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# Inhibitory effect of carvacrol on melanin synthesis via suppression of tyrosinase expression

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ABSTRACT

Carvacrol (2-methyl-5-(1-methylethyl)-phenol) compound derived from oils of Thymus vulgaris is a natural member of monoterpene phenol. It has been shown that carvacrol exhibit anti-microbial, anti-inflammatory, anti-oxidant activities. However, no studies have reported its anti-melanogenesis effect. Therefore, the objective of this study was to investigate the anti-melanogenic potential of carvacrol. Results of this study confirmed that carvacrol could regulate protein expression levels of microphthalmia-associated transcription factor [MITF, a protein closely related to transcription of cAMP response element-binding protein (CREB)] and various enzymes involved in melanin synthesis. Moreover, this study provided evidence that carvacrol could regulate the degradation of MITF protein by extracellularly responsive kinases (ERK) phosphorylation. Carvacrol strongly inhibited the synthesis of CREB protein, tyrosinase-related protein 1 (TRP-1), and tyrosinase known to be important enzymes involved in melanogenesis. These results indicate that carvacrol can inhibit melanin synthase by decreasing enzyme expression levels important for melanin synthesis.

## 1. Introduction

Carvacrol is a monoterpenoid compound derived from oils of *Thymus vulgaris*, *Origanum*, *Carum copticum*. It is a natural member of monoterpene phenol (Hussein, El-Bana, Refaat, & El-Naggar, 2017; Kisk & Roller, 2005; Lampronti, Saab, & Gambari, 2006; Martins, Neves, Silvestre, Silva, & Cavaleiro, 1999). Carvacrol has been used for a wide variety of applications in daily life. It is contained in various products such as cosmetics and wide range of foods. In addition, carvacrol has been shown to exhibit anti-microbial, anti-mutagenic, anti-platelet, analgesic, anti-inflammatory, anti-angiogenic, anti-oxidant, anti-elastase, insecticidal, anti-parasitic, cell-protective and anti-tumor activities (Sokmen et al., 2004; Can Baser, 2008). Carvacrol has also been used as an ingredient in various cosmetics. However, no study has reported the whitening effect of carvacrol.

Many inflammatory cytokines as melanogens have been reported. Histamine, a ubiquitous inflammatory mediator, is a representative melanogen induced by inflammation (Yoshida, Takahashi, & Inoue, 2000). Studies have shown that reactive oxygen species (ROS) generation can activate melanin production (Kim et al., 2014). Based on this knowledge, the objective of this study was to investigate the anti-melanogenesis potential of carvacrol.

Melanin is synthesized by epidermal melanocytes in the skin. Melanin works in physiological defence to protect skin from ultraviolet radiation. However, continuous UV irradiation induces increased accumulation of melanin, leading to skin hyperpigmentation (Agar and Young, 2005). Melanogenesis is induced by isobutylmethylxanthine (IBMX) and α-melanocyte-stimulating hormone (α-MSH). Binding of α-MSH to its receptor, melanocortin-1 receptor (MC1R), induces enhanced expression of microphthalmia-associated transcription factor (MITF) and, cyclic-AMP (cAMP), leading to increased expression of tyrosinase and other melanogenesis-related enzymes such as tyrosinase-related protein 1 (TRP-1), dopachrome tautomerase (TRP-2). Hyperpigmented skin has an unaesthetic appearance, showing melasma and freckles (Plensdorf and Martinez, 2009). TRP-1, TRP-2, and tyrosinase are three representative enzymes that are important for melanin regulation. Tyrosinase is a copper-containing glycoprotein that is important in melanin synthesis. It can catalyze three different reactions:

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the hydroxylation of tyrosine to 3, 4-dihydroxyphenylalanine (DOPA), oxidation of DOPA to dopaquinone and conversion of dopaquinone to dopachrome and then to dihydro-indolizine (DHI) or indole 5,6-quinone-2-carboxylic acid (DHICA) (Ando, Kondoh, Ichihashi, & Hearing, 2007; Chung et al., 2009; Lee et al., 2010; Shimoda et al., 2010). TRP-1 and TRP-2 also play important roles in the synthesis of melanogenesis. TRP-1 catalyzes oxidation of DHICA while TRP-2 catalyzes conversion of dopachrome to DHICA (Sato, Morita, Ichikawa, Takahashi, & Toriyama, 2008). These enzymes are also regulated by a specific transcription factor, MITF (Hasegawa et al., 2010; Widlund & Fisher, 2003). MITF is regulated by a variety of signaling pathways. It is generally known to be controlled by MAPK. Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases that regulate cell differentiation, proliferation, and cellular activities. The MAPK superfamily contains three well-characterized subfamilies: ERKs, p38, and the c-Jun N-terminal kinases (JNKs). MAPKs are known to play a major role in the regulation of melanogenesis (Jiang et al., 2009; Ye et al., 2010). Furthermore, it has been reported that activation of ERK by c-Kit stimulation phosphorylates MITF at its 73rd serine residue. Phosphorylation of MITF at 73rd of serine is followed by MITF ubiquitination and degradation (Hemesath, Price, Takemoto, Badalian, & Fisher, 1998; Xu et al., 2000). Moreover, p38 MAPK pathway activation can increase melanin synthesis (Hirata et al., 2007; Singh et al., 2005). Activation of ERK signaling could also inhibit melanogenesis by inhibiting tyrosinase activity (Jang et al., 2009).

There are numerous melanogenesis inhibiting agents, including arbutin, kojic acid, and linoleic acid. Arbutin is a glycosylated hydroquinone extracted from bearberry plant. It has been used as a cure for hyperpigmentation illnesses in the past. Kojic acid and arbutin are extensively used as cosmetic ingredients due to their anti-tyrosinase activity (Nishimura, Kometani, Okada, Ueno, & Yamamoto, 1995). However, some of these agents can cause skin irritation. It has been described that kojic acid causes skin irritation with side effects such as cytotoxicity, dermatitis, and skin cancer (Busca and Ballotti, 2000). Arbutin has been also banned due to its side effects involving exogenous ochronosis and perdurable depigmentation (Draelos, 2007; O'Donoghue, 2006). Therefore, there were huge interests in finding new potential compounds extracted from natural sources without or with very limited side effects. The present study aimed to investigate such a potential melanogenesis inhibiting compound from a natural source. In this study, we examined the anti-melanogenic effect of carvacrol on IBMX-induced melanogenesis in B16F10 mouse melanoma cells. We also explored the underlying molecular mechanisms involved in this process.

Many previous studies on cavacrol have mainly determined its antibacterial and antioxidant properties. However, in present study, we performed a melanogenesis study using carvacrol for the first time. Results of this study provide basic research data for developing cosmetics using multifunctional resources

## 2. Materials and methods

### 2.1. Chemicals

Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Hyclone (Thermo Scientific, Waltham, MA, USA). Phosphorylated-ERK, tyrosinase, TRP-1, and TRP-2 antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). MITF, phosphorylated-CREB and β-actin antibodies were purchased from Cell Signaling Technology Inc. (Denvers, MA, USA). Carvacrol, IBMX, L-DOPA, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

### 2.2. Cell culture

Mouse melanoma cell line B16F10 was obtained from the Korean cell line bank (Seoul, Korea) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 units/mL of penicillin and 100 μg/mL of streptomycin) at 37 °C in a humidified incubator containing 5% $CO_2$. For maintenance, these cells were sub-cultured every two days.

### 2.3. Determination of cell viability

The effect of carvacrol on viability of mouse melanoma cell line B16F10 viability was evaluated using MTT colorimetric assay. Briefly, B16F10 cells were plated into 96-well plates at a density of $5 \times 10^3$ cells/well. These cells were treated with various concentrations (25, 50, 100, 200, 400 and 600 μM) of carvacrol and stimulated with or without IBMX (100 μM) at 37 °C for 48 h. After treatments, the medium was replaced by 100 μL of DMEM medium containing MTT (200 μg/mL) in each well followed by incubation at 37 °C for 2 h. After discarding MTT solution, the intracellular formazan product in each well was dissolved in 200 μL DMSO. The absorbance was then measured at 540 nm using a microplate reader (Tecan, Grödig, Austria). Values were calculated in comparison with those of control cells.

### 2.4. Measurement of melanin content

Intracellular melanin contents were determined according to published method (Hosoi, Abe, Suda, & Kuroki, 1985) with slight modifications. Briefly, B16F10 cells were stimulated with IBMX and incubated with carvacrol for 48 h. Cell pellets were harvested and then dissolved in 1 N NaOH containing 10% DMSO at 80 °C for 1 h. Melanin contents were analyzed by measuring absorbance at 475 nm using an ELISA reader (Multiskan GO, Thermo Fisher, Massachsetts, USA). For accurate calculation of melanin contents, each level of melanin was normalized to protein content.

### 2.5. Tyrosinase activity

Tyrosinase activity was determined using published method (Hosoi et al., 1985). Briefly, B16F10 cells were co-treated with IBMX and different concentrations of carvacrol. After 48 h incubation, cells were washed with cold PBS and suspended in a lysis buffer (150 mM NaCl, 10 mM Tris pH 7.5, 5 mM EDTA), and Triton-X 100 1.0% in the presence of protease inhibitors (1 μg/mL leupeptin and 100 μg/mL PMSF) and incubated at 4 °C for 20 min to yield cell lysates. Cell lysates were then centrifuged at 12,000 rpm for 10 min. Protein contents in supernatants were then determined using a protein assay kit (Bio-Rad, Laboratories, Inc., Hercules, CA, USA). Then 90 μL of cell extract was transferred to a 96-well containing 10 μL of L-DOPA (final concentration of 1 mmol/L) prepared in 25 mM phosphate buffer (pH 6.8) and incubated at 37 °C for 20 min. Absorbance was then measured at 475 nm using an ELISA reader (Multiskan GO, Thermo Fisher, MA, USA).

### 2.6. Western blot analysis

B16F10 cells were seeded in 60 mm dishes at a density of $3 \times 10^5$ cells/dish. After 24 h of culture, they were co-treated with IBMX (100 μM) and different concentrations (100, 200 and 400 μM) of carvacrol or Kojic acid (1 mM). After incubating at 37 °C for 48 h, cells were subsequently washed with PBS, collected and suspended in a lysis buffer (150 mM NaCl, 10 mM Tris (pH 7.5), 5 mM EDTA, and 1% Triton X-100) containing protease inhibitors (1 μg/mL leupeptin and 100 μg/mL PMSF). After incubating at 4 °C for 20 min, cell lysates were centrifuged at 12,000 rpm for 10 min. Protein concentration in each cell