Natural Product Sciences
19(3) : 258-262 (2013)

Inhibition of Melanin Production and Tyrosinase Expression of Ergosterol Derivatives from *Phellinus pini*

Yun Jung Hong, A Reum Jang, and Ki Sook Yang*

College of Pharmacy, Sookmyung Women's University, Seoul 140-742, Korea

Abstract – Three ergosterol derivatives, ergosta-4,6,8(14),22-tetraen-3-one (**1**), ergosta-7,24(28)-dien-3-ol (**2**), and 5,8-epidioxyergosta-6,22-dien-3-ol(**3**) were isolated from the fruit body of *Phellinus pini*. Their structures were based on spectroscopic methods including IR, MS, and NMR (1D and 2D). These compounds were evaluated for their activity to decrease melanin production in α-MSH (melanocyte stimulating hormone) activated B16F10 cells. Compound **1**, **2**, and **3** reduced melanin content in a dose-dependent manner at concentrations of 5~15 uM. They also suppressed the tyrosinase expression of protein and m-RNA level dose dependently by western blot analysis and RT-PCR experiment in B16F10 murine melanoma cells.
Key words – *Phellinus pini,* Ergosterol derivatives, B16F10 cells, Melanin content, Tyrosinase

Introduction

Phellinus pini Ames (Hymenocaetaceae) is a white-rot fungus that frutifies over the stems of Pinaceae, Cupressaceae etc. This mushroom has been known for its immunomodulating (Jeong *et al*., 2004), hypolipidemic (Yang *et al*., 2002), and anti-inflammatory (Jang and Yang, 2011; Hong *et al*., 2012) activities. The polysaccharide fraction of this mushroom has antitumor and immunostimulating activities (Ikegawa *et al.,* 1968). Chemical compositions such as ergosterol, p-hydroxybenzoic acid, vanillic acid were isolated (Epimenko and Ageenkova, 1965). 2-Farnesyl-5-methylbenzoquinone and 2,4,6-triphenylhex-1-ene were isolated from the fungus of *Phellinus pini* and had a antifungal activities (Ayer *et al*., 1996). Two new ceramides were identified among the chemical constituents of the fungus *Phellinus pini* (Lourenço *et al*., 1996). We determined subacute oral toxicity of the methanol extract from *Phellinus pini* in rat (Hong *et al*., 2011), and isolated ergosta-4,6,8(14),22-tetraen-3-one (**1**) (ETO), ergosta-7,24(28)-dien-3-ol (**2**) (EDO), and 5,8-Epidioxyergosta-6,22-dien-3-ol (**3**) (EPO) from the hexane part of *Phellinus pini*, and investigated inhibition of nitric oxide production, iNOS and COX-2 expression of these ergosterol derivatives from *Phellinus pini* (Hong *et al*., 2012). Although these various activities were reported, whitening effect has not been studied. To elucidate the potential whitening agent from natural sources, we evaluated the inhibitory activities on melanin production in α-MSH activated B16F10 cells and tyrosinase expressions of protein and m-RNA level.

*Author for correspondence
Ki Sook Yang, College of Pharmacy, Sookmyung Women's University, Seoul 140-742, Korea
Tel: +82-2-710-9578; E-mail: ksyang@sookmyung.ac.kr

Experimental

Materials – We isolated ergosta-4,6,8(14),22-tetraen-3-one (**1**) (ETO) (Yuan *et al*., 2003; Gao *et al*., 2003; Liu *et al*., 2005; Lee *et al*., 2005), ergosta-7,24(28)-dien-3-ol (**2**) (EDO) (Morris *et al*., 1974), and 5,8-Epidioxyergosta-6,22-dien-3-ol (**3**) (EPO) (Sheffer *et al*., 1986; Takaishi *et al*., 1992; Sgarbi *et al*., 1997) from the hexane part by column chromatography. The extraction and isolation procedure and the spectral data were published in the previous article (Hong *et al*., 2012).

Cell culture – B16F10 murine melanoma cells were purchased from ATCC. The cells were cultured in DMEM containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, 100 ug/mL streptomycin and 0.25 ug/mL amphotericin. Cells were maintained at 37 ºC under 5% CO_2 in a fully humidified atmosphere.

MTT assay for viability – B16F10 cells were seeded at 1.5×10^4 cells/mL in 48-well plates and incubated for 24 h, then treated with varying concentrations of compounds **1, 2,** and **3**. The cells were incubated for 48 h and then moved to fresh medium containing 0.5 mg/mL

of MTT. The incubation continued for an additional 4 h at 37 ºC. The medium was removed and the MTT-formazan was dissolved in 400 uL of DMSO. The extent of the reduction of MTT to formazan within the cells was quantified by measuring the absorbance of the DMSO solution at 570 nm using an ELISA reader (Mosmann, 1983). Cytotoxicity was calculated as the reduction in cell viability.

Measurement of melanin content – B16F10 cells were plated at a density of 1×10^5 cells/mL in 6-well cell culture plates. Cells were incubated for 24 h then treated with various concentrations of compounds in the presence and absence of 0.4 uM α-MSH for 48 h. Cells were harvested and washed twice with PBS, and then incubated in 1 N NaOH with 10% DMSO for 1 h at 70 ºC and centrifuged at 12000 rpm for 30 min. Optical density of the supernatant was measured at 405 nm using ELISA plate reader.

Assay of tyrosinase activity – B16F10 cells (1×10^5 cells/mL) were incubated for 24 h then treated with various concentrations of compounds in the presence and absence of 0.4 uM α-MSH for 48 h. Cells were harvested and washed twice with PBS, and then lysed with RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxychlolate, 0.1% SDS) and centrifuged at 12000 rpm for 30 min. After quantifying protein levels, the supernatants were mixed with 100 uL of freshly prepared enzyme solution [(0.1% L-DOPA in 0.1 M sodium phosphate buffer (pH 6.8)], then incubated at 37 ºC for 2 h. The absorbance was measured by using a microplate reader at 490 nm.

Tyrosinase zymography – Cell lysates were prepared from B16F10 cells treated with test samples after 2 days of treatment, as described above. The lysates containing 50 ug of protein were mixed with loading buffer without β-mercaptoethanol and separated in SDS-polyacrylamide gel without a boiling step. After electrophoresis, the gel1s were washed with 0.1 M sodium phosphate buffer (pH 6.8) for 30 min 4 times at room temperature and then incubated in 10 mM L-DOPA solution for 2 h at room temperature.

Western blot analysis – B16F10 cells were seeded at 1×10^5 cells/mL in 60 mm cell culture plates containing 4 mL of culture medium. Cells were incubated for 24 h. and then treated with 0.4 uM α-MSH with various concentrations of test samples for 48 h. The cells were rinsed with PBS and lysed with iced-lysis buffer (Complete Lysis-B(2X), Roche) for 1 h and then centrifuged at 12000 rpm for 20 min. The lysates were analyzed protein contents quantified using the Bradford method, and boiled for 5 min at 95 ºC.

Table 1. Sequences of tyrosinase and β-actin primers

Gene	Primer sequence
Tyrosinase	
Forward	CGC CAG CTT TCA GGC AGA GGT
Reverse	TGG TGC TTC ATG GGC AAA ATC
β-actin	
Forward	ACC GTG AAA AGA TGA CCC AG
Reverse	TAC GGA TGT CAA CGT CAC AC

Total cell lysates (20 ug) were applied on 10% SDS-PAGE and transferred to PVDF membranes. The membranes were probed with anti-rabbit tyrosinase (Santacruz, USA) for 1 h and incubated with a horseradish peroxidase conjugated anti-IgG in blocking buffer for 1 h. The blots were visualized using an enhanced chemiluminescence (ECL) detection kit (Amersham Bio-science, England) according to the manufacturer's instruction.

Reverse transcriptase and polymerase chain reaction (RT-PCR) – B16F10 cells were seeded (1×10^6 cells/mL) in 100 mm cell culture plates containing 10 mL of culture medium. Cells were treated with test samples together with 0.4 uM α-MSH. The RNA was isolated with RNA isoplus™ (Takara Biotechnology, Japan) and each RNA extract (2 ug) was reverse transcribed into cDNA using superscript II reverse transcriptase (Gendepot, USA). PCR was performed in 20 uL of a solution containing Top-Taq™ PreMix (CoreBio System, Korea). The primer sequences are listed in Table 1. PCR was performed for 25 amplification cycles in a DNA thermal cycler (TaKaRa PCR Thermal Cycler, Japan). PCR products were separated by electrophoresis on 1.2% agarose gels and visualized using ethidinium bromide staining.

Statistical analysis – All data were presented as the mean value ± standard deviation (S.D.) from three independent experiments. Significant differences between the control and the experimental groups were assessed by the Student's t-test. Results were considered significant at $p < 0.01$.

Results and Discussion

Melanogenesis protects the skin against UV radiation through the production of melanin in melanocytes (Seiberg *et al*., 2000; Hearing, 2005). But, excessive synthesis of melanin may cause various hyperpigmentation disorders. Melanogenesis consists of many enzymatic oxidation steps such as tyrosinase, tyrosinase related protein-1 (TRP-1), and dihydroxyphenylalanine chrome tautomerase (TRP-2) (Chang, 2009; Yamaguchi *et al*., 2009). Tyrosinase is the key regulatory enzyme involved

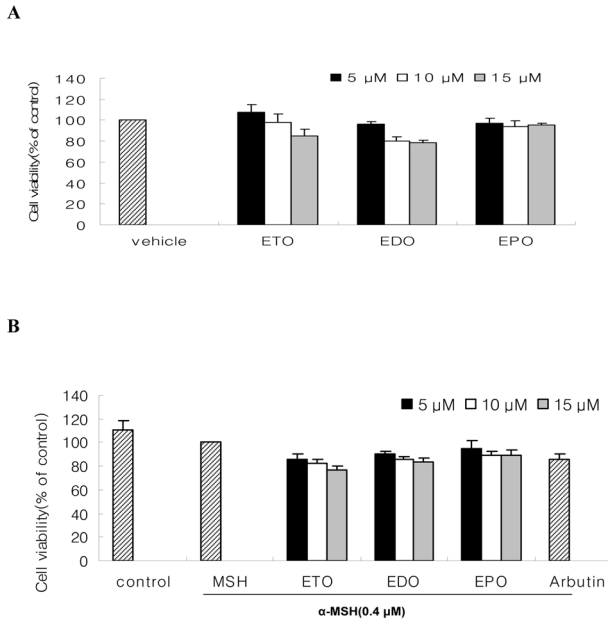

Fig. 1. Effects of ETO, EDO, EPO from *Phellinus pini* on the viability of B16F10 melanoma cells without (A) or with (B) α-MSH (0.4 μM). B16F10 cells (1.5×10^4 cells/mL) were incubated 24 hours in DMEM containing 10% FBS and were treated for 48 hours. Results are expressed as percentage of control. Values are means ± SD and were obtained from three different experiments. (ETO: ergosta-4,6,8(14),22-tetraen-3-one, EDO: ergosta-7,24(28)-dien-3-ol, EPO: ergosterol peroxide).

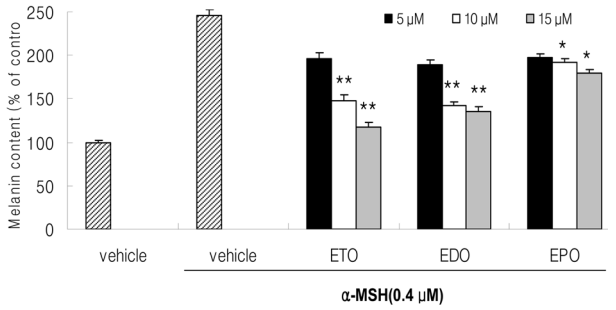

Fig. 2. Melanin content of B16F10 melanoma cells after treatment with ETO, EDO, EPO (5 - 15 μM) and α-MSH (0.4 μM). Cells were seeded at 1×10^5 cells/mL. After 24 hours, cells were treated with various concentrations of compounds (5 - 15 μM) and α-MSH (0.4 μM) and were cultured for 48 hours. Then, melanin content was measured at 490 nm. Each value represents the mean ± S.D of three experiments. *Indicates a significant difference from control, $*p < 0.01$, $**p < 0.001$. (ETO: ergosta-4,6,8(14),22-tetraen-3-one, EDO: ergosta-7,24(28)-dien-3-ol, EPO: ergosterol peroxide).

in the biosynthesis of the melanin (Draelos, 2007). It was affected by hormones (alpha-MSH, IBMX, forskolin), UV, and inflammatory cytokines (Busca *et al.*, 2000).

Several whitening agents from natural sources have been reported that those products inhibit melanin synthesis

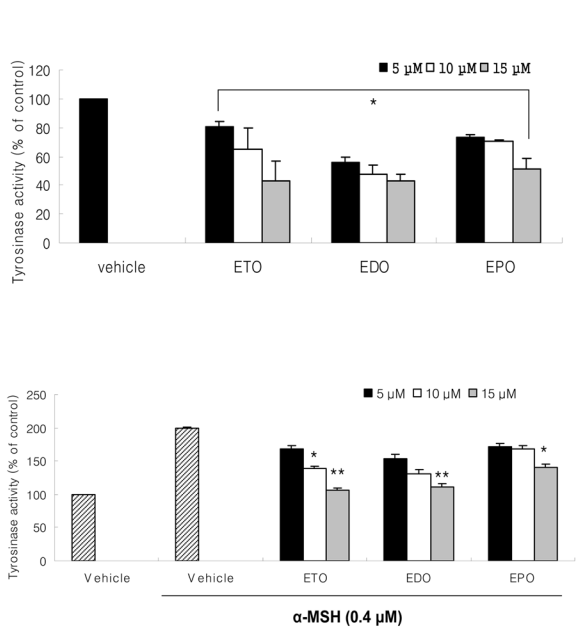

Fig. 3. Inhibitory effects of ETO, EDO, EPO on tyrosinase activity in B16F10 melanoma cells without (A) or with (B) α-MSH (0.4 μM). Cells were seeded at 1×10^5 cells/mL. After 24 hours, cells were treated with various concentrations of compounds 1 - 3 (5 - 15 μM) without (A) or with (B) α-MSH (0.4 μM) and were cultured for 48 hours. Then, tyrosinase activity was measured at 490 nm. Results are expressed as % of control. Each value represents the mean ± S.D of three experiments. *Indicates a significant difference from control, $*p < 0.01$, $**p < 0.001$. (ETO: ergosta-4,6,8(14),22-tetraen-3-one, EDO: ergosta-7,24(28)-dien-3-ol, EPO: ergosterol peroxide).

by regulating these enzymes. Coenzyme Q10 inhibits tyrosinase activity (Zhang *et al*., 2012), and kojic acid from *Aspergillus oryzae* (Saruno *et al*., 1979; Cabanes *et al*., 1994), arbutin from *Arctostaphylos uva-ursi* (Maeda *et al*., 1996), oxyresveratrol from *Morus alba* (Shin *et al*., 1998), chalcone (Nerya *et al*., 2004), and some flavonoids (Shimizu *et al*., 2000) were reported to inhibit melanogenesis.

For the purpose of evaluate whitening effects of the fruit body of *Phellinus pini,* we isolated compounds **1 - 3** using column chromatography. Based on spectroscopic methods including IR, MS, and NMR (1D and 2D), their structures were characterized as ergosterol analogues. We investigated the effects of three ergosterol compounds from *Phellinus pini* on melanin synthesis and tyrosinase activities at concentrations without cytotoxic effect of B16F10 melanoma cells. ETO, EDO, and EPO had no significant cytotoxic effect at concentration of 5-15 uM in the presence and absence of α-MSH (Fig. 1). They had some cytotoxicity at concentrations greater than 15 uM (data not shown). The decrease in the viability of the high concentration, 15 uM was considered the influence of

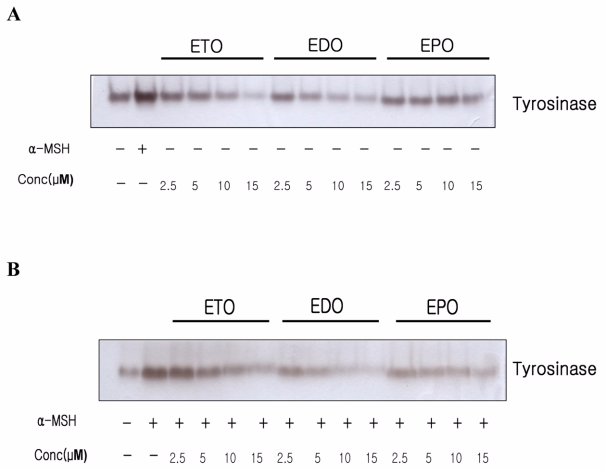

Fig. 4. Gel-dopa staining after treatment with various concentrations of ETO, EDO, EPO without (A) or with (B) α-MSH (0.4 μM) in B16F10 melanoma cells. B16F10 cells were treated with various concentrations of compounds (2.5 - 15 μM) in absence (A) and presence (B) of α-MSH (0.4 μM) for 48 hours. Cell lysates were electrophoresed on SDS polyacrylamide gel. The gel was incubated in L-DOPA solution to visualize tyrosinase activity. (ETO: ergosta-4,6,8(14),22-tetraen-3-one, EDO: ergosta-7,24(28)-dien-3-ol, EPO: ergosterol peroxide).

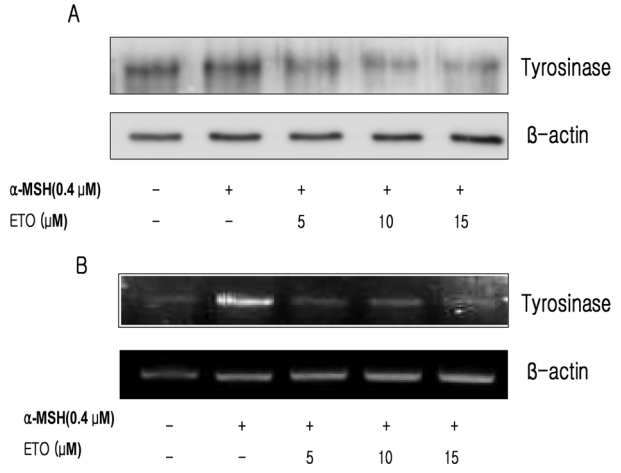

Fig. 5. Effect of ergosta-4,6,8(14),22-tetraen-3-one (ETO) on protein and mRNA expressions of tyrosinase in B16F10 melanoma cells. B16F10 cells were treated for 48 hours with α-MSH (0.4 μM) and ETO (5, 10 and 15 μM). (A) The level of tyrosinase protein in lysates was determined by western blot analysis. (B) The level of tyrosinase mRNA in lysates was determined by RT-PCR analysis.

DMSO in the samples. Melanin biosynthesis was decreased in a dose-dependent manner and significantly inhibited at 10 uM and 15 uM respectively (Fig. 2). ETO showed the greatest activity in melanin production that inhibited about 93% for the α-MSH treated group at 15 uM. The inhibition of melanin production was not due to cytotoxicity, as determined by MTT assay. The addition of ETO, EDO,

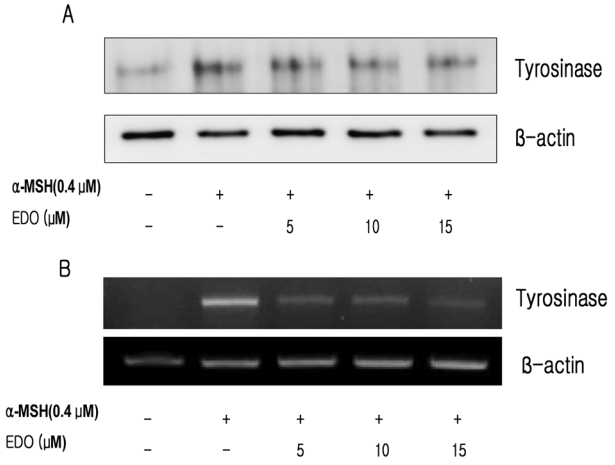

Fig. 6. Effect of ergosta-7,24(28)-dien-3-ol (EDO) on protein and mRNA expressions of tyrosinase in B16F10 melanoma cells. B16F10 cells were treated for 48 hours with α-MSH (0.4 μM) and EDO (5, 10 and 15 μM). (A) The level of tyrosinase protein in lysates was determined by western blot analysis. (B) The level of tyrosinase mRNA in lysates was determined by RT-PCR analysis.

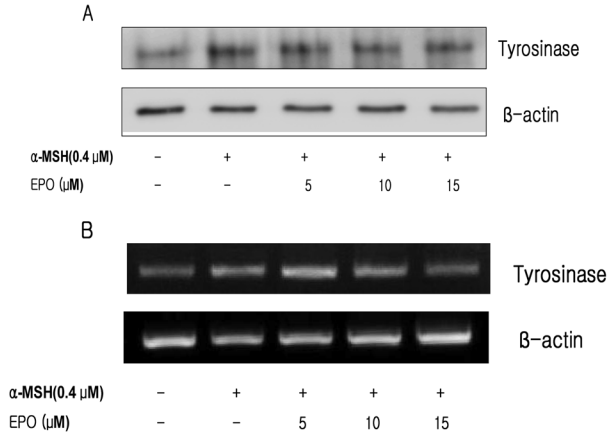

Fig. 7. Effect of ergosterol peroxide (EPO) on protein and mRNA expressions of tyrosinase in B16F10 melanoma cells. B16F10 cells were treated for 48 hours with α-MSH (0.4 μM) and EPO (5, 10 and 15 μM). (A) The level of tyrosinase protein in lysates was determined by western blot analysis. (B) The level of tyrosinase mRNA in lysates was determined by RT-PCR analysis.

and EPO to the cell reduced a significant tyrosinase activity in the presence and absence of α-MSH (Fig. 3). EDO showed the greatest tyrosinase inhibition activity of 60% for non α-MSH treated group, and 95% inhibition rate for the α-MSH treated group at 15 uM. This result was similar to those of tyrosinase zymography (Fig. 4). To investigate the effects of compounds on tyrosinase protein expression, western blot analysis was performed in α-MSH activated B16F10 cells. Next, the effects of ETO, EDO, and EPO on the expression of tyrosinase mRNA were analyzed by RT-PCR. As shown in Fig. 5-7,

the treatment of ETO, EDO, and EPO decreased the expression of tyrosinase activity dose dependently, and ETO, EDO, and EPO showed significant suppression of mRNA expression in a dose dependent manner. EDO showed the greatest activities in tyrosinase activity, and the expressions of tyrosinase protein and mRNA.

In conclusion, these results suggested that these ergosterol compounds inhibited melanin production and down-regulated cellular enzymatic activity of tyrosinase in melanoma cells as well as decreased mRNA and protein levels of the enzyme. So these ergosterol compounds from *Phellinus pini* can be used as potential source of skin whitening agents.

Acknowledgements

This Research was supported by the Sookmyung Women's University Research Grants 2013.

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Received March 20, 2013
Revised April 1, 2013
Accepted August 12, 2013