in B16 mouse melanoma cells as well as mushroom tyrosinase activity in cell-culture free system. Also, we performed western blot analysis

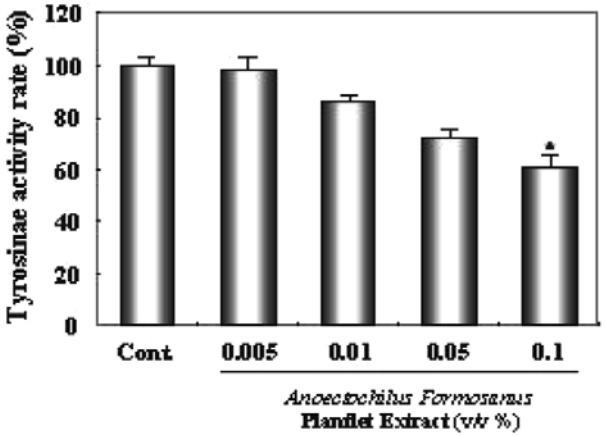


Figure 2. The Effect of *Anoectochilus formosanus* plantlet on Tyrosinase Activity in Cell-culture Free System. Tyrosinase activity were expressed as a percentage of control. The results were represented as mean of standard deviation (S.D.) of three independent experiments. *, $p < 0.05$ versus control.

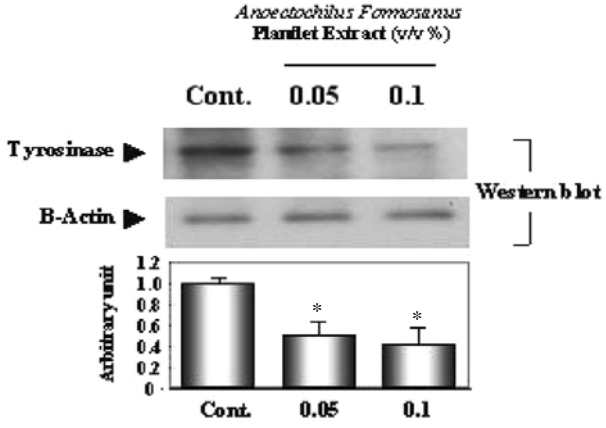


Figure 3. The effect of *Anoectochilus formosanus* plantlet on the Protein Expression of Tyrosinase in B16 Melanoma Cells. B16 melanoma cells were treated with *Anoectochilus formosanus* plantlet at the concentrations indicated in the figure and incubated for 72 h. Tyrosinase protein expression was determined by a western blotting analysis using a specific antibody, as described in the manteials and methods section. Bands were subjected to densitometric scanning using the Scion image NIH image software. The results were represented as mean of standard deviation (S.D.) of three independent experiments. *, $p < 0.05$ versus control.

against the cell lysate obtained from *Anoectochilus formosanus* plantlet extract-treated B16 mouse melanoma cells. As shown in Figure 3, *Anoectochilus formosanus* plantlet extract decreased the expression of

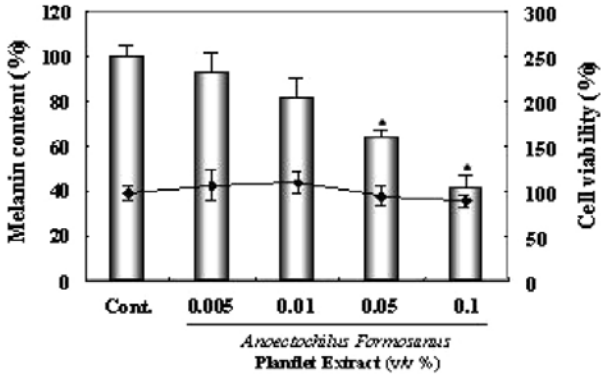


Figure 4. The Effect of *Anoectochilus formosanus* plantlet extract on the Melanin Content and cell viability of Mouse B16 Melanoma Cells. B16 melanoma cells were treated with *Anoectochilus formosanus* plantlet at the concentrations indicated in the Figure 2 and then incubated for 72 h. The melanin content and cell viability were determined as described in the Manteials and Methods section. The results were represented as mean of standard deviation (S.D.) of three independent experiments. *, $p < 0.05$ versus control.

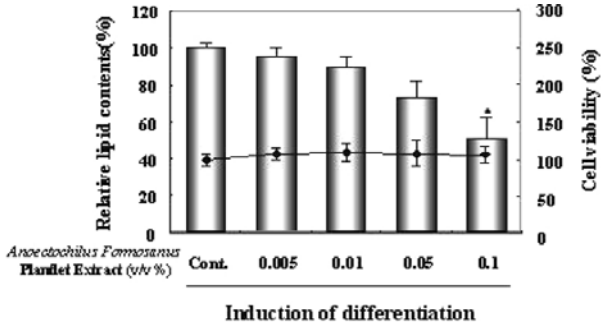


Figure 5. Inhibitory effect of *Anoectochilus formosanus* plantlet on the lipid accumulation and cell viability of 3T3-L1 cells. We compared a lipid contents after cotreatment of *Anoectochilus formosanus* plantlet extract with MID hormone mixture. Lipid accumulation after differentiation for 6 d was measured by Oil Red O staining as shown in Figure 5. The Lipid accumulation and cell viability were determined as described in the Manteials and Methods section. All values are represented as means ± standard deviation (S.D.) of three independent experiments. *, $p < 0.05$ versus control.

tyrosinase protein in a concentration-dependent manner. In addition, we examined melanin content of B16 mouse melanoma cells in order to confirm melanogenesis inhibiting-effect of *Anoectochilus formosanus* plantlet extract. As shown in Figure 4, *Anoectochilus formosanus* plantlet extract inhibited the melanogenesis in a concentration-dependent manner. The melanin content was reduced to 59 % at 0.1 % and to 36 % ant 0.05 %, as compared with the non-treated control cells at a concentration without any cytotoxicity.

3.4. Inhibitory Effect of *Anoectochilus Formosanus* Plantlet on the Lipid Accumulation of 3T3-L1 Cells

To investigate whether *Anoectochilus formosanus* plantlet extract has anti-obesity effect, we examined a cytoplasmic lipid droplet accumulation by the differentiation of preadipocytes into matures adipocytes in presence of MID hormone mixture. After incubation for 6 d with both MID and *Anoectochilus formosanus* plantlet extract, cells reduced a lipid droplet accumulation. We investigated whether *Anoectochilus formosanus* plantlet extract inhibits a cytoplasmic lipid droplet by Oil red O staining assay as described in materials and methods. In result, *Anoectochilus formosanus* plantlet extract, tested at 0.01 %, 0.05 % and 0.1 % inhibited by 11 %, 27 % and 49 % a lipid content, as compared with the only MID hormone mixture control after incubation with both MID hormone mixture and extract at indicated concentrations without any cytotoxicity as shown in Figure 5.

4. Discussion

Generally, tissue-cultured plant has a benefit to solve the drawbacks such as a rareness, long time and high cost for growing herb. Although the *Anoectochilus formosanus* has been well known as herb for remedy a hypertension, diabetes, heart, lung and liver disease as a traditional medicine, we investigated whether *Anoectochilus formosanus* plantlet extract have efficacy as a cosmetic ingredient for skin care in this study. In summary, using B16 mouse melanoma cells and 3T3-L1 preadipocyte, we found that *Anoectochilus formosanus* plantlet extract had a inhibitory effect of melanogenesis by reducing the protein amounts of tyrosinase and suppressive effect of lipid droplet accumulation. As a results, we suggest that *Anoectochilus formosanus* plantlet could be cosmetic ingredient for skin care such as whitening and inhibition of lipogenesis.

Acknowledgments

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