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신규 미백제 : Selina-4(14), 7(11)-dien-8-one

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New Whitening Agent : Selina-4(14), 7(11)-dien-8-one

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요 약: 본 연구자들은 백출의 메탄올 추출물로부터 분리, 정제한 selina-4(14), 7(11)-dien-8-one (Selina)이 B16 멜라노마 세포에 대하여 매우 강력한 미백효과가 있음을 밝혀내었다. 본 연구에서는 Selina의 non-tumorigenic melanocyte cell line인 Melan-a 세포에 대한 작용 메카니즘에 대한 보고를 하고자 한다. 덧붙여 Selina를 함유하고 있는 제품의 임상효과도 연구하였다. Selina는 10 μg/mL의 농도에서 세포독성을 보이지 않으면서 Melan-a 세포의 멜라닌 합성을 50% 저해하였다. 또한 10 μg/mL의 Selina의 처리에 의하여 타이로시네이즈의 활성이 60% 감소시키지만 타이로시네이즈에 대한 직접적인 저해활성은 없는 것으로 밝혀졌다. 이러한 Selina의 작용 기작을 밝히기 위하여 연구자들은 타이로시네이즈, TRP-1, TRP-2의 3가지 유전자의 mRNA 수준과 단백질의 발현량을 RT-PCR과 western blotting을 이용하여 연구하였다. 그 결과 10 μg/mL의 Selina 처리에 의하여 타이로시네이즈의 mRNA와 단백질 양은 현저히 감소하였지만 TRP-1과 TRP-2의 mRNA및 단백질 양에는 변화가 없었다. 이러한 결과는 Selina가 그 미백효과를 주로 타이로시네이즈의 발현조절에 의하여 나타낸다는 것을 알 수 있다. 0.2%의 Selina를 함유하는 화장품 제형을 이용, 7주간 20명의 피시험자를 대상으로 한 임상시험에서도 어떠한 부작용도 없이 통계적으로 유의한($p < 0.05$) 수준의 미백효과를 볼 수 있었다. 이상의 결과를 종합해 볼 때 Selina는 깨끗하고 밝은 피부를 위한 유용하면서도 안전한 신규 미백 원료임을 알 수 있다.

Abstract: We had previously reported that Selina (selina-4(14), 7(11)-dien-8-one) was isolated from methanol extract of *Atractylodes* rhizome and has strong whitening activity in B16 melanoma cells. In this report, we demonstrated its action mechanism in melan-a cells, non-tumorigenic melanocytes. We also investigated the clinical efficacy of cosmetic preparation containing Selina. Selina reduced the melanin synthesis of Melan-a cells by 50% at a concentration of 10 μg/mL without any apparent cytotoxicity. We also found that the treatment of cells with Selina decreased tyrosinase activity by 60% at a concentration of 10 μg/mL but Selina was not a direct inhibitor of tyrosinase activities. To elucidate the action mechanism of Selina, we investigated the changes in mRNA and protein level of tyrosinase, TRP-1 and TRP-2 using RT-PCR and western blotting, respectively. As a result, the mRNA and protein level of tyrosinase were markedly reduced at 10 μg/mL of Selina without any effect on TRP-1 and TRP-2. These results suggest that Selina exerts its whitening effect mainly through regulating expression of tyrosinase. A 7 week-clinical trial using formulation containing 0.2% selina-4(14), 7(11)-dien-8-one with 20 volunteers resulted in statistically significant whitening effect ($p < 0.05$), without any adverse effect. Based on these results, Selina (selina-4(14), 7(11)-dien-8-one) can be a useful and safe ingredient for the cleanness and brightness of skin.

Keywords: *melanogenesis, skin whitening, selina-4(14), 7(11)-dien-8-one*

1. Introduction

The levels of ultraviolet (UV) radiation emitted by the sun are increasing due to the destruction of ozone layer. Our skin is exposed more to UV radiation and often suffers from various harmful effects from UV light. Melanin pigmentation in human skin is a major defense mechanism against UV. Melanins are synthe-

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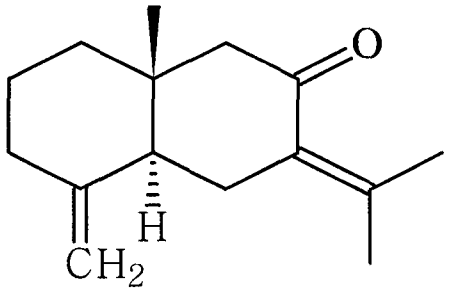

Figure 1. Structure of selina-4(14), 7(11)-dien-8-one.

sized in melanosomes by a coordinative action of various melanogenic enzymes such as tyrosinase, TRP-1 and TRP-2. Then, they are transferred to keratinocytes and eventually disappear with desquamation of the skin. However, abnormal hyperpigmentation such as freckles, chloasma, lentigines and other forms of melanin hyperpigmentation sometimes can be serious aesthetic problems [1]. Therefore, potent active agents for the improvement of hyperpigmentation are sought for their cosmetic use. Many chemicals such as hydroquinone, arbutin, kojic acid, and ascorbic acid are well known for their melanogenic inhibitory functions [2,3]. Even though these chemicals are melanogenic inhibitory in melanoma cells and widely used as whitening agents in many cosmetic formulations, there were some controversies about their whitening effects in cultured normal primary melanocytes [4,5,6].

In our previous study, we have isolated Selina (selina-4(14), 7(11)-dien-8-one) (Figure 1) from methanol extract of Atractylodis rhizome Alba and found that it has a strong melanogenic inhibitory effect in B16 melanoma cell-based screening assays [7]. To verify the potential usefulness of Selina as a cosmetic active ingredient, we evaluated the effects of Selina on melan-a cells which are non-tumorigenic immortalized melanocytes. We also performed a clinical study with a cosmetic formulation containing 0.2% Selina. In this report, we demonstrated that Selina can be a useful whitening agent.

2. Materials and Methods

2.1. Chemicals

All tissue culture medium and components were purchased form GIBCO BRL (Long Island, NY, USA). L-tyrosine, L-Dopa, PMA, MTT, Triton X-100, PMSF,

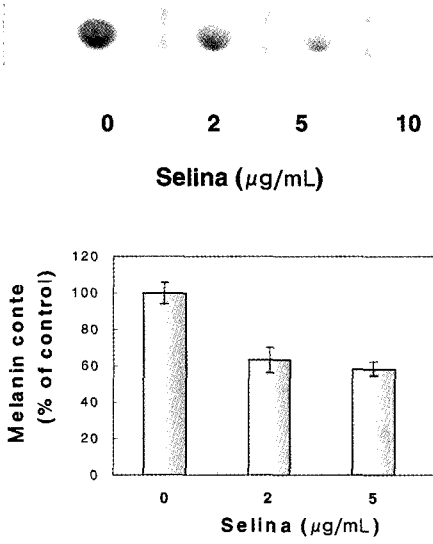

Figure 2. Selina decreased the pigmentation of Melan-a cells. Melan-a cells were treated with or without Selina. After 4 days, cells were harvested. (A) Harvested cells were pelleted and photographed. (B) Their melanin contents were assayed as described in Materials and Methods. Melanin contents were expressed as percent of control (Selina 0 μg/mL). Values are the averages of three determinations ± S.D.

sodium phosphate, beta-mercaptoethanol, HCl and charcoal were from Sigma Chemical Co. (St. Louis, MO, USA). Protease inhibitor cocktail was from Boehringer-Mannheim (Indianapolis, IN, USA), and L-[U-^{14}C] tyrosine and [^{3}H] tyrosine were from Amersham (Piscataway, NJ, USA).

2.2. Cells and Culture

Melan-a melanocytes were a kind gift from Dr. D.C. Benette (St. George's Hospital, London). Melan-a cells were originally derived from C57BL mice, and grown in a humidified incubator at 37℃ under 5% CO_2. Cells were routinely passaged in RPMI 1640 supplemented with 5% fetal calf serum, 100 μM beta-mercaptoethanol, 50 U/mL penicillin, 50 μg/mL streptomycin, 2 mM L-glutamine and 200 nM PMA.

2.3. Cell Treatment

Melan-a cells were seeded into 6-well plate at a density of 2.5 × 10^4 cells per well and allowed to attach

for 24 h. After then, triplicate cultures were fed with fresh medium containing various concentrations of compound. After 48 h, the medium was replaced with the same, fresh test medium. After further 48 h, cells are harvested with 0.5 mL of 0.25% trypsin/EDTA. After dislodging the cells with occasional agitation, 2 mL of medium were immediately added to inactivate the trypsin, and 100 μL aliquot were seeded into 96-well plate for MTT assay, as described below. The remainder cell suspensions were centrifuged for 5 min at 1500 g, washed with PBS and then solubilzed in 200 μL of extraction buffer (1% Nonidet P-40, 0.01% SDS, 0.1 M Tris-HCl pH 7.2 and protease inhibitor cocktail). Extracts were solubilized at 4℃ for at least 1 h and then assays were conducted for each sample, in triplicate.

2.4. Measurement of Melanin Content

After the treatment and extraction as described above, cell extracts were centrifuged for 5 min at 15,000 rpm. The resulting pellets were lysed with 200 μL of 1 N NaOH and transferred to 96-well plate in triplicate. Relative melanin content was determined by absorbance at 405 nm in PowerWave X340 ELISA reader (Bio-tech Instrumnets, Inc.).

2.5. Measurement of Cell Proliferation

MTT assay was used to determine cell proliferation. After the treatment as described above, 100 μL aliquots of harvested cells were plated. Cells were allowed to attach and grow overnight at 37℃. The media were discarded, 100 μL of 0.5 mg/mL MTT was added to each well and incubated at 37℃ for 4 h. After the incubation, plate was centrifuged for 10 min at 3,000 rpm to down the cells. Supernatants were discarded and 200 μL of isopropanol was added and the plate was incubated at RT for 4 h. The formazan precipitates were quantitated by absorbance at 562 nm in PowerWave X340 ELISA reader (Bio-tech Instrumnets, Inc.).

2.6. Measurement of Tyrosinase Activity in Compound Pre-treated Cell Extract

Tyrosinase activity was measured according to the method of Virador with some modifications [8]. Briefly, tyrosinase assay was performed in 96-well plate by adding 30 μL of cell extracts which were prepared as described above, 10 μL of L-[U-^{14}C] tyrosine and 10 μL of 0.25 mM L-Dopa cofactor in 1 M sodium phosphate buffer, pH 7.2, containing 0.01% albumin. Reactions were incubated for 4 h at 37℃ and stop the reaction by cooling. The contents of each well were transferred to 2.5 cm diameter pre-labeled 3MM filter disk and air-dried. The disks were washed three times with 0.1 N HCl containing excess cold L-tyrosine, twice with 95% ethanol and once with acetone. The disks were air-dried and put into liquid scintillation vial, mixed with scintillation cocktail, and the radioactivity was determined by LS 6500 scintillation system (Beckman, USA).

2.7. Measurement of Tyrosinase Activity in Solubilized Tyrosinase

To prepare solubilized tyrosinase, 5×10^6 cells were removed from stock culture flasks and centrifuged at 100 g for 10 min, and the cell pellet was sonicated in 0.5 mL of 0.1 M sodium phosphate buffer (pH 6.8) containing 1% Triton X-100 and 0.1 mM PMSF. After a 1 h incubation at 4℃ to release tyrosinase from the melanosome membrane, the resulting extract was centrifuged at 40,000 g for 20 min at 4℃. 50 μL extract was added to 1 mL of a reaction mixture containing 0.1 mM tyrosine, 2 uCi [^{3}H]tyrosine per mL, 0.1 mM L-DOPA, 0.1 mM PMSF and various concentrations of compound in 0.1 M sodium phosphate buffer (pH 6.8). After a 3 h incubation at 37℃, reaction were terminated by the addition of 1 mL of charcoal (10% wt/vol in 0.1 N HCl). Samples were centrifuged at 2000 g for 10 min, and the supernatant were removed and mixed with scintillation cocktail, and the radioactivity was determined by LS 6500 scintillation system (Beckman, USA).

2.8. RNA Preparation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Melan-a cells were seeded into T-75 flask at a density of 2×10^5 cells per flask and allowed to attach for 48 h. Compounds were treated as described above. Total cellular RNA was prepared using RNAeasy Mini Kit (Qiagen) according to the supplier's instruction. Primers used for RT-PCR analysis in this study were as follows; tyrosinase, 5′TGCCAACGATCCTATCTTCC3′ (5′primer), 5′TGAGGAGTGGCTGCTTTTCT3′ (3′primer); tyrosinase-related protein-1 (TRP-1), 5′CCCTTGCGCT TCTTCAATAG3′ (5′primer), 5′TTGCAACATTT CCT

GCATGT3′ (3′primer); tyrosinase-related protein-2 (TRP-2), 5′CCGACTACGT GATCACCACA3′ (5′primer), 5′TGGCAATTTCATGCTGTTTC3′ (3′primer); glyceroaldehyde-3phosphate dehydrogenase (G3PDH), 5′ATGT TCGTCATGGGTGTGAA′(5′primer), 5′GGGGTCTACA GGCAACTG3′ (3′primer). These primers were synthesized by Bioneer co., Korea. For cDNA synthesis, 1 μg of the total RNA was reverse transcribed in 20 μL of reaction mixture containing 2 μL of 10 × reverse transcription buffer (Perkin-Elmer), 4 μL of 25 mM $MgCl_2$ (Perkin-Elmer), 2 μL of 10 mM each dNTPs (Clontech), 1 μL of 50 mM Oligo d(T)16 (Perkin-Elmer), 20 units of RNasin (Promega) and 50 units of MuLV reverse transcriptase (Perkin-Elmer). Reverse transcription reaction mixture was incubated at RT for 10 min, 42℃ for 15 min, 99℃ for 5 min, and 5℃ for 5 min (GeneAmp PCR system 2400 thermal cycler, Perkin-Elmer). For PCR amplification of cDNA, 1 μL of the cDNA product was amplified in a total reaction volume of 50 μL containing 5 units of DNA Polymerase (AmpliTaq DNA Polymerase, Perkin-Elmer), GeneAmp 10 × PCR buffer (Perkin-Elmer), 4 μL of 25 mM $MgCl_2$ (Perkin-Elmer), 1 μL of 10 mM each dNTPs (Clontech), 20 pmole upstream primer, and 20 pmole downstream primer. DNA amplification was performed using a Perkin Elmer Gene Amp PCR system 2400 thermal cycler. The PCR cycle conditions were melting for 15 s at 95℃, annealing for 30 s at 60℃, extension for 90 s at 72℃. PCR products were resolved on 1% agarose gel and visualized by ethidium bromide staining and photographed.

2.9. Western Blotting

Cells were treated as described above. At the end of each treatment period, cells were washed in PBS and were lyzed in extraction buffer containing 1% Nonidet P-40, 0.01% SDS, and the protease inhibitor cocktail. Protein contents were determined with a BCA assay kit (Pierce, Rockford, IL) and equal amounts of each protein extract (10 μg per lane) were resolved using 8% SDS polyacrylamide gel (Koma Biotech, Korea), and transblotted onto nitrocellulose membranes (Amersham, Piscataway, NJ) and the membranes were blocked with 5% nonfat milk in TBS buffer. Following the blocking, the membranes were incubated with α PEP7 (anti-Tyrosinase), α PEP1 (anti-TRP1), or α PEP8 (anti-TRP2) (each at a 1 : 1000 dilution). α PEP7, α PEP1, and α PEP8 were kind gifts from Dr. Vincent J. Hearing (NCI, NIH). The membranes were then incubated with HRP-conjugated anti-rabbit IgG at a dilution of 1 : 2000. Immunoreactive bands were detected with enhanced chemiluminescence using an ECL kit (Amersham, Piscataway, NJ) according to the manufacturer's instructions.

2.10. Clinical Study

A conventional cosmetic preparation containing 0.2% selina-4(14), 7(11)-dien-8-one was used in the clinical study. 20 healthy female volunteers aged 18 to 48 (mean 36.5 yrs) were participated, with informed consents. The study was performed in randomized, placebo-controlled double-blind trial. With UVB light, each volunteer's minimal erythemal dose (MED) was determined and two artificial tanning (1.3 × 1.3 cm) was induced by irradiating 2 MED of UVB on the upper arm. After checking the conspicuous erythema, one site was treated twice daily for 7 weeks with selina -4(14), 7(11)-dien-8-one cream and the other with the control cream (placebo). The clinical efficacy was evaluated on weeks of 1, 3, 5, 7, with both the dermatologist's naked eye (10 point-scale) and the colorimeter, CR-400 (Konica-Minolta, Japan).

2.11. Statistics

All data were expressed as means ± S.D. The statistical significance for the comet assay was evaluated with student's t-test and for the clinical study with one-way ANOVA. $P < 0.05$ was considered significant.

3. Results and Discussion

3.1. Effect of Selina on Pigmentation of Melan-a Cells

Selina screened from B16 melanoma cell-based assay was tested on melan-a, non-melanoma cells and showed a remarkable whitening effect on melan-a cells (Figure 2A). This visual determination showed highly related results with direct measurement of melanin content (Figure 2B). At a concentration of 5 μg/mL and 10 μg/mL of Selina, melanin content decreased by almost 40% and 50%, respectively.

3.2. Effect of Selina on Cell Proliferation

When selecting whitening compounds, one of important

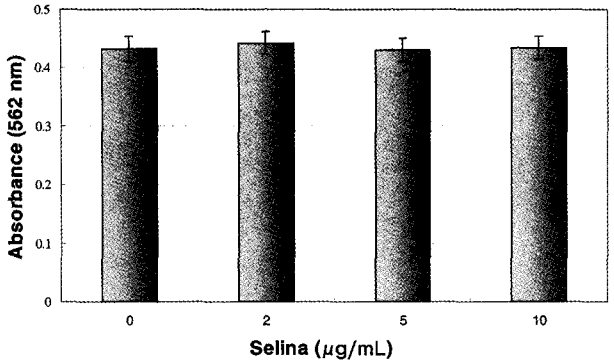

Figure 3. Selina did not show any inhibitory effect on cell proliferation. Melan-a cells were treated with or without Selina. After 4 days, cells were harvested. MTT assay was performed as described in Materials and Methods. Values are the averages of three determinations ± S.D.

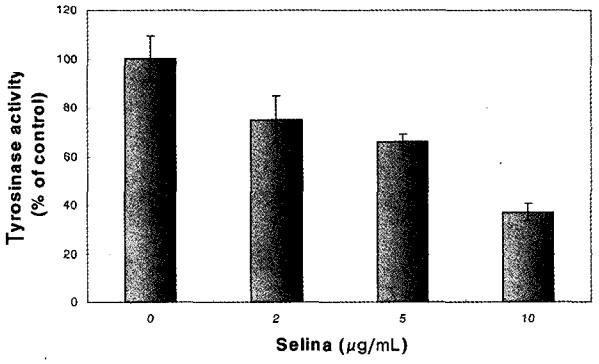

Figure 4. Selina inhibited tyrosinase activity. Melan-a cells were treated with or without Selina. After 4 days, cells were harvested. Radiometric tyrosinase assay was performed as described in Materials and Methods. Changes in tyrosinase activity were expressed as % of control (Selina 0 μg/mL). Values are the averages of three determinations ± S.D.

points is that they should have minimal effect on melanocyte cell proliferation. Thus, the proliferation of cells treated with Selina was evaluated by MTT assay. Selina showed little inhibitory effect on cell proliferation at the tested concentration (Figure 3). This result suggests that Selina can be a safe whitening agent candidate.

3.3. Effect of Selina on Tyrosinase Activity

Tyrosinase is a rate-limiting enzyme in melanin synthesis, and some melanin production-inhibiting agents such as arbutin and kojic acid are known to inhibit the

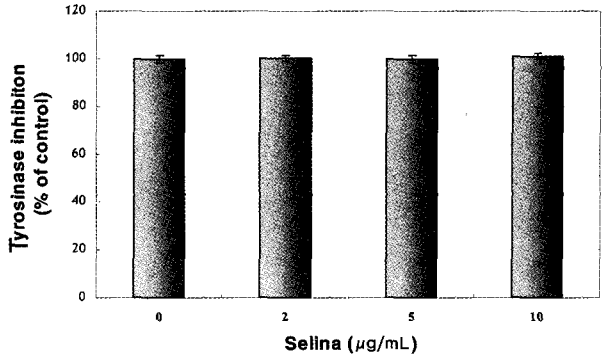

Figure 5. Selina has no direct inhibitory effect on isolated tyrosinase. Tyrosinase was released from the melanosome by detergent treatment as described in Materials and Methods. The supernatant containing soluble tyrosinase were incubated without or with Selina for 3 h incubation at 37℃ in the reaction buffer. The amount of tritiated water produced during this time was determined as described in Materials and Methods. Inhibition of tyrosinase was expressed as % of control (Selina 0 μg/mL). Values are the averages of three determinations ± S.D.

tyrosinase activity (6). To determine the effect of Selina on tyrosinase activity, we measured tyrosinase activity by counting [^{14}C] tyrosine incorporation into nascent insoluble melanin as described in Materials and Methods. Compared with the untreated control, Selina reduced tyrosinase activity by 40% and 60% at 5 μg/mL and 10 μg/mL, respectively (Figure 4).

To examine whether this reduced tyrosinase activity is caused by direct inhibition of tyrosinase action, we performed tyrosinase assay using solubilized tyrosinase as described in Materials and Methods. Because Selina showed no inhibition on solubilized tyrosinase, we concluded that Selina is not a direct inhibitor of tyrosinase (Figure 5).

3.4. Effect of Selina on Expression of Tyrosinase, TRP-1 and TRP-2

To explore the mechanism responsible for the decreased pigmentation, we examined changes in the mRNA levels and protein levels of three important melanogenic enzymes (tyrosinase, TRP-1 and TRP-2) using RT-PCR (Figure 6A) and western blotting (Figure 6B), respectively. Melan-a cells were treated with 5 μg/mL and 10 μg/mL of Selina for 4 days and then, each mRNA level and protein level was examined.

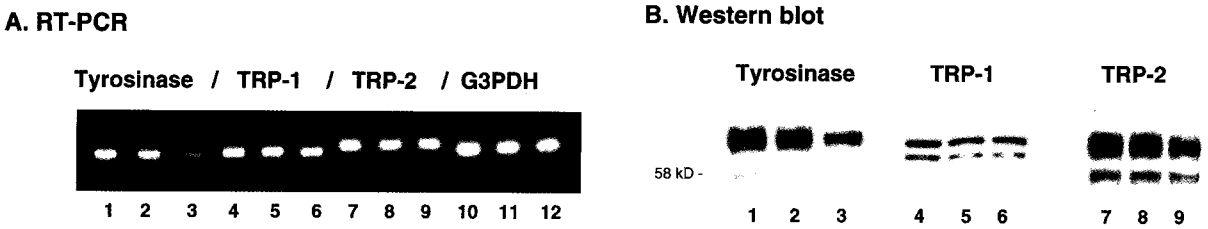

Figure 6. Selina inhibited tyrosinase expression. Fig. A. Total RNA was extracted and applied to RT-PCR as described in Materials and Methods. Gel electrophoresis of the RT-PCR products. Lane 1, 4, 7, 10: PCR product of tyrosinase (400 bp), TRP-1 (450 bp), TRP-2 (500 bp) and G3PDH (400 bp) of untreated control, respectively; Lane 2, 5, 8, 11: PCR product of tyrosinase, TRP-1, TRP-2 and G3PDH of 5 μg/mL Selina treated melan-a, respectively; Lane 3, 6, 9, 12: PCR product of tyrosinase, TRP-1, TRP-2 and G3PDH of 10 μg/mL Selina treated melan-a, respectively. (B) Total protein was extracted and applied to western blotting as described in Materials and Methods. Specific detection of tyrosinase, TRP-1, TRP-2 was performed using α PEP7 (anti-Tyrosinase), α PEP1 (anti- TRP1), or α PEP8 (anti-TRP2). Lane 1, 4, 7: tyrosinase, TRP-1 and TRP-2 of untreated control, respectively; Lane 2, 5, 8: tyrosinase, TRP-1 and TRP-2 of 5 μg/mL Selina treated melan-a, respectively; Lane 3, 6, 9: tyrosinase, TRP-1 and TRP-2 of 10 μg/mL Selina treated melan-a, respectively.

Table 1. Whitening Effect Measured by Dermatologist's Naked Eyes with 10 Point-Scale

Weeks	Placebo (mean ± S.D)	0.2% Selina cream (mean ± S.D)	p-Value
1	4.55 ± 1.61	3.80 ± 1.94	0.19020
3	3.95 ± 1.36	2.80 ± 1.47	*0.01425
5	3.05 ± 0.89	1.55 ± 0.89	*0.0000045
7	2.20 ± 0.77	0.70 ± 0.73	*0.0000002

* p-Value < 0.05

When compared with untreated control, 10 μg/mL Selina inhibited tyrosinase mRNA and protein expression dramatically without any effect on TRP-1 and TRP-2. However, at 5 μg/mL concentration, there was no change in expression level of tyrosinase. These results suggest that whitening potency of Selina results mainly from down regulation of expression of tyrosinase and other unknown mechanism also may be concerned in the action of Selina, because 5 μg/mL concentration which did not decrease expression of tyrosinase also showed whitening effect.

3.5. Clinical Study

From a seven-week clinical study with 0.2% selina-4(14), 7(11)-dien-8-one cream, it was found that there were statistically significant reductions ($P < 0.05$) in skin colors after 3, 5, 7 weeks of sample application, measured by both dermatologist's naked eyes with 10 point-scale and colorimeter. Table 1 and Table 2 demonstrate the whitening effect of selina-4(14), 7(11)-dien-8-one cream.

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Table 2. Whitening Effect Measured by Colorimeter

Weeks	Placebo L*value (mean ± S.D)	0.2% Selina cream L*value (mean ± S.D)	ΔL*value	p-Value
1	60.17 ± 2.26	60.35 ± 2.34	0.18 ± 0.95	0.4053
3	61.62 ± 2.17	62.03 ± 2.05	0.41 ± 0.81	*0.0337
5	62.01 ± 2.08	62.52 ± 1.94	0.51 ± 0.49	*0.0002
7	62.10 ± 2.19	62.72 ± 1.88	0.62 ± 0.76	*0.0018

* p-Value < 0.05

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