**Natural Product Sciences**
22(4) : 252-258 (2016)
https://doi.org/10.20307/nps.2016.22.4.252

# Inhibition of Melanogenesis by Abietatriene from *Vitex Trifolia* Leaf Oil

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**Abstract** – *Vitex trifolia* L. has been used traditionally to treat various illnesses, such as inflammation, headache, migraine, and gastrointestinal infections. We analyzed and evaluated the composition of *V. trifolia* leaf oil. Based on the results, we isolated abietatriene from *V. trifolia* leaf oil and investigated the effect of *V. trifolia* leaf oil and its active compound abietatriene on melanogenesis in B16F10 melanoma cells. They significantly decreased melanin contents and melanogenic factors, such as tyrosinase, TRP-1, TRP-2, and MITF dose-dependently in both protein and mRNA levels. Protein and mRNA expressions were determined by Western blot analysis and quantitative real time RT-PCR. Findings indicate that *V. trifolia* leaf oil and abietatriene reduce melanogenesis by regulating the expression of melanogenic factors. These results suggest that *V. trifolia* leaf oil and abietatriene could comprise a useful therapeutic agent for treating hyperpigmentation and used as effective skin-whitening agents.
**Key words** – *Vitex trifolia,* Abietatriene, Melanogenesis, B16F10 melanoma

## Introduction

Melanogenesis is the physiological process by which melanocytes produce the pigment melanin to protect against ultraviolet (UV) radiation and diverse free radicals.[1,2] Tyrosinase is the key enzyme in melanogenesis, initiating a cascade of reactions that convert tyrosine to melanin.[3] Tyrosinase catalyzes the rate-limiting steps of tyrosine hydroxylation to 3,4-dihydroxyphenyalanine (DOPA) and DOPA oxidation to dopaquinone. Dopaquinone spontaneously converts to dopachrome, and tyrosinase-related protein-2 (TRP-2) then converts dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA). Tyrosinase-related protein-1 (TRP-1) then catalyzes the oxidation of DHICA to indole-5,6-quinone-2-carboxylic acid to produce brown-black eumelanins.[4] Tyrosinase inhibitors have been clinically used for the treatment of some skin disorders associated with melanin hyperpigmentation and are also important in cosmetics for skin whitening effects. MITF is a key transcription factor for the regulation of tyrosinase gene expression as well as for the tissue-specific expression of tyrosinase.[5,6] MITF has been reported as an important regulator of melanogenesis, and mutations in the MITF gene cause abnormal pigmentation of skin and hair.

Melanin plays a crucial role in the absorption of free radicals generated within the cytoplasm and shielding the host from UV light; However, the over-production and accumulation of melanin in skin can be a serious problem resulting in a large number of skin disorders such as melasma, freckles, senile lentigines and sites of actinic damage.[7] Since the 1980s, the melanin biosynthetic pathways have been studied by skin cancer researchers, which have led to the development of whitening cosmetics and medicine. At that time, arbutin, kojic acid and its derivatives were developed.[8] Use of natural plant extracts is on the rise for the treatment of skin diseases and other medicinal purposes.[9]

*Vitex trifolia* L. (*V. trifolia*, Verbenaceae) grows widely through-out Southeast Asia, Korea, China, Micronesia, Australia and East Africa. The fruits of this plant are called “Viticis Fructus” and are used as a folk medicine for headaches, colds, migraine and eyepain.[10-15] *V. trifolia* has been reported to exhibit a broad range of pharmacological activities.[16-19] Previous phytochemical research has resulted in the isolation of diverse compounds such as flavonoids, sterols, and terpenoids.[20-25]

The composition of *V. trifolia* essential oil has been

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reported.[26,27] However, the effect of *V. trifolia* leaf oil on melanogenesis has not been examined. In this study, we checked the composition of *V. trifolia* leaf oil, confirmed melanogenesis inhibitory effect of the main component such as α-pinene, β-pinene, sabinene, 1,8-cineole. But we got unsatisfactory results. We tried to search a potent compound, and isolated abietatriene from *V. trifolia* which has melanogenesis inhibitory activity. Inhibitory effect of *V. trifolia* leaf oil and its active compound abietatriene on melanin biosynthesis and the protein and mRNA expression of melanogenic factors were evaluated by using B16F10 murine melanoma cells.

## Experimental

**General experimental procedures** – $^1$H NMR and $^{13}$C NMR spectra were recorded in $CDCl_3$ solution using Avance III 600 (600MHz) spectrometer (Bruker, Germany). EI-MS were collected on Clarus 600 GC/MS (PerkinElmer, U.S.A.). Column chromatography of silica gel used Kieselgel 60 (no. 9385, Merck, Germany) and TLC plate was used Keselgel 60F254 (Merck, Germany) or RP-18 254 precoated plate (Merck, Germany). B16F10 murine melanoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.) and all cell culture media and components were purchased from Gibco (Rockville, MD, U.S.A.). All chemicals were obtained from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Cell Counting Kit-8 (CCK-8) assay kit was purchased from Dojindo Laboratories (Kumamoto, Japan). Arbutin, BCA solution, and β-mercaptoethanol were from Sigma Chemical Co. (MO, U.S.A.). M-PER® (Mammalian protein extraction reagent), protease inhibitor cocktail were from Thermo Fisher Scientific (Waltham, MA, U.S.A.). Antibodies were obtained from Santa Cruz Biotechnology (CA, U.S.A.), and enhanced chemoluminescence (ECL) solution was purchased from Amersham (Piscataway, NJ, U.S.A.). Nitrocellulose membranes and SuperScript® III First-Strand were from Invitrogen (Carlsbad, CA, U.S.A.), and RNeasy Mini Kits were from Qiagen (Hilden, Germany).

**Preparation of Essential Oil from Leaf of *V. trifolia*** – *V. trifolia* leaf was collected from Gwangseung-ri waterfront on August 26, 2014 (Gochang-gun, Jeonbuk-do, Korea). These samples were identified by Yoon Ki Hong (Jeonbuk Agriculral Research & Extension Services). The samples (VT-140826-1) were air-dried and deposited in Herbal Experiment Station, Medicinal Resources Research Institute, Jeonbuk Agriculral Research & Extension Services (Namwon-si, Jeollabuk-do, Korea). They were put into a three-necked flask filled with hot water (1 kg) in a steam distillation apparatus and distilled for 4 h. *V. trifolia* leaf oil was obtained as the upper phase in the distilled fluid and dehydrated by adding sodium sulfate. Overall yield was about 0.81% (8.1 g)

**Gas chromatography - mass spectrometry** – Volatile substances were analyzed by GC-MS under the following method. A capillary DB-FFAP column (30 m × 0.25 mm; 0.25 μm) was equipped on a chromatogragh (Agilent 7693 GC) coupled to a mass spectrometer (Agilent 5975 mass selective detector). 1.0 μl sample was injected with a pulsed splitless mode (injector temperature 230 °C, injector pulse pressure 8.6 psi) on capillary column. The carrier gas (He) was flowed at the rate of 1.0 ml/min. The oven temperature was increased from 40 to 210 °C for 60 min. Mass spectrometry condition was as follows: full scan mode, mass range (40 - 550 amu). Each compound was identified by comparisons of the m/z value and relative abundance of molecular and fragment ion peaks, which were compared with the spectra of standard substances in the database.

**Isolation of Abietatriene** – *V. trifolia* leaf oil was chromatographed over Si gel (hexane-EtOAc gradient) to afford fractions 1 - 8. Fraction 1 (10 mg) separated over Si gel column with a hexane as the eluent to give compound **1** (1.4 mg).

**Abietatriene(1)** – White amorphous powder. $^1$H-NMR (600 MHz, $CDCl_3$): δ 7.17 (1H, d, $J$ = 8.4 Hz, H-11), 6.98 (1H, dd, $J$ = 8.4, 1.8 Hz, H-12), 6.89 (1H, br s, H-14), 2.92 (1H, dd, $J$ = 16.2, 6 Hz, H-7a), 2.87 (1H, m, H-7b), 2.83 (1H, m, H-15), 2.27 (1H, br d, $J$ = 12.6 Hz, H-1a), 1.87 (1H, dd, $J$ = 13.2, 7.8 Hz, H-6a), 1.75 (1H, m, H-2a), 1.71 (1H, m, H-6b), 1.59 (1H, m, H-2b), 1.47 (1H, d, $J$ = 13.2 Hz, H-3a), 1.39 (1H, td, $J$ = 12.9, 3.6 Hz, H-1b), 1.34 (1H, br d, $J$ = 12.6 Hz, H-5), 1.22 (6H, d, $J$ = 6.6 Hz, H-16, H-17), 1.18 (3H, s, H-20), 1.21 (1H, m, H-3b), 0.94 (3H, s, H-18), 0.93 (3H, s, H-19); $^{13}$C-NMR (150 MHz, $CDCl_3$): δ 147.6 (C-9), 145.4 (C-13), 134.9 (C-8), 126.8 (C-14), 124.3 (C-11), 123.8 (C-12), 50.4 (C-5), 41.7 (C-3), 38.9 (C-1), 37.5 (C-10), 33.4 (C-4, C-15), 33.3 (C-18), 30.5 (C-7), 24.9 (C-20), 24.0 (C-16, C-17), 21.6 (C-19), 19.3 (C-2), 19.1 (C-6). EIMS *m/z*: 270 $[M]^+$, 255 $[M-CH_3]^+$, 173, 159.

**Cells and Culture** – B16F10 murine melanoma cells (passages 2 - 4) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin A, and 100 U/ml streptomycin. Cells were cultured at 37 °C in a humidified atmosphere of 5% $CO_2$. B16F10 cells were grown to 60 to 80% confluence and then treated with *V. trifolia* leaf oil and abietatriene for 48 h in

serum-free DMEM.

**Determination of Cell Viability** – Cell viability was measured using the CCK-8 assay. B16F10 cells ($5 \times 10^4$ cells/well) were seeded in a 96-well plate and incubated for 24 h to allow the cells to attach and treated with various concentrations of *V. trifolia* leaf oil and abietatriene (1 - 100 μg/mL). After 48 h incubation, 10 μL of CCK-8 solution was added to each well of the plate and incubated for 2 h. Cell viability was determined by absorbance at 450 nm in a SPECTRA MAX 190 (Molecular devices, Sunnyvale, CA, U.S.A.). The percentage of cell viability was calculated as the absorbance of sample-treated cells divided by the absorbance of control cells. Cell viability of the control group was calculated as 100%.

**Measurement of Melanin Content** – The amount of melanin in B16F10 cells was measured according to a previously published method[28] with slight modification. The cells were treated with *V. trifolia* leaf oil and abietatriene for 48 h at 37 °C. After treatment, cell pellets were dried at 60 °C and dissolved in 1 N NaOH (10% DMSO) for 1 h at 60 °C. Spectrophotometric analysis of melanin content was performed at 400 nm absorbance. Each experiment was performed in triplicate.

**Western Blot Analysis** – Media were discarded and B16F10 cells were harvested with 1 mL of PBS using a cell scraper and collect the cells. Supernatant was discarded by centrifugation for 10 min at 10,000 rpm. Cells were lysed by M-PER® with protease inhibitor cocktail. The lysate protein concentrations were determined by the BCA assay. Equal amounts of protein extract (10 μg per lane) in each sample were loaded, separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature and then incubated overnight with specific primary antibodies against tyrosinase, TRP1, TRP2, MITF, and β-actin (dilution, 1:1000) at 4 °C. Bound antibodies were detected by horseradish peroxidase-conjugated secondary antibodies for 2 h. Proteins were detected by ECL solution and visualized with Fusion Fx 5 image system (Vilber Lourmat, France).

**Quantitative real time RT-RCR** – B16F10 cells treated for 24 h with *V. trifolia* leaf oil and abietatriene were washed with PBS and harvested, and total RNA was isolated by RNeasy Mini Kit. Extracted total RNA (500 ng) was converted to cDNA using SuperScript® III First-Strand. Gene expression was analyzed using StepOnePlus™ real-time PCR system (Applied Biosystems, U.S.A.). All reactions were performed in triplicate for 42 cycles. The relative fold expression levels of tyrosinase, TRP1, TRP2, and MITF were calculated. The threshold cycle (Ct) was determined and normalized to the average of housekeeping gene's (GAPDH) level (ΔCt). The ΔCt of chemical-treated cells were then subtracted from non-treated control cells (ΔΔCt), and the relative quantification in gene expression was determined using the 2-ΔΔCt method. [29] The results were expressed as % of control.

**Statistics** – Results are expressed as means ± SD of three independent experiments. The significance of differences between two independent groups was analyzed using the Student's t-test. For all tests, p-value < 0.05 was considered statistically significant.

## Result and Discussion

**GC-MS analysis** – Gas chromatogram of the essential oil isolated from leaf of *V. trifolia* is shown in supporting information (Fig. S3). The mass spectra of identified compounds were in good agreement with the mass spectra in database (Table 1). Less than 1% of the peak area and unidentified compounds were not included. Seven compounds were identified. In Table 1, mass spectra of the standard compounds that have been compared with each compound of the peak were noted as the chemical abstract number (CAS#). These are α-pinene (11.38%), α-pinene (2.84%), sabinene (10.25%), eucaluptol (8.60%), camphene (12.69%), Manoyl oxide (16.11%), abietatriene (9.03%).

**Identification of Abietatriene** – Compound **1**, called abietatriene, and obtained as a white amorphous powder, was concluded to have molecular formula $C_{20}H_{30}$ by the $^1$NMR spectra and by EIMS, which showed an $[M]^+$ ion peak at *m/z*: 270. The $^1$H NMR spectrum of **1** indicated signals due to five tertiary methyl groups at $\delta_H$ 1.22 (3H, d, $J$ = 6.6 Hz) × 2, 1.18 (3H, s), 0.94 (3H, s), 0.93 (3H, s) and a 1,2,4-trisubstituted benzene ring at $\delta_H$ 7.17 (d, $J$ = 8.4 Hz), 6.98 (dd, $J$ = 8.4, 1.8 Hz), 6.89 (br s). The $^{13}$C

**Table 1.** Main composition of the essential oil isolated from leaf of *V. trifolia*

| RT (min) | Compounds | Peak area (%) |
|---|---|---|
| 13.038 | α-pinene | 11.38 |
| 15.506 | β-pinene | 2.84 |
| 15.815 | sabinene | 10.25 |
| 18.256 | eucaluptol | 8.60 |
| 28.433 | camphene | 12.69 |
| 41.495 | Manoyl oxide | 16.11 |
| 50.807 | abietatriene | 9.03 |

NMR spectrum of **1** showed signals due to six $sp^2$ carbons at $\delta_C$ 147.6, 145.4, 134.9, 126.8, 124.3, 123.8, five methyl carbons at $\delta_C$ 33.3, 24.9, 24.0 × 2, 21.6, and two quaternary carbon at $\delta_C$ 37.5, 33.4. The $^1H$ and $^{13}C$ NMR signals were assigned with the aid of NMR techniques similar to those previously reported[30,31], and an abeoabietane-type diterpene, was characterized as shown in Fig. 1.

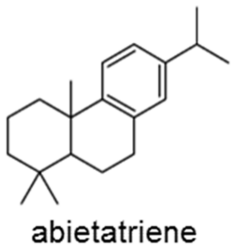


**Fig. 1.** Structure of Compound 1.

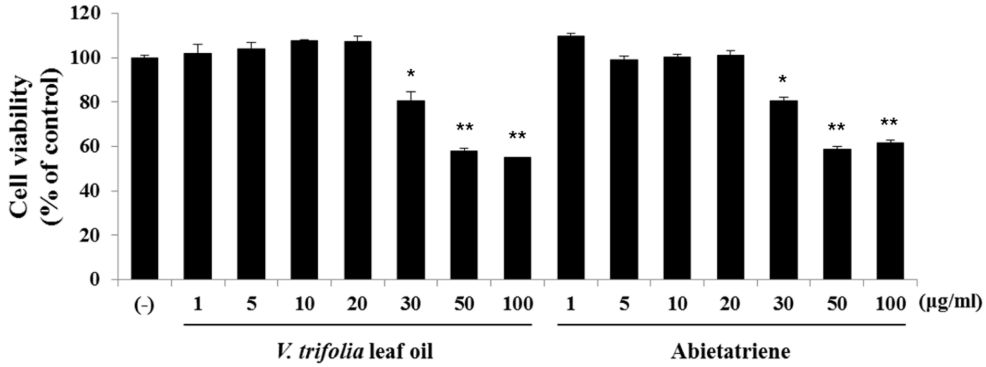


**Fig. 2.** The cell viability of *V. trifolia* leaf oil and abietatriene. B16F10 melanoma cells were treated with *V. trifolia* leaf oil and abietatriene (1 - 100 μg/mL) for 48 h. Cell viability was measured by CCK-8 assay. Data are expressed as mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$ compared with DMSO control (–).

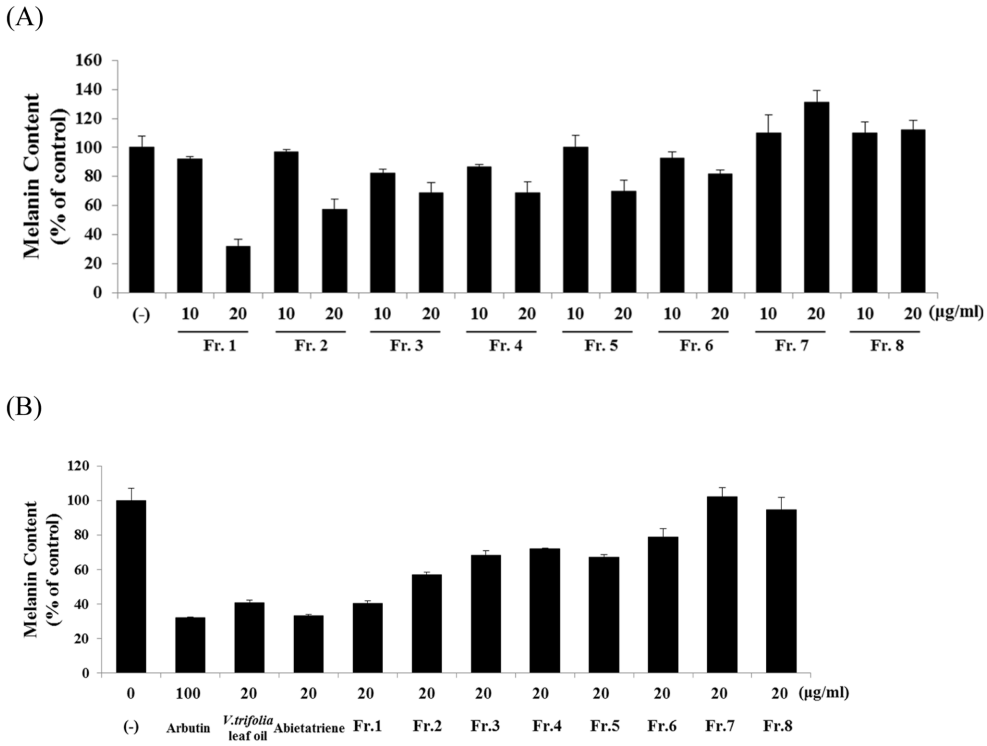


**Fig. 3.** Effect of *V. trifolia* leaf oil fractions 1 - 8 on melanin content. B16F10 cells were treated with *V. trifolia* leaf oil fractions 1 - 8, *V. trifolia* leaf oil and abietatriene or arbutin for 48 h. (A) Melanin content of *V. trifolia* leaf oil fractions 1 - 8 (10, 20 μg/mL). (B) Melanin content of *V. trifolia* leaf oil fractions 1 - 8, *V. trifolia* leaf oil and abietatriene 20 μg/mL or arbutin 100 μg/mL. Melanin content was determined by spectrophotometry. Data are expressed as % of DMSO control (–).

**Cytotoxicity of *V. trifolia* leaf oil and Abietatriene in B16F10 cells** – To investigate the cytotoxicity, B16F10 cells were treated with various concentrations of *V. trifolia* leaf oil and abietatriene. Neither *V. trifolia* leaf oil nor

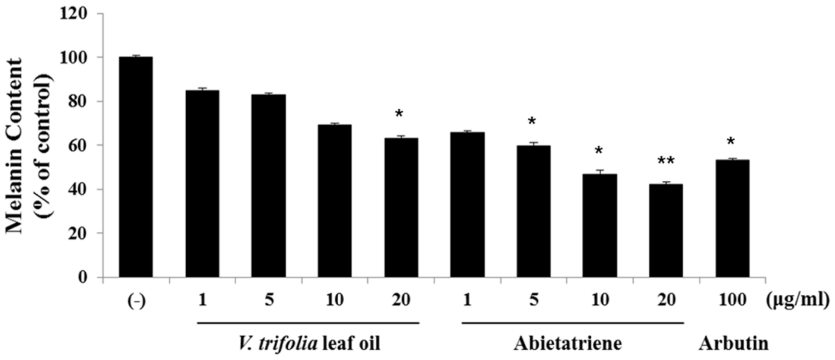


**Fig. 4.** Effect of *V. trifolia* leaf oil and abietatriene on melanin content. B16F10 cells were treated with *V. trifolia* leaf oil and abietatriene (1 - 20 μg/mL) or arbutin 100 μg/mL for 48 h. Melanin content was determined by spectrophotometry. Data are expressed as % of control (DMSO) and expressed as mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$ compared with DMSO control (–).

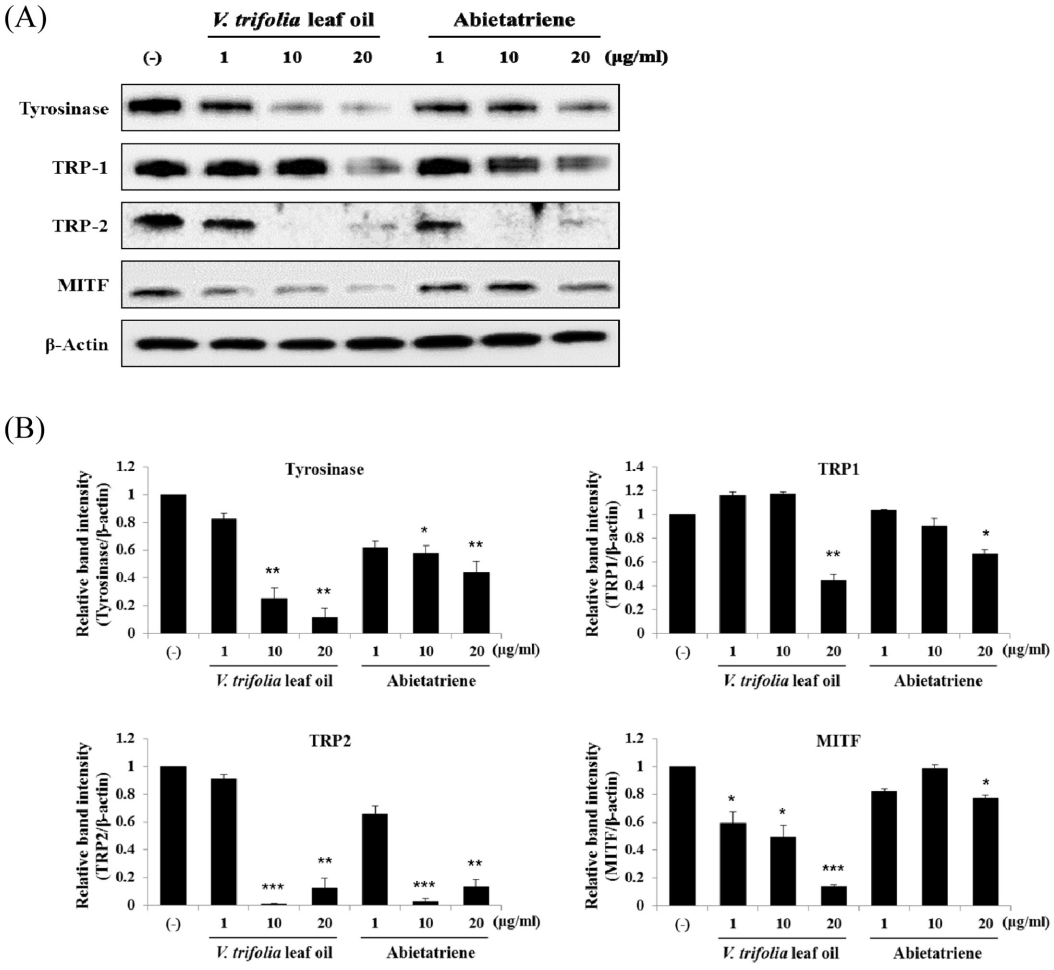


**Fig. 5.** Effect of *V. trifolia* leaf oil and abietatriene on the protein expression of melanogenic factors. B16F10 cells were treated with *V. trifolia* leaf oil and abietatriene (1, 10, and 20 μg/mL) and incubated for 48 h. Cell lysates were evaluated by Western blot with antibodies against tyrosinase, TRP-1, TRP-2, and MITF. Equal protein loading was confirmed by staining with antibodies against â-actin. (A) The protein expression of melanogenic proteins. (B) Relative protein expression of each melanogenic proteins. Band intensity was quantified using ImageJ software. The results are expressed as % of control and each column represents the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$ compared with DMSO control (–).

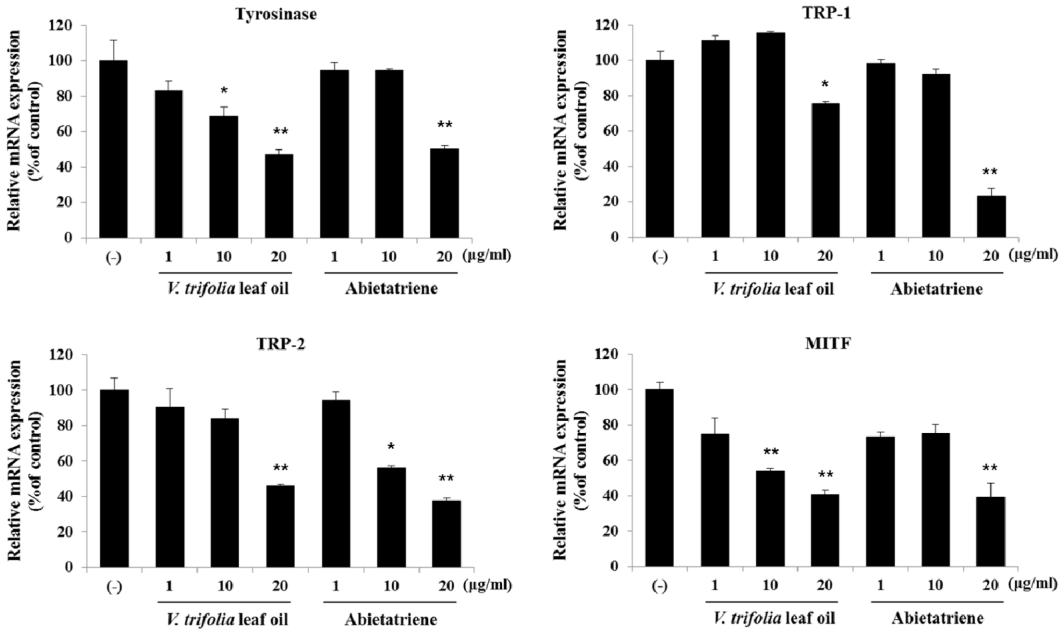


**Fig. 6.** Effect of *V. trifolia* leaf oil and abietatriene on the mRNA expression of melanogenic factors. B16F10 cells were treated with *V. trifolia* leaf oil and abietatriene (1, 10 and 20 μg/mL) and incubated for 24 h. Cell lysates were evaluated by qPCR with Taqman probe against tyrosinase, TRP-1, TRP-2, and MITF. Equal mRNA loading was confirmed by GAPDH. The results are expressed as % of control, the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$ compared with DMSO control (–).

abietatriene exhibited any cytotoxicity effects at the concentrations ranging from 1 - 20 μg/mL (Fig. 2). Thus, 1 - 20 μg/mL of *V. trifolia* leaf oil and abietatriene were used for the experiments.

**Effect of *V. trifolia* leaf oil and Abietatriene on melanogenesis** – Melanin is end-product of a cascade of melanogenesis. Melanin content was measured in B16F10 cells to examine the effect of *V. trifolia* leaf oil fractions 1-8 on melanogenesis. The melanin content of cells was decreased in a dose-dependent manner. *V. trifolia* leaf oil fraction 1-treated cells each showed 37% in melanin content at a concentration of 20 μg/mL (Fig. 3). After isolation of abietatriene from fraction 1, melanin content of *V. trifolia* leaf oil and abietatriene were also determined. Arbutin was used as a positive control. As shown in Fig. 4, the melanin content of cells was decreased in a dose-dependent manner. *V. trifolia* leaf oil and abietatriene-treated cells showed 37% and 47% decrease in melanin content, respectively, at a concentration of 20 μg/mL.

**Effect of *V. trifolia* leaf oil and Abietatriene on the protein expression of melanogenic factors** – Tyrosinase, and tyrosinase related protein TRP-1 and TRP-2 are known to regulate the melanogenesis. MITF is a master transcription factor that regulates melanogenesis. To investigate the effects of *V. trifolia* leaf oil and abietatriene on the expression of these melanogenic proteins, Western blot analysis was done on to these proteins after treatment with 1, 10, and 20 μg/mL for 48 h on B16F10 melanocytes. *V. trifolia* leaf oil and abietatriene both decreased protein levels of tyrosinase, TRP-1, TRP-2 and MITF in a dose-dependent manner (Fig. 5). These results indicate that both *V. trifolia* leaf oil and abietatriene can contribute to inhibited melanin biosynthesis by regulating the expression of tyrosinase, TRP-1, TRP-2, and MITF.

**Effect of *V. trifolia* leaf oil and Abietatriene on the mRNA expression of melanogenic factors** – To investigate change in protein level, mRNA expression of melanogenic factors, such as tyrosinase, TRP-1, TRP-2, and MITF were analyzed after treatment with *V. trifolia* leaf oil and abietatriene on B16F10 cells using quantitative RT-PCR. The treatment of 20 μg/mL *V. trifolia* leaf oil decreased the mRNA levels of tyrosinase, TRP-1, TRP-2, and MITF by 53, 24, 54, and 59%, respectively. Together, abietatriene 20 μg/mL decreased each factor by 50, 77, 63, and 61%, respectively, compared to control (Fig. 6).

## Conclusion

The skin forms an effective barrier between the organism and the external environment, and relies on melanocytes to provide photo-protection and thermo-regulation by producing melanin in the outer skin layer.[32]

Melanocytes subsequently migrate throughout the developing organism to the skin, eyes, and the hair bulbs. Almost-normal pigmentation is due to varying amounts, types, and distribution of melanin throughout the epidermis. Therefore, the biosynthesis of the melanin in the human epidermis presents a challenging field of interest to scientists and physicians as they try to understand skin phenotypes. Melanin plays a crucial role in protecting humans from harmful UV radiation; However the over-production and accumulation of melanin in skin may cause various skin disorders, such as melasma, freckles, senile lentigines and sites of actinic damage. Therefore, the use of skin-whitening agents has been rising consistently. The melanin biosynthetic pathways have been studied by skin cancer researchers, which had led to the development of whitening cosmetics and medicines. Numerous pharmacologic and cosmeceutic agents that inhibit tyrosinase or other melanogenic pathways have been reported.

In this study, we analyzed and evaluated the composition of *V. trifolia* leaf oil and potent anti-melanogenesis compound abietatriene was isolated from *V. trifolia* leaf oil. The activity of *V. trifolia* leaf oil and its active compound abietatriene in depigmentation was investigated using *in vitro* assays. *V. trifolia* leaf oil and abietatriene reduced the melanin content dose-dependently in B16F10 cells at non-cytotoxic concentration. In Western blot analysis, *V. trifolia* leaf oil and abietatriene decreased the protein expression of melanogenesis-related proteins, such as tyrosinase, TRP-1, TRP-2, and MITF. These results indicate that *V. trifolia* leaf oil and abietatriene reduce melanin synthesis in B16F10 cells through down-regulating the melanogenic proteins, such as tyrosinase, TRP-1, TRP-2 and MITF by regulating the expression of mRNA and protein. The present study suggests that *V. trifolia* leaf oil and abietatriene could be used as a natural anti-melanogenesis and anti-hyperpigmentation agent.

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Received March 30, 2016
Revised June 14, 2016
Accepted June 18, 2016