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참나물추출물의 멜라닌 생성저해 효과

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New Whitening Agent From *Pimpinella brachycarpa*

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요 약: 천연물로부터 새로운 미백 화장품 소재를 개발하기 위해 참나물(*P. brachycarpa*)을 선택하여 B16 멜라노마 세포에서 tyrosinase 활성 저해 효과, melanin 생성 저해 효과를 측정하였다. 먼저 참나물추출물의 4가지 극성별 용매 분획화 실험을 실시하였으며, 항산화효과와 티로시나아제 저해효과를 측정하였다. 참나물의 미백 활성 메카니즘을 알아보기 위해 Western blotting과 RT-PCR을 이용하여 tyrosinase, TRP-1, TRP-2의 단백질 발현과 mRNA 변화를 연구하였다. 또한, HaCaT 각질형성세포에서 UVB 조사 후 엔도세린-1 (ET-1)의 발현은 사람 ET-1 항체를 이용하여 quantitative enzyme immunoassay (EIA)로 측정하였다. 그 결과 참나물추출물과 4가지 분획(hexane, EtOAc, butanol and aqueous)은 100 μg/mL 농도에서 각각 87.2, 2.5, 97.2, 80.5, 49.8 %의 프리라디칼 소거효과를 나타내었으며, tyrosinase 저해효과는 100 μg/mL 농도에서 각각 18.3, 15.1, 55.4, 13.1, 0 %로 나타났다. 각 극성별 분획 중 EtOAc 분획 100 μg/mL 농도에서 58 % 이상의 가장 우수한 멜라닌 생성 저해 효과가 나타났다. 참나물 EtOAc 분획은 B16 멜라노마 세포에서 티로시나아제 활성 및 발현을 모두 저해하였으며, RT-PCR 결과에서도 tyrosinase, TRP-1의 mRNA 발현 저해효과가 우수하게 나타났다. 또한 HaCaT 각질형성세포에서 UVB 조사 후 생성된 엔도세린-1의 생성 실험에서도 참나물 EtOAc 분획 12.5 ~ 50 μg/mL 농도에서 엔도세린-1의 생성이 컨트롤에 비해 40 % 정도로 우수하게 저해되었다. 결론적으로 참나물추출물은 멜라닌 합성과정에서 우수한 tyrosinase 저해효과와 엔도세린-1 발현저해효과를 가지는 새로운 천연 미백 소재로 적용될 수 있을 것이라 기대된다.

Abstract: To develop a new whitening agent for cosmetics from natural products, *Pimpinella brachycarpa* was selected for its inhibitory effect on melanogenesis in B16 melanoma cells. Crude ethanolic extract of *P. brachycarpa* and its four fractions - hexane, ethyl acetate (EtOAc), butanol and aqueous were evaluated for antioxidative effects and tyrosinase inhibitory activity. To elucidate the mechanism of active compounds of *P. brachycarpa*, we investigated the changes in protein level of tyrosinase, TRP-1 and TRP-2 using Western blotting and the changes in mRNA level of tyrosinase using RT-PCR technique. Following UV irradiation, expression of ET-1 in HaCaT keratinocytes was measured by quantitative enzyme immunoassay (EIA) using human ET-1 antibody. Crude ethanolic extract of *P. brachycarpa* and its four fractions - hexane, EtOAc, butanol and aqueous had free radical scavenging effect by 87.2, 2.5, 97.2, 80.5, 49.8 % at 100 μg/mL and tyrosinase inhibitory effect by 18.3, 15.1, 55.4, 13.1, 0 % at 100 μg/mL. *P. brachycarpa* EtOAc fraction significantly inhibited melanin production in B16 melanoma cells. Treatment with *P. brachycarpa* extract for 72 h suppressed the biosynthesis of melanin up to 58 % at 100 μg/mL. Especially, the EtOAc fraction of *P. brachycarpa* reduced the tyrosinase activity and tyrosinase expression in B16 melanoma cells in a dose-dependent manner. mRNA levels of tyrosinase and TRP-1 were markedly reduced by the EtOAc fraction of *P. brachycarpa*. Moreover, at the concentrations of 12.5 ~ 50 μg/mL of the fraction, the production of UV- induced ET-1 in HaCaT keratinocytes (24 h after 8 mJ/cm^2 UVB irradiation) was reduced about 40 % ($p < 0.05$). *P. brachycarpa* could be used as a new natural skin-whitening agent due to the inhibitory effect of on melanin biosynthesis and endothelin-1 expression.

Keywords: *pimpinella brachycarpa, melanogenesis, whitening, tyrosinase, endothelin*

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1. Introduction

Variation in human pigmentation is due to the rate of melanin synthesis by epidermal melanocytes, the relative amounts of eumelanin and pheomelanin synthesized by melanocytes, and the manner and rate of melanosome transfer from melanocytes to keratinocytes. Dark skin differs from light skin in having higher melanin content, higher eumelanin to pheomelanin ratio, and higher mRNA and protein levels of tyrosinase, TRP-1 and TRP-2[1]. Melanin production plays an important role in prevention of sun-induced skin injury[2]. But abnormal hyperpigmentation such as freckles, chloasma, lentigines and other forms of melanin hyperpigmentation could be serious aesthetic problems[3].

To develop plant materials protecting from hyperpigmentation, we have screened herb medicine extracts on the inhibition of melanogenesis and the activity of tyrosinase, respectively. From the results of these screening procedures, *P. brachycarpa* was selected for its inhibitory effect on melanogenesis in B16 melanoma cells. To elucidate the action mechanism of *P. brachycarpa*, we investigated the changes in protein and mRNA level of tyrosinase, TRP-1 and TRP-2 and inhibition of ET-1 expression.

2. Materials and Methods

2.1. Preparation of Plant Extract

P. brachycarpa was purchased from Kyeong-dong market (Seoul, Korea). It was extracted in 95 % aqueous EtOH under reflux for 4 h. Crude ethanolic extract of *P. brachycarpa* was then fractionated with hexane, EtOAc, and buthanol (BuOH), respectively. The ethanolic extract and 4 fractions were filtered and concentrated *in vacuo*.

2.2. Tyrosinase Activity Assay

The test extract was added to designated concentrations of L-tyrosine as substrate and in the presence or absence of mushroom tyrosinase (Sigma, USA)[4].

2.3. Free Radical Scavenging Activity Assay

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging effect was evaluated according to the method of Blois, *et al.* with minor modifications[5].

2.4. Cell Culture

B16F10 mouse melanoma cell line procured from the Korean Cell Line Bank was used in this study. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS, Bio Whittaker, USA), 1 % penicillin-streptomycin (Gibco BRL, USA), and 200 nM α-MSH (Sigma, USA) at 37 ℃ in a humidified incubator with 5 % CO_2.

2.5. Measurement of Melanin Content in B16F10 Mouse Melanoma Cells

Estimation of melanin content was performed using a modification of the method reported by Funasaka[6]. The cells were collected and counted. After centrifugation, the cell pellet was dissolved in 1 mL of homogenization buffer and added 200 μL 1 N NaOH and melanin concentration was calculated by measuring the absorbance value at 405 nm.

2.6. Measurement of Tyrosinase Activity in B16F10 Mouse Melanoma cells

Cellular tyrosinase activity was measured using a modification of the method reported by Pawelek *et al.*[7]. The cells were collected and resuspended. After incubation with 0.1 M sodium phosphate buffer, pH 6.8, containing 0.2 % L-DOPA at 37 ℃ or 2 h, the amount of DOPAchrome in the reaction mixture was determined by measuring the absorbance at 490 nm.

2.7. Western Blot Analysis

Following treatment with various concentrations of the extracts, B16F10 melanoma cells were treated with radio-immunoprecipitation assay (RIPA) buffer. The solubilized proteins were separated on 12 % SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membranes were saturated with 3 % powdered milk in saline buffer and proteins was detected with the tyrosinase (sc-7833), TRP-1 (sc-10443), TRP-2 (sc-10452) and actin (sc-1616) antibodies in saturation buffer, and with a secondary alkaline phosphatase-conjugated anti-mouse antibody. Proteins were visualized with the 5-bromo-4-chloro-3-indolyl-1-phosphate/nitro blue tetrazolium (BCIP/NBT).

2.8. RT-PCR Analysis

Total cellular RNA was extracted by using RNeasy mini kit (Qiagen, MD, USA) from B16F10 melanoma cells. 10 μg of RNA was reverse transcribed by using Qiagen reverse transcription system. 20 ~ 29 cycles of PCR amplification of the resulting cDNA allowed to quantify tyrosinase, TRP-1, TRP-2 and GAPDH mRNA (50 ~ 55 ℃ 30 s; 72 ℃ 1 min; 94 ℃ 30 s). The PCR products were electrophoresed on 1.5 % agarose gels and stained with ethidium bromide before visualization under UV light.

2.9. Endothelin-1 (ET-1) Enzyme-linked immunosorbent assay (ELISA)

The cells (HaCaT keratinocytes) were irradiated by 8 mJ/cm^2 of UVB source emitting wavelengths in the 280 ~ 360 nm range (Sankyo Denki, Japan). The expression of ET-1 was assayed by ELISA[8].

2.10. Statistical Analysis

Results were presented as mean ± S.D. Experimental results were statistically analyzed by using SPSS. p values < 0.05 were regarded as indicating significant differences.

3. Results and Discussion

3.1. Inhibition of Tyrosinase Activities

Tyrosinase is the rate-limiting enzyme in melanin synthesis. The results of tyrosinase activity assay are shown in Figure 1. The EtOAc fraction of *P. brachycarpa* showed relatively high inhibitory effect of mushroom tyrosinase.

3.2. Free Radical Scavenging Activity

The DPPH radical scavenging activity of the extract is shown in Figure 2. The EtOAc fraction of *P. brachycarpa* showed relatively high DPPH radical scavenging activity.

3.3. Effect of P. *brachycarpa* on Melanogenesis in B16 Melanoma Cells

We quantitatively examined the effect of *P. brachycarpa* fractions on melanogenesis. The results are shown in Figure 3. At a concentration of 100 μg/mL, the EtOAc, EtOH, Hexane, BuOH, and aqueous fractions of *P. brachycarpa* decreased the melanin contents to 58.7, 39.9, 37.1, 41.7, and 35.0 %, respectively, compared to those of control cells. *P. brachycarpa* EtOAc fraction of *P. brachycarpa* significantly decreased the intracellular melanin contents compared to the other fractions (Figure 3).

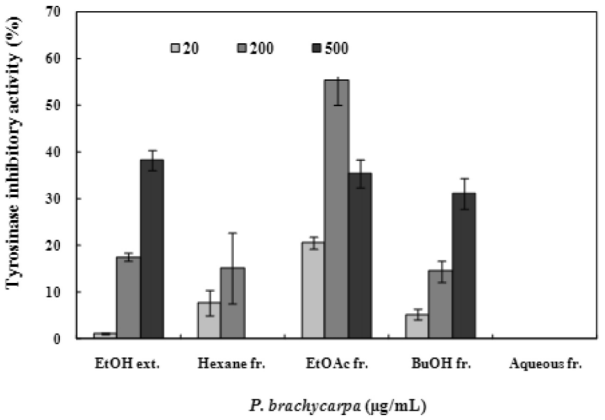

Figure 1. Inhibitory effect of *P. brachycarpa* on mushroom tyrosinase. The results were expressed as the average of triplicate samples with S.D.

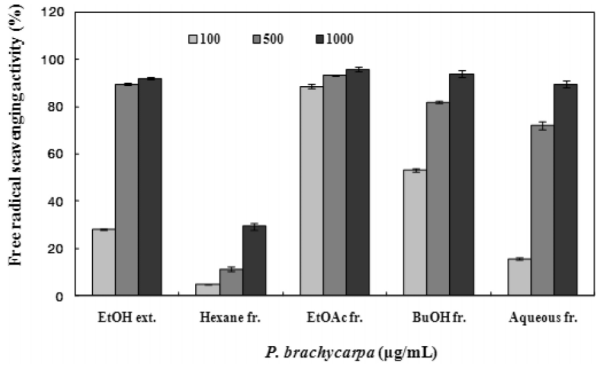

Figure 2. Antioxidant effect of *P. brachycarpa* in the DPPH assay. The results were expressed as the average of triplicate samples with S.D.

3.4. Effect of P. *brachycarpa* EtOAc Fraction on Activity of Tyrosinase in B16 Melanoma Cells

To clarify the inhibitory mechanism of *P. brachycarpa* EtOAc fraction on melanogenesis, we examined the effect of *P. brachycarpa* EtOAc fraction on tyrosinase activity which was extracted from B16 melanoma cells. The results are shown in Figure 4. *P. brachycarpa* EtOAc fraction decreased the intracellular tyrosinase

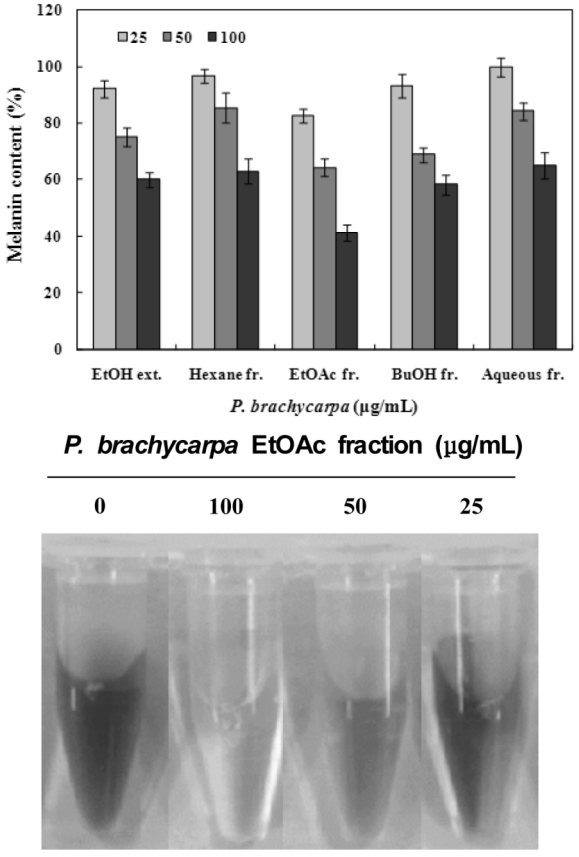

Figure 3. Inhibitory effect of *P. brachycarpa* on melanin contents in B16 melanoma cells. B16 melanoma cells were cultured in the presence of *P. brachycarpa* at concentrations of 25, 50, 100 μg/mL for 72 h. Values are the averages of three determinations ± S.D.

activity at all testing concentrations. At a concentration of 100 μg/mL, *P. brachycarpa* EtOAc fraction decreased the tyrosinase activity about 35 ± 2.5 % compared to those of control B16 melanoma cells. From these results, we supposed that *P. brachycarpa* EtOAc fraction inhibited melanin biosynthesis by regulating tyrosinase activity with low cytotoxicity.

3.5. Effect of P. brachycarpa EtOAc Fraction on Synthesis of Tyrosinase, TRP-1 and TRP-2

The tyrosinase protein synthesis was examined by Western blot analysis. The results are shown in Figure 5. *P. brachycarpa* EtOAc fraction at the concentrations of 100, 50, and 25 μg/mL reduced the tyrosinase synthesis to 72 ± 1.5 %, 41 ± 1.5 %, and 11 ± 1.5 %

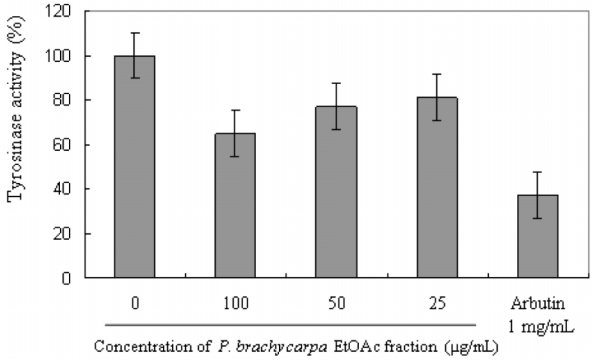

Figure 4. Effect of *P. brachycarpa* EtOAc fraction on tyrosinase activity in B16F10 melanoma cells. B16F10 melanoma cells were treated with various concentrations of *P. brachycarpa* EtOAc fraction for 24 h. The lysates of the cells containing tyrosinase were incubated with DOPA for 2 h. Results are expressed as a percentage of the control. Values are the averages of three determinations ± S.D.

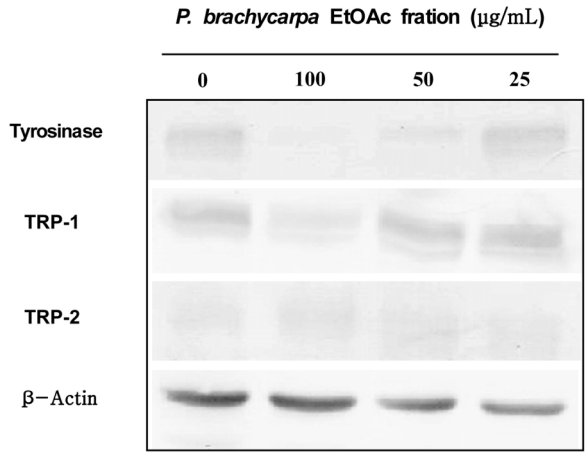

Figure 5. Effect of *P. brachycarpa* EtOAc fraction on Tyrosinase, TRP-1, and TRP-2 protein expression in B16F10 melanoma cells. B16F10 melanoma cells were treated with various concentrations of *P. brachycarpa* EtOAc fraction for 48 h. Solubilized total protein (50 μg) was electrophoresed in 12 % SDS-PAGE gels and transferred to nitrocellulose membrane. Specific detection of proteins was performed with the polyclonal antibody against tyrosinase, TRP-1, and TRP-2. Similar results were observed in three independent experiments.

of the control value, respectively. The fraction also reduced TRP-1 synthesis at the concentration of 100 μg/mL. These results suggest the possibility that *P.*

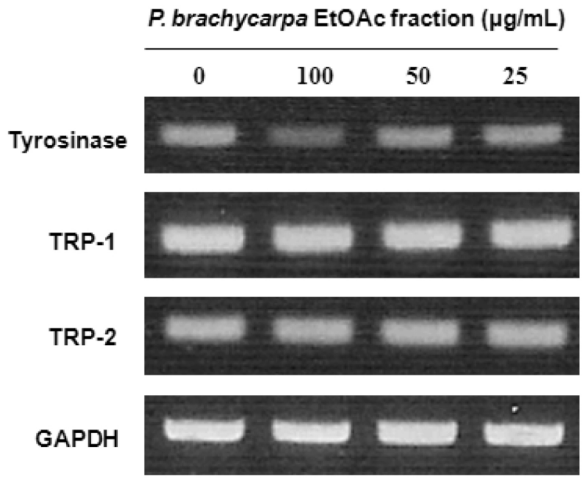

Figure 6. Effect of *P. brachycarpa* EtOAc fraction on Tyrosinase, TRP-1, and TRP-2 mRNA expression in B16F10 melanoma cells. B16F10 melanoma cells were treated with various concentrations of *P. brachycarpa* EtOAc fraction for 24 h. Total RNA extracted from B16F10 melanoma was analyzed by RT-PCR.

brachycarpa EtOAc fraction effectively inhibited tyro sinase and TRP-1 protein synthesis in B16 melanoma cells.

3.6. Effect of *P. brachycarpa* EtOAc Fraction on mRNA Expression of Tyrosinase, TRP-1, and TRP-2

To elucidate the action mechanism of *P. brachycarpa* EtOAc fraction, we investigated the changes in mRNA level of tyrosinase, TRP-1, and TRP-2 using RT-PCR technique. B16 melanoma cells were treated with 100, 50, 25 μg/mL of *P. brachycarpa* EtOAc fraction for 48 h, respectively and then, each mRNA level was examined. The results are shown in Figure 6. When normalized with the mRNA level of β-actin, the mRNA level of tyrosinase was decreased at 100 μg/mL. Theses results suggest that *P. brachycarpa* EtOAc fraction might act on the common upstream event that controls the transcription of tyrosinase gene.

3.7. Determination of Melanogenic Cytokine, Endothelin-1 (ET-1)

ET-1, one of the paracrine melanogenic cytokines, plays pivotal roles in skin pigmentation including UVB-induced pigmentation.

In order to investigate the whitening effect of the *P. brachycarpa* EtOAc fraction on the UVB response in human skin, we studied the effect of the fraction on UVB-modulated ET-1 expression in normal human keratinocytes (HaCaT). After irradiation UVB 8 mJ/cm^2, the expression of ET-1 was about 16 pg/mL, and in the concentration of the extract at 50 μg/mL, ET-1 production was inhibited about 40 % compared with UVB irradiated cells (Figure 7).

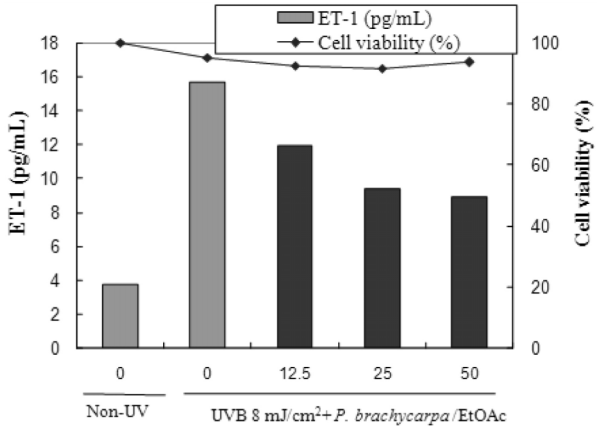

Figure 7. Inhibitory effect of *P. brachycarpa* EtOAc fraction on ET-1 expression in UVB irradiated HaCaT keratinocytes. HaCaT keratinocytes were cultured in the presence of *P. brachycarpa* EtOAc fraction at concentrations of 12.5, 25, 50 μg/mL for 24 h. Values are the averages of three determinations ± S.D.

4. Conclusions

We found that the EtOAc fraction of *P. brachycarpa* had a strong inhibitory activity against melanogenesis and exerted its melanogenic inhibitory effect through the modulation of protein and mRNA levels of tyrosinase and inhibition of ET-1 expression.

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