# Inhibitory Effects of Herb and Seeds of
# *Oenothera odorata* on the Melanogenesis

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## 월견초 및 월견자의 멜라닌 생성 억제효과

### 최미은 · 우원홍 · 문연자

**목적 :** 월견초는 다량의 불포화 지방산인 리놀렌산과 감마 리놀렌산을 함유하고 있으며, 천식성 기침이나 아토피 피부염에 효능이 있는 것으로 알려져 있다. 본 연구는 월견초의 전초와 종자 추출물의 피부 멜라닌 합성에 대한 억제효과를 조사하였다.

**방법 :** B16F10 멜라닌세포주를 이용하여 멜라닌, tyrosinase 활성 및 세포생존율을 측정하였다. 또한 멜라닌 합성-관련효소인 tyrosinase, TRP-1, TRP-2의 단백질발현과 α-MSH를 처리하여 색소침착을 유도 한 뒤 단백질 발현을 조사하였다.

**결과 :** 월견자는 B16F10 세포의 멜라닌 합성을 5 ㎍/㎖와 10 ㎍/㎖ 농도에서 각각 대조군의 81.3%, 68.3%로 억제하였고 tyrosinase의 활성도 이와 유사하게 억제하였다. 멜라닌 합성-관련효소들의 단백질발현을 관찰한 결과 월견초와 월견자는 tyrosinase 발현을 억제하였으며 TRP-1과 TRP-2의 발현에는 영향을 주지 않았다. 특히 α-MSH에 의한 과색소 유도 시 tyrosinase 발현이 현저하게 감소되었으며, 월견자의 멜라닌 합성 억제 효과가 월견초 보다 높게 나타났다.

**결론 :** 이상의 연구 결과 월견자는 멜라닌세포의 tyrosinase 단백질 발현과 tyrosinase 활성을 억제하여 멜라닌 생성을 감소시키는 것으로 사료된다.

Key words : *Oenothera odorata,* tyrosinase activity, melanin synthesis, TRP-1, TRP-2

## Ⅰ. Introduction

Acquried pigmentary skin diseases such as abnormal melanogenesis, vitiligo, chloasma and

inflammatoy pigmentation are related to regulate the melanin production. Melanin is related to the color of the skin and hair, and plays a significant role in protecting the skin from UV. Melanin synthesis is regulated by melanogenic enzyme such as tyrosinase, tyrosinase-related protein 1 (TRP-1) and tyrosinase-related protein 2 (TRP-2)[1]. Tyrosinase is the key enzyme in melanin biosynthesis. Therefore, tyrosinase inhibitors have become increasingly significant medicinal and cosmetic agents[2]. Recently the consumers desire and social atmosphere preferred natural product to artificial, and various extracts of traditional herb medicine are getting more attention in developing cosmetic products. Natural substances such as herb have been increased in the demand for new agents of cosmeceutical and antioxidants, anti-wrinkles, anti-aging and skin whitening purposes in the global market[3,4].

*Oenothera odorata*, known as evening primrose is a perennial plant belonging to the genus Oenothera. It was considered to be effective in healing asthmatic coughs, gastro-intestinal disorders and whooping cough. The mature seeds contain approximately 7-10% gamma-linoleic acid (GLA), a rare essential fatty acid. Evening primrose oil is one of the commercial sources of GLA (18:3n-6). GLA is an essential n-6 polyunsaturated fatty acid (PUFA) and a precursor of dihomo-gamma-linolenic acid (20:3n-6) and arachidonic acid (20:4n-6)[5,6]. GLA has the potential to increase tissue biosynthesis of 1-series prostaglandins and is known to play an important role in the

treatment of diabetes[7], hypertension[8], thromboembolic disease[9], atopic eczema[10], and in the regulation of the inflammatory response[11].

In this study, we investigated the effects of whole plant of *Oenothera odorata* (WOO) and seeds of *Oenothera odorata* (SOO) on the melanogenesis in B16F10 melanoma cells.

## Ⅱ. Materials and Methods

### 1. Extractions of WOO and SOO

The whole plant extract of *Oenothera odorata* (WOO) was purchased from the Plant extract bank (Korea, 2009) as the methanol extract of *Oenothera odorata*. The seeds of *Oenothera odorata* (SOO) was purchased from the Hanyakjaemart (Yeong Cheon of Gyeong-buk Province, Korea, 2009). After cleaning, SOO were air-dried at room temperature. The dried SOO (200 g) was extracted with 2,000 ml of ethanol (100%) at room temperature for 3 days. The extract was filtered through Whatman (No. 2) filter paper and concentrated using the rotary evaporator. After lyophilized using freeze dryer, the yield of the ethanol extract was approximately 12.1% of the powder and dissolved in DMSO (Sigma, USA), and th final concentration of DMSO was less than 0.1%.

### 2. Cell line and culture

B16F10 mouse melanoma cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, USA) supplemented with 5% fetal

bovine serum (FBS, Gibco, USA) and 1% antibiotics in air containing 5% $CO_2$ at 37℃.

## 3. Measurement of melanin contents

Melanin contents were determined by the modified method of Hosoi et al (1985)[12]. B16F10 cells were seeded at $3×10^5$ cells in 10 ㎝ dishes. After 24 hours, cells treated with WOO and SOO at 5 ㎍/㎖, 10 ㎍/㎖ for 72 hours. After washing with PBS, the cells were harvested and collected at $2×10^6$ cells by centrifugation at 13,000 rpm and 4℃ for 2 min. After centrifugation, carefully removed supernatants were dissolved in 200 ㎕ of 1 N NaOH containing 10% DMSO at 90℃ for 1 hours. The amount of melanin was determined by absorbance at 405 nm using ELISA reader.

## 4. Tyrosinase activity assay of B16F10 cells

Tyrosinase activity was assayed as 3,4-dihydroxyphenylalanine (DOPA, Sigma, USA) oxidase activity using a modified method described by Martinez-Esparza (1998)[13]. B16F10 cells were seeded at $8×10^4$ cells in 6-well plates with DMEM containing 5% FBS and antibiotics. After 24 hours, each cells were treated with WOO and SOO at 5 ㎍/㎖, 10 ㎍/㎖ and $α$-MSH at 10 nM for 72 hours incubated. Cells were washed twice with PBS and scraped with 200 ㎕ of lysis buffer [0.1 M sodium phosphate buffer (SPB, pH 6.8) containing 5 mM EDTA and 0.1 M PMSF and 1% triton X-100] and sonicated in to an ice bath for 30 min. After centrifugation at 13,000 rpm for 20 min at 4℃, the supernatant was corrected by the protein contents of the samples. Protein concentration was determined by the method of Bradford (1976). The reaction mixture containing 150 ㎕ of calculated samples and 0.1 M SPB (pH 6.8) and 50 ㎕ of substrate solution (0.1% L-DOPA in 0.1 M SPB) was incubated at 37℃. The absorbance change was measured during the 1 hours of the reacted in 30 minute interval at 405 nm using ELISA reader.

## 5. Cell viability using MTT assay

The general viability of cultured cells was determined by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT, Sigma, USA) to formazan[14]. B16F10 cells ($5×10^4$ cells in 1 ㎖/well) were seeded in 24-well plates with DMEM containing 5% FBS and antibiotics. After 24 hours, each cells were treated with WOO and SOO at 1.25 ～ 20 ㎍/㎖ for 24 hours cultured with serum starvation. MTT (5 ㎎/㎖ in phosphate buffered saline) was added to each well at a 1/10 volume of media. Cells were incubated at 37℃ for 3 hours. After removed media, DMSO added in order to dissolve the formazan crystals. The absorbance was then measured at 540 nm using ELISA reader (Bio-Tek Instrument, USA).

## 6. Protein preparation and Western blot analysis

Western blotting was performed as described previously. Cells were lysed with lysis buffer (1 × RIPA buffer 1 ㎖, 1 mM phenylmethylsulphonyl

fluoride (PMSF), 1 $\mu$g/$ml$ aprotinin, 1 $\mu$g/$ml$ leupeptin, 2 mM DTT, 50 mM) for 30 min at 4℃. Insoluble debris was removed by centrifugation at 15,000 rpm for 20 min, and protein content was determined using Bradford reagent. Equal amounts of protein were resolved on gradient (7.5~10%) SDS-polyacrylamide gel and then electrophoretically transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% non-fat skim milk in TBS/T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.05% Tween 20) for 1 hour, and then incubated primary antibodies at a final dilution of 1:1000 overnight, 4℃. The blot was washed several times with TBS/T and incubated with the appropriate secondary antibody for 2 hours. After the membrane was washed several times with TBS/T and then detected by western blotting detection reagent procedure. The protein expression levels were determined by analysing the signals captured on membrane using a ChmiDoc image analyzer (Bio-Rad, USA).

## 7. Statistical analysis

Results were presented as means ± S.D. Experimental results were statistically analyzed by using ANOVA test (Origin 5.0) or Student's t-test (SigmaPlot 2000). P values 〈0.05 were regarded as indicating significant differences.

## Ⅲ. Results

### 1. Effects of WOO and SOO on melanin production in B16F10 cells

Melanocytes are normally present in skin, being responsible for the production of the dark pigment melanin. To provide direct evidence that WOO and SOO inhibit melanogenesis, we determined its effects on melanin production in B16F10 cells. The cells were incubated with WOO and SOO (5, 10 $\mu$g/$ml$) for 3 days and then melanin content was determined. WOO treatment slightly decreased the melanin production at 10 $\mu$g/$ml$. However, SOO significantly inhibited melanin formation as compared with control in a dose-dependent manner (81.3% at 5 $\mu$g/$ml$ and 68.3% at 10 $\mu$g/$ml$) (Fig. 1).
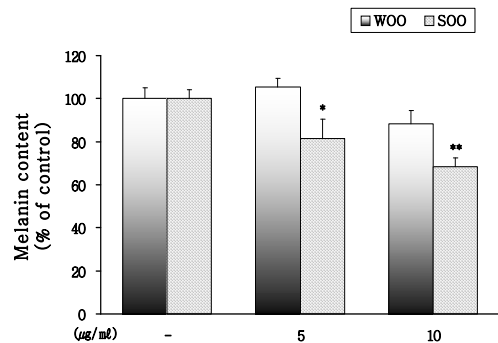


Fig. 1. Effects of WOO and SOO on melanin production in B16F10 cells.

B16F10 cells were treated with 5, 10 $\mu$g/$ml$ of WOO and SOO for 3 days. Melanin contents were measured as described in materials and methods. Results are expressed as percent of control. Each determination was made in triplicate; the data shown represent means ± S.D. *P〈0.05, **P〈0.01 compared with the control.

## 2. Effects of WOO and SOO on cell tyrosinase activity in B16F10 cells

In this study, tyrosinase activities in B16F10 cells treated with WOO and SOO also decreased in a manner correlating with the inhibitory effect on melanin production. To examine the action of WOO and SOO on skin hyper-pigmentation, we investigated its effects in B16F10 cells stimulated with $\alpha$-melanocyte stimulating hromone ($\alpha$-MSH), which are the best characterized biologically relevant agent inducing melanogensis. After 3 days incubation with $\alpha$-MSH, tyrosinase activity increased 2.3-fold compared with control (100%). However, these increases were markedly suppressed in the presence of SOO (Fig. 2).

## 3. Effects of WOO and SOO on B16F10 melanoma viability

To assess the effects of WOO and SOO on cytotoxicity in B16F10 cells, the cells were preincubated with WOO and SOO (1.25 - 20 $\mu$g/$ml$) for 24 hours, and then MTT assay were performed. As shown in Fig. 3, WOO did not alter cell viability at the range of 1.25 - 20 $\mu$g/$ml$. However, incubation with 10, 20 $\mu$g/$ml$ SOO significantly decreased cell viability.

## 4. Effects of WOO and SOO on tyrosinase, TRP-1 and TRP-2 protein expressions

The synthesis of melanin polymer requires at least three enzymes: tyrosinase, tyrosinase-related protein-1 (TRP-1) and tyrosinase-related protein-2 (TRP-2). To know the effects of WOO and SOO on tyrosinase, TRP-1 and

TRP-2 expressions, western blot analysis was done on to these proteins after treated with 5, 10 $\mu$g/$ml$ WOO and SOO for 3 days on B16F10 cells. There was marked reduction of tyrosinase expression with SOO treatment compared with control treatment. TRP-1 and TRP-2 expressions was not reduced by WOO and SOO treatment (Fig. 4).
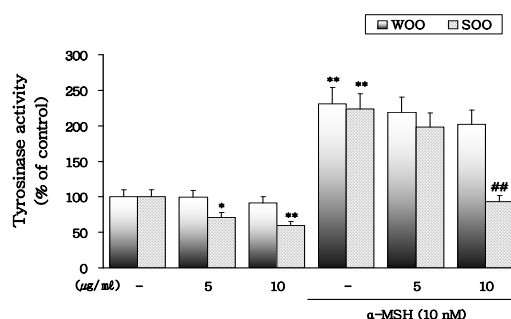
Fig. 2. Effects of WOO and SOO on tyrosinase activity in B16F10 cells.

The effect on tyrosinase activity was tested with 5, 10 $\mu$g/$ml$ of WOO, SOO and $\alpha$-MSH in B16F10 cells for 3 days. Results are expressed as percent of control. Each column represents the means ± S.D. of triplicate determinations. *P⟨0.05, **P⟨0.01 compared with the control, and ##P⟨0.01 compared with $\alpha$-MSH.
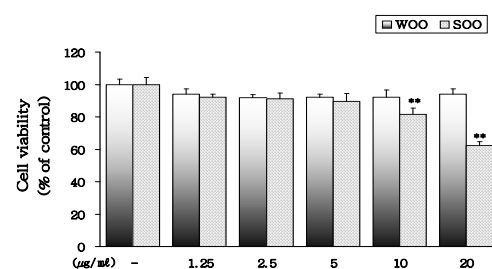
Fig. 3. Effects of WOO and SOO on B16F10 cell viability.

Cells were ($5 \times 10^4$) serum-starved for 24 hours. WOO and SOO was treated in serum free-media at 1.25 - 20 $\mu$g/$ml$ for 24 hours. Cell viability was determined by MTT assay as described in materials and methods. Data are mean ± S.D. of three experiments performed in triplicate. **P⟨0.01 compared with the control.
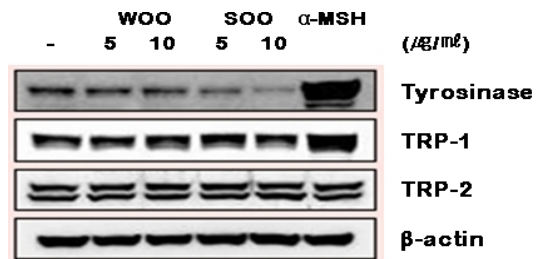
Fig. 4. Effects of WOO and SOO on the tyrosinase, TRP-1 and TRP-2 protein expressions in B16F10 cells.

Cells were incubated with WOO and SOO. After 3 days, cells were analysed using Western blotting as described in materials & methods. Western blot analysis using antibodies against tyrosinase, TRP-1 and TRP-2. Equal protein lading was confirmed by reaction with $\beta$-actin, tyrosinase, TRP-1, TRP-2 antibodies. Lane 1: control, Lane 2: WOO 5 ㎍/㎖, Lane 3: WOO 10 ㎍/㎖, Lane 4: SOO 5 ㎍/㎖, Lane 5: SOO 10 ㎍/㎖, Lane 6: α-MSH 10 nM.
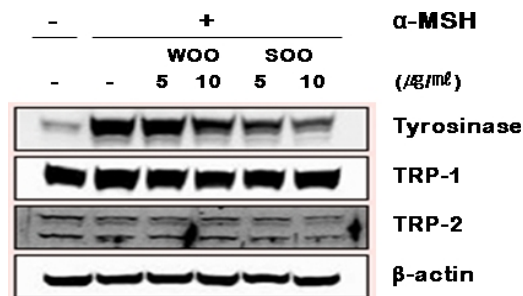


Fig. 5. Effects of WOO and SOO on melanogenic enzyme expressions in B16F10 cells stimulated with α-MSH.

Cells were incubated with WOO, SOO and α-MSH. After 3 days, cells were analysed using Western blotting as described in materials & methods. Western blot analysis using antibodies against tyrosinase, TRP-1 and TRP-2. Equal protein lading was confirmed by reaction with $\beta$-actin, tyrosinase, TRP-1, TRP-2 antibodies. Lane 1: control, Lane 2: α-MSH (10 nM), Lane 3: α-MSH + WOO 5 ㎍/㎖, Lane 4: α-MSH + WOO 10 ㎍/㎖, Lane 5: α-MSH + SOO 5 ㎍/㎖, Lane 6: α-MSH + SOO 10 ㎍/㎖.

α-MSH is one of the major pigmentation hormones, and synthesis of α-MSH is an important determinant of constitutive pigmentation[15]. α-MSH has been reported to increase melanogenic enzyme protein levels in melanocytes. It was confirmed that α-MSH (10 nM) stimulated tyrosinase, TRP-1 and TRP-2 expressions in B16F10 cells. WOO and SOO inhibited the expression of the tyrosinase, TRP-1 of the melanocyte stimulated with α-MSH (Fig. 5). These findings indicate that WOO and SOO decreased tyrosinase expression and they may be more effective on active melanocytes.

## Ⅳ. Discussion

Melanin is the most important determinant of the skin color. Major role of melanin is to protect the skin and underlying tissues from UV-induced skin injury. However, excessive melanin formation and accumulation of melanin in the skin causes negative hyperpigmenting effects such as melasma, freckles, and geriatric pigment spots[15-17]. The present study was to investigate the inhibitory effects of WOO and SOO on melanin production, which is closely related to pigmentation of th skin. Our study demonstrated that melanin production was suppressed by SOO.

Melanin synthesis proceeds from L-tyrosine through a series of enzymatic and chemical steps initiated by tyrosine hydroxylation to yield L-3,4- dihydroxyphenylalanine (DOPA). L-Tyrosine is oxidized to DOPA, which is oxidized to DOPA-quinone, which rearranges to leucoDOPAchrome and then to DOPAchrome.

In the presence of oxygen, DOPAchrome loses its carboxyl group to become 5,6-dihydroxyindole (DHI), which further oxidizes to indole-5,6-quinone and then to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) to indole-5,6-quinone carboxylic acid. Tyrosinase catalyze the first step reaction as the key enzyme in the pathway of melanin synthesis[2]. TRP-1 catalyze the oxidation of DHICA to indole-5,6-quinonec carboxylic acid and tautomerization of DOPAchrome to DHICA[18].

Tyrosinase inhibitors are important constituents of cosmetics and skin lightening agent[19]. Several substances such as arbutin, kojic acid and hydroquinone have been used as skin whitening agents in the cosmetic industry. Kojic acid is extremely irritable and exhibits cell toxicity. In this study, tyrosinase activities in B16F10 cells treated with WOO and SOO also decreased in a manner correlating with the inhibitory effect on melanin production. The synthesis of melanin polymer requires at least three enzymes: tyrosinase, TRP-1 and TRP-2. We examined the effects of WOO and SOO on tyrosinase, TRP-1 and TRP-2 expressions. As expected, WOO and SOO decreased the level of tyrosinase protein.

Melanin synthesis is stimulated by a large number of effectors, including cAMP elevating agents (forskolin, IBMX, $\alpha$-MSH), cholera toxin, UV light, placental total lipid fraction, lupeol, lipopolysaccharide[20,21]. The balance of a variety of signal transduction pathways regulates melanogenesis[22]. In humans, UV-induced skin darkening involves an increase in the melanocyte number as well as a stimulation of melanin biosynthesis and melanocytes dendricty. This induced a subsequent accumulation of melanosomes in melanocytes and transfer to neighboring keratinocytes[23,24]. $\alpha$-MSH is one of the major pigmentation hormones, and synthesis of $\alpha$-MSH is an important determinant of constitutive pigmentation[20]. We also showed that WOO and SOO markedly inhibit the tyrosinase activity of B16F10 cells stimulated by $\alpha$-MSH. These observations imply that the depigmenting effect of SOO may be more effective on active melanocytes.

In conclusion, our results demonstrate that SOO is an effective inhibitor of tyrosinase and tyrosinase protein expression in B16F10 cells, which eventually slow melanin synthesis. These results indicate that SOO may be a useful inhibitor of melanogenesis and serve as a new candidate in the design of new skin-whitening or therapeutic agents.

## Acknowlegments

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