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Effect of xanthohumol on melanogenesis in B16 melanoma cells

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Abbreviations: DHICA, 5,6-dihydroxyindole-2-carboxylic acid; IBMX, isobutylmethylxanthine; MITF, microphthalmia-associated transcription factor; PKG, protein kinase G; TRP, tyrosinase-related protein; XH, xanthohumol; α-MSH, α-melanocyte stimulating hormone

Abstract

Xanthohumol (XH), the principal prenylflavonoid of the hop plant (*Humulus lupulus* L.), dose-dependently inhibited isobutylmethylxanthine (IBMX)-induced melanogenesis in B16 melanoma cells, with little cytotoxicity at the effective concentrations. Decreased melanin content was accompanied by reduced tyrosinase enzyme activity, protein and mRNA expression. The levels of tyrosinase-related protein 1 and 2 mRNAs were decreased by XH. XH also inhibited α-melanocyte stimulating hormone- or forskolin-induced increases in melanogenesis, suggesting an action on the cAMP-dependent melanogenic pathway. XH downregulated the protein and mRNA expression of microphthalmia-associated transcription factor (MITF), a master transcriptional regulator of key melanogenic enzymes. These results suggest that XH might act as a hypo-pigmenting agent through the downregulation of MITF in the cAMP-dependent melanogenic pathway.

Keywords: cell differentiation; melanins; melanocytes; microphthalmia-associated transcription factor; monophenol monooxygenase; xanthohumol

Introduction

Melanin is a unique pigmented biopolymer synthesized by melanocytes, dendritic cells that exist in the dermal-epidermal border of the skin. Melanin has a number of important functions, including the determination of phenotypic appearance, protective coloration, balance and auditory processing, absorption of toxic drugs and chemicals, and neurologic development during embryogenesis (Hearing, 1998). Melanogenesis itself is a complex process, with at least 125 distinct genes involved in the regulation of melanogenesis either directly or indirectly (Yamaguchi *et al*., 2007). Mutations of these genes are associated with different pigmentary diseases, including various forms of ocular and oculocutaneous albinism, piebaldism, Hirschsprung's disease, and Waardenberg's syndrome (Hearing, 1999).

The tyrosinase gene family plays an pivotal role in the regulation of melanogenesis (Pawelek and Chakraborty, 1998). The tyrosinase gene family consists of tyrosinase, tyrosinase-related protein 1 (TRP-1), and tyrosinase-related protein 2 (TRP-2) (Hearing, 1999). Tyrosinase is a bifunctional enzyme that modulates melanin production, first by catalyzing the hydroxylation of tyrosine to DOPA and secondly by catalyzing the oxidation of DOPA to DOPAquinone (Hearing and Jimenez, 1987). TRP-2, which functions as a DOPAchrome tautomerase, catalyzes the rearrangement of DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Yokoyama *et al*., 1994), and TRP-1 oxidizes DHICA to a carboxylated indole-quinone (Kobayashi *et al*., 1994). Microphthalmia-associated transcription factor (MITF) is a master regulator of melanocyte development and melanogenesis (Levy *et al*., 2006). It is a melanocyte-specific transcription factor, and

regulates the transcription of three major pigmentation enzymes: tyrosinase, TRP-1, and TRP-2. The promoters of these genes contain the MITF consensus E-box sequence and are expressed in melanocytes.

Understanding the mechanisms of melanogenesis is of great interest pharmaceutically and cosmeceutically. Inhibitors of melanin synthesis are related to localized hyper-pigmentation in humans, such as melasma, lentigines, nevus, ephelis, and post-inflammatory state. In human epidermis, α-melanocyte stimulating hormone (α-MSH) and adrenocorticotropic hormone are produced and released by keratinocytes after UV radiation, and are involved in the regulation of melanogenesis and/or melanocyte dendrite formation (Wakamatsu *et al*., 1997). α-MSH and adrenocorticotropic hormone bind to a melanocyte-specific receptor, MC1-R (Cone *et al*., 1996), which activates adenylate cyclase through G proteins to elevate intracellular cAMP (Costin and Hearing, 2007). Cyclic AMP increases the expression of melanogenic enzymes in part through PKA (Busca and Ballotti, 2000). UV radiation also affects melanogenesis through the activation of the diacylglycerol/PKC pathway, nitric oxide/cGMP pathway, or the SOS responses to UV-induced DNA damage (Costin and Hearing, 2007).

Flavonoids are constituents of food and drinks that include flavones (7,8-benzoflavone), flavonols (quercetin), flavanol (cathechin), flavanones (naringenin), isoflavones (genistein), and chalcones (xanthohumol). Xanthohumol (XH) has been isolated from hop "cones," the female inflorescences of the hop plant (*Humulus lupulus* L.) that are largely used in the brewing industry as a preservative and flavoring agent to add bitterness and aroma to beer. Chemically, XH belongs to the prenylated chalcones (open C-ring flavonoids) class, and is the main prenylflavonoid of hops (0.1-1% on dry weight) (Stevens and Page, 2004). XH is an antioxidant (Miranda *et al*., 2000) and a broad-spectrum cancer chemopreventive agent that prevents carcinogenesis in the initiation, promotion, and progression phase (Gerhauser *et al*., 2002; Pan *et al*., 2005; Plazar *et al*., 2007). It also inhibits adipogenesis (Yang *et al*., 2007) and osteoporosis (Tobe *et al*., 1997), and potentially influences AIDS (Wang *et al*., 2004) and malaria (Frolich *et al*., 2005), at least *in vitro*. However, to our knowledge, there is no report about the effect of XH on melanogenesis. In this study, we observed that XH can effectively inhibit isobutylmethylxanthine (IBMX)- induced melanogenesis in B16 melanoma cells.

Materials and Methods

Cells and Materials

The B16/F10 murine melanoma cell line was obtained from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37°C in a humidified 95% air/5% CO_2 atmosphere as described previously (Jung *et al*., 2001). XH was obtained from Alexis Biochemicals (Lausen, Switzerland), and α-MSH, IBMX, and forskolin were obtained from Sigma (St. Louis, MO). Drug treatment began 24 h after seeding, and cells were harvested after 2 days of incubation.

Melanin content measurement

The melanin content of the cultured B16 cells were measured as described previously (Yang *et al*., 2006). The cells were washed twice with PBS and lysed with 20 mM Tris-0.1% Triton X-100 (pH 7.5). Cell lysates were precipitated with the same amount of 20% trichloroacetic acid. After washing twice with 10% trichloroacetic acid, the pellets were treated with ethyl alcohol:diethyl ether (3 : 1) and diethyl ether successively. Samples were air-dried, dissolved in 1 ml of 0.85 M KOH, and boiled for 15 min. After cooling, absorbance was measured with a spectrophotometer at 440 nm. The amount of cellular melanin was corrected according to the protein content of the samples. The protein content was determined by the method of Bradford (Bradford, 1976).

Tyrosinase activity assay

Tyrosinase activity was assayed as DOPA oxidase activity (Lerch, 1987) with some modifications as described previously (Lv *et al*., 2007). Cell lysate was obtained after washing twice with PBS. Tyrosinase activity was analyzed spectrophotometrically by following the oxidation of DOPA to DOPAchrome at 475 nm. The reaction mixture containing 100 μl of freshly prepared substrate solution (0.1% L-DOPA in 0.1 M sodium phosphate, pH 6.0) and 50 μl of enzyme solution was incubated at 37°C. The absorbance change was measured during the first 10 min of the reaction while the increase of the absorbance was linear, and corrections for auto-oxidation of L-DOPA in controls were made. The tyrosinase activity was corrected according to the protein content of the samples and presented as % of IBMX-treated control cells.

MTT assay

Viability of cultured cells was determined by reduction of MTT (Sigma) to formazan (Mosmann, 1983). Cells were seeded in 96-well plates and cultured for 24 h. After drug treatment, MTT (5 mg/ml in PBS, 100 μl) was added to each well. Cells were incubated at 37°C for 30 min and DMSO (100 μl) was added to dissolve the formazan crystals. The absorbance was measured at 570 nm with a spectrophotometer (Spectra Max Plus, Molecular Devices, Sunnyvale, CA).

Western blot analysis

Cells were homogenized in ice-cold lysis buffer. The homogenates containing 10 μg of protein were separated by SDS-PAGE with a 10% resolving and 3% acrylamide stacking gel (Laemmli, 1970), and transferred to a nitrocellulose membrane (Millipore, Billerica, MA) in a Western blot apparatus (Bio-Rad, Hercules, CA) run at 100 V for 1.5 h. The nitrocellulose membrane was blocked with 2% BSA (Sigma), and then incubated overnight with 1 μg/ml goat anti-murine tyrosinase IgG (sc-7834, Santa-Cruz, CA), goat anti-human MITF IgG (sc-10999, Santa-Cruz) or monoclonal anti-actin IgG (A5441, Sigma). The binding of antibody was detected with anti-goat or anti-murine IgG conjugated with HRP (Sigma). Immunoblots were developed using an Enhanced Chemiluminescence Plus kit (Amersham Biosciences, Buckinghamshire, UK), and the intensity of the bands was measured by an LAS-1000 (Fujifilm, Tokyo, Japan).

RT-PCR

Total cellular RNA was prepared using Trizol solution (Invitrogen, Praisley, UK) according to the manufacturer's instructions. After the preparation of cDNA with oligo $d(T)_{16}$ as a reverse transcriptase primer from the extracted RNA, amplification with PCR was performed using GeneAmp kit (Perkin Elmer, Foster City, CA) according to the manufacturer's manual. The oligonucleotide primers used for PCR are as follows: tyrosinase upstream 5'-GGCCAGCTTTCAGGCAGAGGT-3'; downstream 5'-TGGTGCTTCATGGGCAAAATC-3': TRP-1 upstream 5'-GCTGCAGGAGCCTTCTTTCTC-3'; downstream 5'-AAGACGCTGCACTGCTGGTCT-3': TRP-2 upstream 5'-GGATGACCGTGAGCAATGGCC-3'; downstream 5'-CGGTTGTGACCAATGGGTGCC-3': MITF upstream 5'-GTATGAACACGCACTCTCTCGA-3'; downstream 5`-CTTCTGCGCTCATACTGCTC-3`; β-actin upstream 5'-ACCGTGAAAAGATGACCCAG-3'; downstream 5'-TACGGATGTCAACGTCACAC-3'. cDNA amplification used the product of about 1 μg of the total RNA. The reaction was cycled 28 times (for tyrosinase), 25 times (for TRP-1 and -2) and 35 times (for MITF) for 60 s at 94°C, 60 s at 56°C and 60 s at 72°C. Fifty percent of the reaction mixture was analyzed by electrophoresis on 1% agarose gels and stained by ethidium bromide. The intensity of the bands was measured by LAS-1000 (Fujifilm, Tokyo, Japan).

Statistical analysis

Statistical analysis of the data was performed using ANOVA and Duncan's test. Differences with $P < 0.05$ were considered statistically significant.

Results

When B16 cells were incubated with IBMX, an inhibitor of phosphodiesterase (Beavo *et al*., 1970), the cell suspension turned black, indicating increased cellular melanogenesis (Figure 1A). XH dose-dependently decreased this IBMX-induced black color (Figure 1A), with significant inhibition observed from 0.5 μM XH (Figure 1B). No cytotoxicity was observed until 2.5 μM of XH as determined by

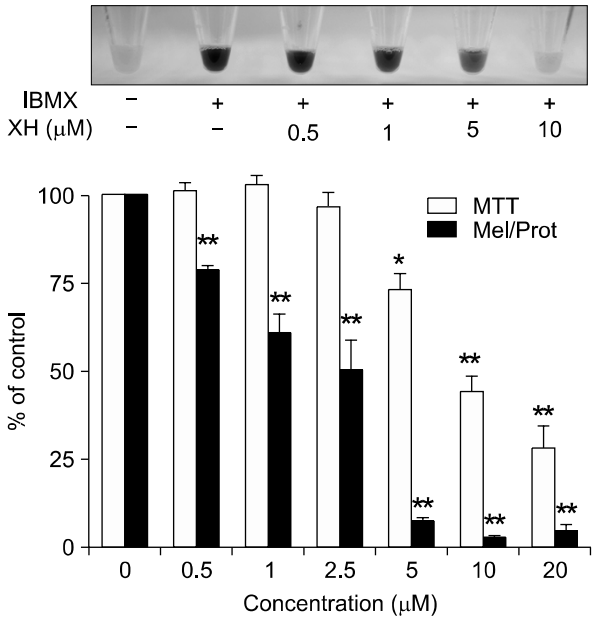

Figure 1. Effect of XH on melanin content and cytotoxicity in B16 melanoma cells. Cells (5×10^6 cells/well) were incubated with various concentrations of XH in the presence of 0.1 mM IBMX for 2 days. Melanin and protein content were determined as described in "Materials and Methods". Cell viability was determined by MTT assay. Data are expressed as a percentage of IBMX-treated control and presented as mean ± SEM of three separate experiments. *$P < 0.05$ and **$P < 0.01$ vs. IBMX-treated control.

the MTT assay. Even at 5 μM XH, 73.0% ± 4.6% of cells were still viable, while the cellular melanin content was decreased to 6.51% ± 1.13% of IBMX-treated cells.

XH dose-dependently decreased cellular tyrosinase activity (Figure 2), the rate-limiting step in melanin biosynthesis, in parallel with the decreased melanin content (Figure 1). However, *in vitro* preincubation of enzyme with XH for 30 min at 4°C did not affect the tyrosinase activity. At 20 μM concentration of XH, the remaining activity was 95.4 ± 5.9% of control, indicating that the decrease in cellular tyrosinase activity by XH was not due to the direct inhibition of enzyme activity.

IBMX treatment increased tyrosinase protein expression, and this induction could be dose-dependently inhibited by XH (Figure 3). XH also decreased tyrosinase mRNA levels (Figure 4). These results indicate that XH inhibited tyrosinase at the transcriptional level. XH decreased the mRNA expression of TRP-1 and TRP-2, members of the tyrosinase gene family, as well (Figure 4).

Cellular melanin content was significantly increased in cells treated with 5 μM α-MSH or 5 μM forskolin (Figure 5). α-MSH produced by keratinocytes increases adenylate cyclase activity of melanocytes through G proteins (Busca and Ballotti, 2000), and forskolin is a direct activator of adenylate cyclase (Tamagawa *et al*., 1985). XH significantly inhibited the melanogenesis induced by both α-MSH and forskolin (Figure 5), suggesting that XH regulates the expression of the tyrosinase gene family through a cAMP-dependent pathway.

cAMP-mediated activation of PKA induces the expression of MITF, a master transcriptional regulator for melanogenic enzymes (Levy *et al*., 2006), and tyrosinase family proteins are important targets of MITF. The presence of XH significantly decreased the expression of MITF mRNA (Figure 4) and protein (Figure 3) expression, suggesting that XH worked by down-regulating MITF transcription.

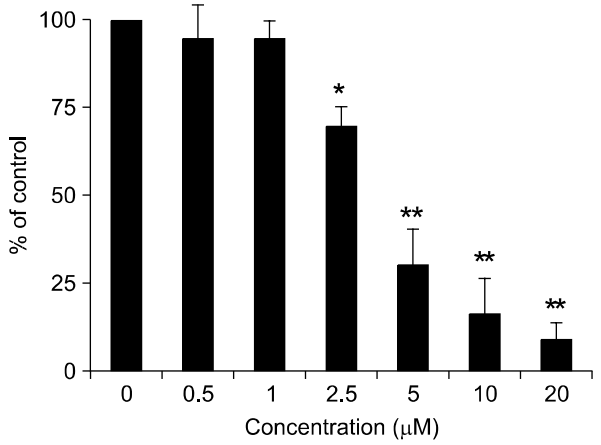

Figure 2. Effect of various concentrations of XH on cellular tyrosinase activity. Cells (5×10^6 cells) were treated with various concentrations of XH in the presence of 0.1 mM IBMX for 2 days. Tyrosinase activity in cellular lysates was determined as described in "Materials and Methods". Data are expressed as a percentage of IBMX-treated controls and presented as mean ± SEM of three separate experiments. *$P < 0.05$ and **$P < 0.01$ vs. IBMX-treated control.

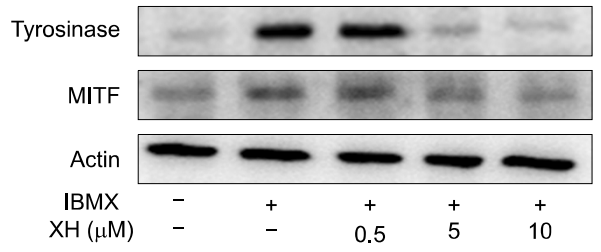

Figure 3. Effect of XH on tyrosinase and MITF protein expression. Cells (5×10^6 cells) were treated with a range of concentrations (0.5-10 μM) of XH in the presence or absence of 0.1 mM IBMX for 2 days. Tyrosinase and MITF protein was analyzed by Western blotting as described in "Materials and Methods". Experiments were performed three times with similar results and typical one is presented. Lane 1, control; lane 2, 0.1 mM IBMX; lane 3-5, 0.1 mM IBMX with 0.5 μM (lane 3), 5 μM (lane 4) and 10 μM (lane 5) XH.

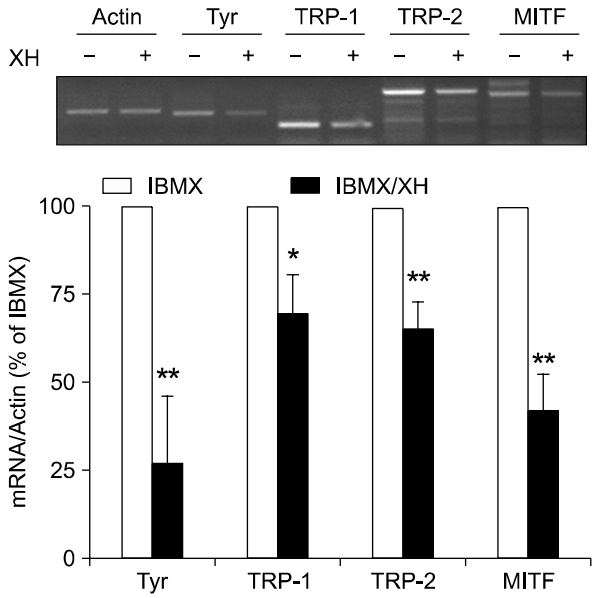

Figure 4. Effect of XH on mRNA expression of melanogenesis-related genes. (A) Cells (5×10^6 cells) were treated with 0.1 mM IBMX for 2 days in the presence or absence of 5 μM XH. Then cells were harvested and total RNA was extracted. mRNA expression was visualized by RT-PCR and quantitated as described in "Materials and Methods". The sizes of amplified gene products were 528 bp for β-actin, 477 bp for tyrosinase, 268 bp for TRP-1, 1044 bp for TRP-2, and 910 bp for MITF. Data are expressed as a percentage of IBMX-treated cells and presented as the mean ± SEM of three separate experiments. *$P < 0.05$ and **$P < 0.01$ vs IBMX-treated control.

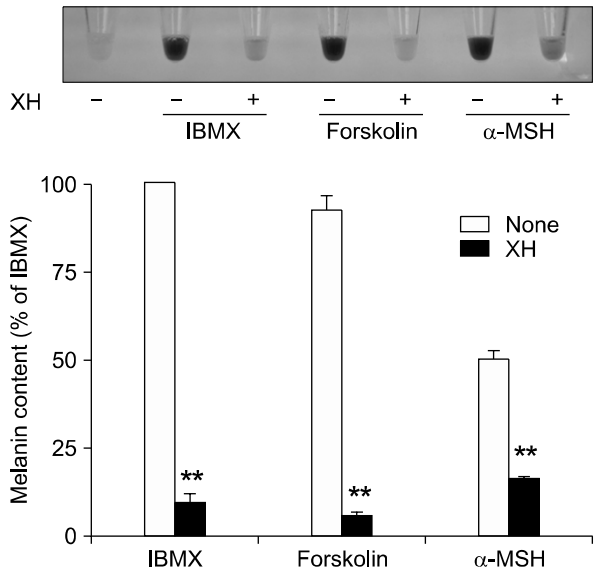

Figure 5. Effect of XH on α-MSH-, IBMX- and forskolin-induced melanogenesis. (A) Cells (5×10^6) were incubated with 5 μM XH in the presence of IBMX (0.1 mM), α-MSH (5 μM), or forskolin (5 μM) for 2 days. Melanin and protein content were determined as described in "Materials and Methods". Data are expressed as a percentage of IBMX-treated control and presented as mean ± SEM of three separate experiments. $^{**}P < 0.01$ vs. each drug control.

Discussion

Here, IBMX-treated B16 melanoma cells were used to investigate the effect of XH on melanogenesis. Exposure of skin to UV radiation is the most common environmental stimuli for skin pigmentation. UV-induced hyperpigmentation occurs in two stages, an immediate darkening and a delayed tanning reaction. Immediate pigment darkening results from oxidation of pre-existing melanin and redistribution of melanosomes. In contrast, the delayed tanning response is photoprotective against subsequent UV injury, begins as the immediate pigmentation reaction fades, and progresses for at least 3-5 days after UV exposure (Eller and Gilchrest, 2000). Delayed tanning is preceded by increased tyrosinase activity in melanocytes (Eller and Gilchrest, 2000; Costin and Hearing, 2007). In B16 cells treated with IBMX, increased melanogenesis was associated with increased tyrosinase activity, protein and mRNA expression, which are similar with delayed tanning response after UV irradiation.

The melanocyte-keratinocyte complex of the skin responds quickly to a wide range of environmental stimuli, often in paracrine and/or autocrine manners. IBMX increases cellular cAMP through the inhibition of the cAMP-degrading enzyme, phosphodiesterase (Beavo *et al*., 1970). XH blocked IBMX-induced increases in melanogenesis at the transcriptional level of tyrosinase, suggesting an action on the cAMP-dependent pathway. α-MSH (Wakamatsu *et al*., 1997), a peptide acting on the MC1-R of melanocytes (Cone *et al*., 1996; Wakamatsu *et al*., 1997), and forskolin, an activator of adenylate cyclase (Tamagawa *et al*., 1985), both increased cellular melanin content in B16 cells. XH significantly inhibited the melanogenesis induced by both of these factors, supporting the idea that XH works on a cAMP-dependent pathway.

PKA phosphorylates and activates the cAMP response element binding protein that binds to the cAMP response element in the M promoter of the MITF gene (Tachibana, 2000; Levy *et al*., 2006). MITF is a tissue restricted, basic helix–loop–helix leucine zipper (b-HLH-Zip), dimeric transcription factor, and its mutation leads to defects in melanocytes, the retinal pigmented epithelium, mast cells, and osteoclasts in mice (Levy *et al*., 2006). In humans, mutations affecting the MITF pathway lead to pigmentary and auditory defects that are known collectively as Waardenburg syndrome (Lin and Fisher, 2007). The increase in MITF-M expression induces the up-regulation of tyrosinase gene family, which leads to increased melanin synthesis (Busca and Ballotti, 2000; Levy *et al*., 2006). XH significantly decreased the expression of MITF mRNA, suggesting that XH inhibits MITF transcription. Suppression of MITF mRNA was followed by decreased expression of tyrosinase and TRP-1 and -2 mRNAs.

In addition to the cAMP/PKA pathway, melanogenesis requires the cross-talking of several signaling pathways including diacylglycerol/PKC, nitric oxide/protein kinase G (PKG), tyrosine kinase pathway, or the SOS response to UV- induced DNA damage (Costin and Hearing, 2007). PKC-induced activation of tyrosinase occurs through phosphorylation rather than the synthesis of new enzyme (Park *et al*., 1993). However, PKG can increase the expression of tyrosinase protein (Sasaki *et al*., 2000). Alteration of melanocyte ERK activity by various paracrine cytokines can affect the MITF degradation (Xu *et al*., 2000; Kim *et al*., 2006). Induction of p53 increases the expression of hepatocyte nuclear factor-1α, which directly increases the transcription of MITF and tyrosinase (Schallreuter *et al*., 2003; Schallreuter, 2007). The additional effects of XH on other pathways and their cross-talking require further study.

In addition to the roles of protecting skin from harmful solar UV radiation or toxic chemicals, melanin determines racial and phenotypic appearance. The accumulation of melanin in specific parts of the skin as more pigmented patches such as me-

lasma, freckles, ephelides, or senile lentigines might become an aesthetic problem (Solano *et al*., 2006). Elucidating the molecular mechanisms underlying hyperpigmentation could lead to technology that allows unwanted pigmentation to be decreased and photoaging to be preserved, as well as the design of tanning products with the potential to reduce the risk of skin cancer. Recently, natural herbal extracts and compounds have gained attention as putative hypo-pigmenting agents (Parvez *et al*., 2006; Solano *et al*., 2006). In this study, we showed that XH could inhibit IBMX-induced melanogenesis by inhibiting tyrosinase and related enzyme expression via down-regulating MITF expression, a key regulatory transcription factor of melanogenesis. Safety after long-term application is important, and no toxicity was observed after oral administration of XH (5×10^{-4} M) at libitum for 4 weeks in laboratory mice (Vanhoecke *et al*., 2005). Lifelong treatment of XH at a daily dose of 100 mg/kg body weight in a two-generation study did not affect the development of rats (Hussong *et al*., 2005). These results suggest that XH may be a safe hypo-pigmenting agent.

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