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Research Note

# Comparative Depigmentation Effects of Resveratrol and Its Two Methyl Analogues in α-Melanocyte Stimulating Hormone-Triggered B16/F10 Murine Melanoma Cells

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**ABSTRACT:** Previous research showed that resveratrol (*trans*-3,4',5-trihydroxystilbene) and pinostilbene (*trans*-3-methoxy-4',5-dihydroxystilbene) were able to inhibit tyrosinase directly; however, anti-melanogenic effects of pterostilbene (*trans*-3,5-dimethoxy-4'-hydroxystilbene) and resveratrol trimethyl ether (RTE) have not been compared. To investigate the hypopigmentation effects of pterostilbene and RTE, melanin contents and intracellular tyrosinase activity were determined by western blot analysis. Firstly, pterostilbene showed the inhibitory effects on α-melanocyte stimulating hormone (MSH)-induced melanin synthesis stronger than RTE, resveratrol, and arbutin. Pterostilbene inhibited melanin biosynthesis in a dose-dependent manner in α-MSH-stimulated B16/F10 murine melanoma cells. Specifically, melanin content and intracellular tyrosinase activity were inhibited by 63% and 58%, respectively, in response to treatment with 10 μM of pterostilbene. The results of western blot analysis indicated that pterostilbene induced downregulation of tyrosinase protein expression and suppression of α-MSH-stimulated melan-A protein expression stronger than RTE or resveratrol. Based on these results, our study suggests that pterostilbene can induce hypopigmentation effects more effectively than resveratrol and RTE, and it functions via downregulation of protein expression associated with hyperpigmentation in α-MSH-triggered B16/F10 murine melanoma cells.

**Keywords:** pterostilbene, methyl resveratrol, depigmentation, tyrosinase, melan-A

## INTRODUCTION

Acquired hyperpigmentation disorder is caused by external stimuli, including ultraviolet ray (UVR)-induced pigmentation, postinflammatory pigmentation, chemical/drug-induced pigmentation, and foreign material deposition (1). The anti-melanogenic process against hyperpigmentation can be accomplished by suppressing the transcription and activity of tyrosinase, tyrosinase related protein (TRP)-1, TRP-2, and/or sustained extracellular signal-regulated kinase (ERK) 1/2 by inhibiting related signaling pathways. Tyrosinase and TRP-1 are indispensable enzymes involved in eumelanogenesis. Hypopigmentation is caused by inhibition of the uptake and distribution of melanosomes in keratinocytes, which takes place through induction of melanin and melanosome degradation or expeditious turnover of pigmented keratinocytes. Most hypopigmentation agents act specifically to impede the function of tyrosinase via several mechanisms. For instance, hydroquinone and arbutin work as competitive inhibitors, $C_2$-ceramide and tretionin inhibit through blocking transcription, and linoleic acid and α-linolenic acid act by degrading tyrosinase (2). Additionally, the extracts of several plants and algae have been reported to suppress α-melanocyte stimulating hormone (MSH)-stimulated melanogenesis through sustained ERK 1/2 activation (3-6). There have been several investigations on anti-melanogenesis of methylated compounds, such as diosgenin, α-tocopheryl ferulic acid, and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (7-9). Moreover, other reports show that melanogenesis is stimulated by methoxylated compounds, such as nobiletin, tangeretin, sinensetin, ferulic acid, and scoparone (10-15).

Resveratrol (*trans*-3,4',5-trihydroxystilbene), a non-flavonoid polyphenol in the stilbene group, has been reported in various food sources, such as grapes, berries, red wine, chocolate, and peanuts (16-18). The di- and trimethylated anaolgues of resveratol, pterostilbene (*trans*-

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3,5-dimethoxy-4'-hydroxystilbene) and resveratrol trimethyl ether (RTE; *trans*-3,4',5-trimethoxystilbene) are stilbenoids shown to have higher bioavailability than resveratrol which are found in grapes, blueberries and heartwood (*Pterocarpus marsupium*) (19). Pterostilbene has been shown to exert anti-inflammatory, anti-proliferative, and anti-aging effects (20-23) and RTE showed anti-proliferative and/or apoptosis-inductive effects in various cancer cells with potency relatively higher than resveratrol (24-30). Our study shows that the differential hypopigmentation effects of resveratrol and its two methyl analogues can be associated with expression of melan-A and tyrosinase protein in α-MSH-triggered B16/F10 melanoma cells.

## MATERIALS AND METHODS

### Materials

All solvents were of analytical grade and used without further purification. α-MSH, arbutin, pterostilbene, resveratrol, RTE, and 3,4-dihydroxy-L-phenylalanine (L-DOPA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Arbutin was dissolved in 50% ethanol at a concentration of 360 mM and pterostilbene, resveratrol, and RTE were dissolved in dimethyl sulfoxide at a concentration of 10 mM. These compounds were used for an *in vitro* assay.

### Antibodies

Antibodies against tyrosinase (M-19, sc-7834-R), melan-A (A103, sc-20032) were purchased from Santa Cruz Biotech. (Santa Cruz, CA, USA). Antibody against β-actin (A2228-0.1) was obtained from Sigma-Aldrich Co..

### Cell culture

B16/F10 murine melanoma cells were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco BRL), 1% penicillin-streptomycin (10,000 U/mL and 10,000 μg/mL, Gibco BRL). Cells were maintained in a humid atmosphere of 5% $CO_2$ at 37°C.

### Assessment of cytotoxicity

Cytotoxicity was determined by a lactate dehydrogenase (LDH) release assay. The cytotoxic effects of RTE or arbutin in the presence of α-MSH were estimated by the measurement of LDH in culture media. Leakage of LDH is a well-known marker of damage to the cellular membrane. The cytotoxicity was expressed as the percentage of LDH released (LDH release in media of RTE or arbutin treatment in the presence of α-MSH/maximal LDH release×100). Maximal LDH release was measured after lysis of cells with 0.5% Triton X-100.

### Determination of intracellular melanin contents and tyrosinase activity

The cells were seeded into 6 well plates at a density of $1\times10^5$ cells/well. The cells were then treated with or without α-MSH and test compounds at 37°C for 2 days. The cells were then washed with 1× phosphate buffered saline and then collected in 1× trypsin-ethylenediaminetetraacetic acid (EDTA), after which they were lysed with 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 1% Triton X-100 in 67 mM sodium phosphate buffer (pH 6.8). The samples were sonicated and centrifuged at 12,000 rpm for 15 min at 4°C and the supernatants were transfered into new eppendorf tube to measure intracellular tyrosinase activity, and the remaining pellets were used to determine melanin. To extract the melanin from the pellets, 1 N sodium hydroxide (NaOH) was added to the pellets, which was subsequently incubated at 70°C for 30 min. The absorbance was then measured at 405 nm and the corresponding total protein was determined and used to normalize the absorbance. The tyrosinase activity was determined based on the amount of DOPA chrome produced in response to the use of various substrates, including L-tyrosine and L-DOPA. To assess this, 100 μL of supernatants and 100 μL of 12.5 mM L-DOPA were then mixed and incubated at 37°C for 30 min. The absorbance was then measured at 475 nm and the corresponding total protein was determined and used to normalize the absorbance.

### Western blot analysis

Cells were collected and lysed in 1× radio immunoprecipitation assay (RIPA) buffer [10× RIPA lysis buffer (Upstate, Boston, MA, USA), 0.1 mM PMSF, 0.1 M $Na_3VO_4$, 0.5 M NaF, 5 mg/mL aportinin, and 5 mg/mL leupeptin]. Thirty micrograms of protein per lane were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently blotted onto nitrocellulose membranes. The nitrocellulose membranes were then blocked with 5% dried milk in tris-buffered saline containing 0.05% Tween 20. Next, the blots were incubated with primary antibodies at a dilution of 1:1,000 and then further incubated with horseradish peroxidase-conjugated secondary antibody. The bound antibodies were then detected using an enhanced chemiluminescence kit (Amersham Cat. No. RPN2106V2, Amersham Life Science, Arlington Heights, IL, USA).

### Statistical analysis

All experiments were performed in triplicate. Treatment effects were analyzed using the Student's *t*-test. $P<0.05$ was considered to be statistically significant.

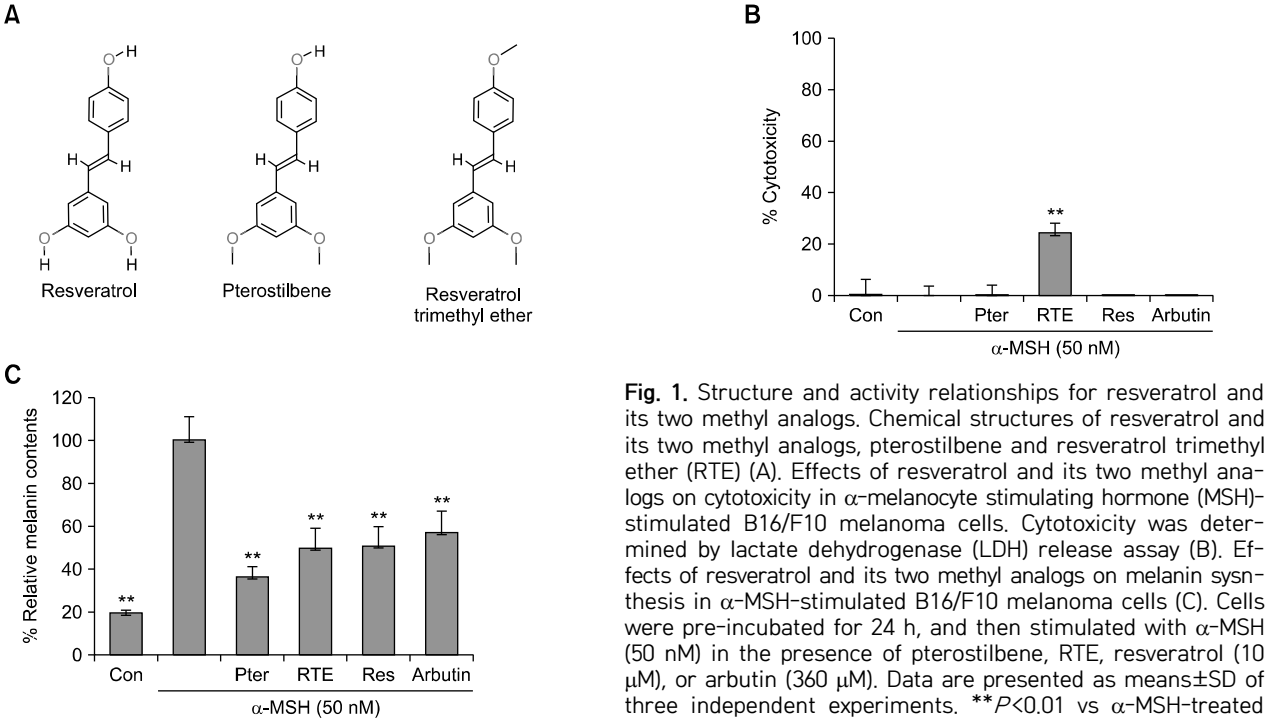


**Fig. 1.** Structure and activity relationships for resveratrol and its two methyl analogs. Chemical structures of resveratrol and its two methyl analogs, pterostilbene and resveratrol trimethyl ether (RTE) (A). Effects of resveratrol and its two methyl analogs on cytotoxicity in α-melanocyte stimulating hormone (MSH)-stimulated B16/F10 melanoma cells. Cytotoxicity was determined by lactate dehydrogenase (LDH) release assay (B). Effects of resveratrol and its two methyl analogs on melanin sysnthesis in α-MSH-stimulated B16/F10 melanoma cells (C). Cells were pre-incubated for 24 h, and then stimulated with α-MSH (50 nM) in the presence of pterostilbene, RTE, resveratrol (10 μM), or arbutin (360 μM). Data are presented as means±SD of three independent experiments. $^{**}P<0.01$ vs α-MSH-treated cells.

## RESULTS AND DISCUSSION

Previous research reported that the anti-melanogenic mechanism of oxyresveratrol suppresses tyrosinase in a noncompetitive manner with L-tyrosine as the substrate (31). In addition, resveratrol and pinostilbene (*trans*-3-methoxy-4',5-dihydroxystilbene) have been shown to exert inhibitory effects against tyrosinase, while pterostilbene and RTE did not suppress tyrosinase directly (31). To investigate the mechanism of action on hypopigmenting effects of pterostilbene and RTE in α-MSH-stimulated B16/F10 melanoma, cells were incubated with pterostilbene, RTE, resveratrol, or arbutin in the presence of α-MSH at indicated concentrations for 48 h (Fig. 1A). Arbutin was used as a reference. Treatment with 10 μM pterostibene or resveratrol for 48 h did not affect cytotoxicity in α-MSH-stimulated B16/F10 melanoma cells; however, treatment with 10 μM of RTE for 48 h induced cytotoxicity (Fig. 1B). Pterostilbene showed a greater suppressive effect on melanin biosynthesis than RTE, resveratrol, or arbutin (Fig. 1C), and these effects occurred in a dose-dependent manner, with up to 63% of the amount of melanin (Fig. 2A) and 58% of the tyrosinase activity being inhibited in response to treatment with 10 μM of pterostilbene (Fig. 2B). These results show that pterostilbene suppressed melanin synthesis via inhibition of nonspecific tyrosinase, meanwhile, resveratrol and arbutin suppressed melanin synthesis in a tyrosinase-specific manner.

To examine the relationship between proteins associa-

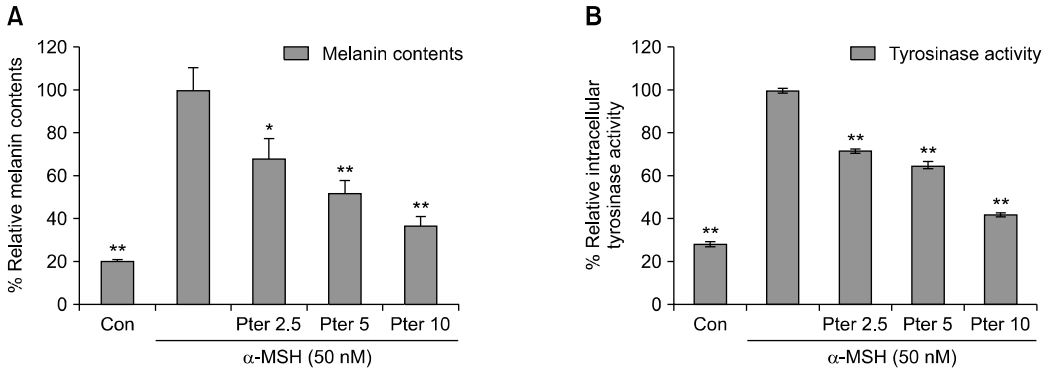


**Fig. 2.** Effects of pterostilbene on melanin sysnthesis (A) and intracellular tyrosinase activity (B) in α-melanocyte stimulating hormone (MSH)-stimulated B16/F10 melanoma cells. Cells were pre-incubated for 24 h, and then stimulated with α-MSH (50 nM) in the presence of pterostilbene (2.5, 5, and 10 μM). Data are presented as mean±SD of three independent experiments. $^{*}P<0.05$ and $^{**}P<0.01$ vs α-MSH-treated cells.

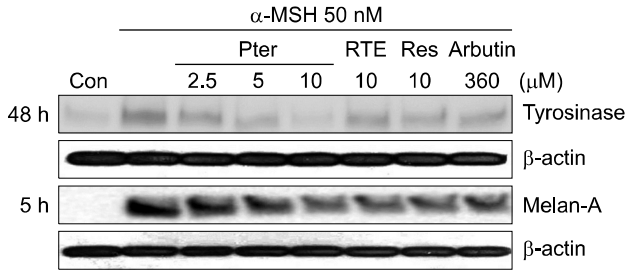


**Fig. 3.** Effects of resveratrol and its two methyl analogs on melanogenic proteins, tyrosinase and melan-A protein levels in α-melanocyte stimulating hormone (MSH)-stimulated B16/F10 melanoma cells. Cells were pre-incubated for 24 h, and then stimulated with α-MSH (50 nM) in the presence of pterostilbene (2.5, 5, and 10 μM), RTE (10 μM), resveratrol (10 μM), or arbutin (360 μM) for 5 h and 48 h. The protein levels of tyrosinanse, melan-A, and β-actin were determined by Western blotting. β-Actin was used as a loading control.

ted with melanogenesis in α-MSH-induced B16/F10 murine melanoma, the cells were treated with resveratrol, pterostilbene or RTE in the presence of α-MSH for 5 h or 48 h. The total protein was then isolated and subjected to Western blot analysis. The examination of the protein expression revealed that pterostilbene suppressed tyrosinase protein expression, which is associated with eumelanogenesis, and downregulated melan-A protein expression, which is associated with α-MSH-induced differentiation of B16/F10 murine melanoma cells (Fig. 3). Previous studies have reported that protein melan-A also known as melanoma antigen recognized by T cells 1 regulated melanosomal matrix protein Pmel17 processing and the maturation of melanosomes (32). Moreover, recent reports have shown that melanin biosynthesis is induced by methoxylated compounds, such as nobiletin, tangenretin, sinensetin, 4'-*O*-methylfisetin, scoparone, ferulic acid, while suppressed by diosgenin, α-tocopheryl ferulate, and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (8-15,33,34). In the case of scoparone and ferulic acid, addition of methoxy groups to original compounds (coumarin and *p*-coumaric acid, respectively) is responsible for stimulating melanogenesis in B16 melanoma cells (13,15).

Taken together, this study suggests that pterostilbene can ameliorate acquired hyperpigmentation disorders without cytotoxic effects, and functions via downregulation of tyrosinase protein expression and inhibition of melan-A protein expression in α-MSH-triggered B16/F10 melanoma cells. Furthermore, this research also suggests that pterostilbene can regulate α-MSH-induced melanogenic gene expression more efficiently than resveratrol and RTE.

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## AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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