Hexane Extract of *Kaempferia galanga L.* Suppresses Melanogenesis via p38, JNK and Akt

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Kaempferia galanga L. is one of the plants in Zingiberaceae family. It is used by people in many regions of Asia and Africa for relieving toothache, abdominal pain, muscular swelling and rheumatism. Tyrosinase is a key enzyme for melanogenesis, and hyperpigmentation is associated with abnomal accumulation of melanin pigment. This study aimed to investigate the inhibition of melanogenesis by hexane extract of *Kaempferia galanga* L. (HKG) in B16F10 melanoma cells. Cell-free tyrosinase, melanin contents, intracellular tyrosinase activity and western blot analysis were performed to elucidate the effects on anti-melanogenesis. Cytotoxicity of the extracts was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and determined the concentration of 12.5, 25 μg/ml. HKG significantly inhibited to activities of intracellular tyrosinase and melanin synthesis in the absence or presence of α-melanocyte stimulating hormone (α-MSH) with dose-dependent manner. And HKG inhibited the expression of tyrosinase, tyrosinase-related protein 1 (TRP-1) and tyrosinase-related protein 2 (TRP-2), regardless of the presence or absence of α-MSH. HKG also down-regulated phosphorylation of p38 and JNK, and up-regulated phosphorylation of Akt. These effects were not related to its cytotoxicity action. These results indicate that HKG has the potential to be a useful therapeutic agent for treating hyperpigmentation disorders and as a beneficial additive in whitening agents in cosmetics industry.

keywords : *Kaempferia galanga L.*, MAPKs, Melanin, α-MSH

Introduction

Melanin serves a number of valuable functions, the most important being photoprotection of the skin from ultraviolet (UV). It is also an important factor that affects the color of skin and hair[1]. Although melanin production play an important role in protecting the skin from the UV irradation, excessive melanin production in the skin has negative hyperpigmentation effects, such as melasma, freckles, age or liver spot, and actinic damage. Melanin is synthesized in melanocytes in the innermost layer of the epidermis and melanin-containing granules are known as melanosomes[2]. Melanin synthesis, called as melanogenesis, is a biosynthetic pathway that is regulated by enzymes such as tyrosinase, tyrosinase-related protein-1 (TRP-1) and tyrosinase related-protein(TRP-2)[3]. Tyrosinase (monophenol, L-dihydroxyphenylalanine (L-DOPA) is the key enzyme in the first two steps of melanin biosynthesis, in which L-tyrosine is hydroxylated to L-DOPA, and L-DOPA is further oxidized into the corresponding dopaquinone[4,5]. TRP-2 catalyzes the conversion of DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA), and TRP-1 catalyses the oxidation of DHICA to indole-5,6-quinone-2-carboxylic acid. Oxidative polymerization of several dopaquinone derivatives gives rise to melanin[6,7]. Hence, down-regulation of tyrosinase activity has been proposed to be responsible for reduced melanin production.

Mitogen-activated protein kinases (MAPKs) which include p38 MAPK, c-jun N-terminal kinase (JNK) and ERK are known to be involved in the signal transduction of cell proliferation, differentiation, apoptosis, and stress response[8]. Besides, several reports showed that signaling pathways, such as the extracellular signal-regulated kinase (ERK) and PI3K/Akt pathways, are involved in melanogenesis[9]. Also, It was reported that the p38 MAPK signaling pathway is involved in stress-induced melanogenesis[10], and inhibition of PI3K/Akt results in an

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increase in melanin synthesis in melanocytes[11,12].

Substances that inhibit tyrosinase activity may be useful ingredients to be incorporated into cosmetic fields. Some products, such as arbutin, kojic acid and hydroquinone, are the most popular agents to inhibit melanogenesis in the skin. In addition, as a whitening activity studies using natural plant, Ficus deltoidea extract exerted anti-melanogenic activity by preventing tyrosinase activity in B16F10 melanoma cells[13], and Pini nodi lignum extract also had inhibitory effects of melanogenic in HM_3KO melanoma cells[14]. However, the effects of hexane extract of *Kaempferia galanga L.* on the anti-melanogenic has not yet been reported.

Kaempferia galanga L. (Zingiberaceae), one of the perennial traditional aroma medicinal herbs, is mainly distributed in the tropics and subtropics of Asia, including Southern China, Malaysia and India. It is commonly used in traditional medicine for the management of swelling, rheumatism, cough, dysentery, diarrhea, stomachache, headache, toothache, inflammatory tumours, hypertension and also has a long history in the treatment of restlessness, stress, anxiety[15,16]. Previous studies reported properties of the extracts of K. galanga including anti-microbial[17], anti-oxidant[18], anti-allergic[19], anti-inflammatory[20]. In spite of these valuable effects, Kaempferia galanga L is comparatively little known for its biological effects on the skin. This study was conducted to determine effect of hexane extract of *Kaempferia galanga L.* (HKG) on melanogenesis in B16F10 melanoma cells.

Materials and Methods

1. Materials and cell cultures

Kaempferia galanga L. was purchased from Sinwoo Oriental Herb shop(Seoul, Korea). Tyrosinase, TRP-1 and TRP-2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). B16 melanoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum and 100 units/ml penicillin, 100 μg/ml streptomycin. The cells were cultured at 37℃ in a humidified chamber with 5% CO_2.

2. Preparation of *Kaempferia galanga L.* extract

The *Kaempferia galanga L.* (300 g) was soaked in 3 L of 95% methanol for 3days at room temperature with sonication for 30 min once a day. The solvent was then decanted and filtered. The filtrate solvent from extraction was evaporated at 40℃ under reduced pressure using a ratory evaportator to give an methanolic crude extract (16.55 g). It was further extracted with 1 L of hexane and 900 ml of water to give a hexane-soluble fraction (2.9 g).

3. MTT assay

The general viability of cultured cells was determined through the reduction of 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrabromide (MTT) to formazan. After HKG treatment, cells were incubated for 3 days at 37℃ in a 5% CO_2 atmosphere. MTT (5 mg/ml in PBS) was added to each well at a 1/10 volume of media. Cells were incubated at 3 7℃ for 3h, and dimethyl sulfoxide was added in order to dissolve the formazan crystals. The absorbance was then measured at 570 nm using a spectrophotometer.

4. Measurement of tyrosinase activity

Tyrosinase activity was determined using a modification of the method described[21]. B16 cells were cultured at 8×10^4 cells/well (6-well plates). After 24h, cells were pretreated with concentrations of HKG (12.5, 25 μg/ml) for 1 h and then stimulated with α-MSH (100 nM) for 3 days. The cells were harvested and the pelleted cells washed twice with phosphate-buffered saline (PBS). The cells were lysed in 200 μl of 0.1 M sodium phosphate buffer (pH 6.8) contatining 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (PMSF). After incubation for 30 min to release tyrosinase from the melanosome membrane, cellular extracts were clarified by centrifugation at 15,000rpm for 30 min at 4℃. The supernatant (50 μl) and 100 μl of 0.1 M sodium phosphate buffer (pH 6.8) were put in a 96-well plate, and the enzymatic assay was started by the adding 50 μl of 0.1% L-DOPA in sodium phosphate buffer (pH 6.8). Absorbance at 405 nm was read every 30 min for 1h at 37℃ using an ELISA plate reader.

5. Measurement of melanin content

Melanin content of cultured B16 melanoma cells were measured according to the method of slight modification[22]. The colors of cell pellets were evaluated visually, and pellets were solubilized in boiling 1 M NaOH containing 10% DMSO. The samples were incubated at 90℃ for 1 h and solubilized the melanin. Spectro- photometric analysis of melanin content was performed at 405 nm absorbance.

6. Western blot analysis

B16 cell lysates were separated by SDS-PAGE (10% polyacrylamide gels) and then transferred to polyvinylidene fluoride (PVDF). The membranes were then probed with

tyrosinase, TRP-1, TRP-2, P-p38, p38, P-JNK, JNK, P-Akt, Akt. Briefly, the cultured B16 melanoma cells were washed with PBS, then incubated with RIPA lysis buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 NaCl, 0.01 M sodium phosphate (pH 7.2), 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) for 30 min on ice. Following incubation, the cell lysates were cleared by centrifugation at 15,000 rpm for 30 min and the resultant supernatants collected and used to estimate protein concentration by Bradford assay. Then 10 μg of total protein lysates were resolved on 10% sodium dodecyl sulphate pholyacrylamide gels (SDS-PAGE) and the separated proteins were transferred to PVDF membrane. The level of protein expression in each sample was detected using specific primary antibodies diluted in TBST solution containing 5% (w/v) of skim milk or 3% (w/v) of BSA. The membranes were incubated with a specific HRP-conjugated secondary antibody and developed using the enhanced chemiluminescent substrate from West zol-plus then, they were stripped and reproved with β-actin primary antibody as a protein loading control.

7. Statistical analysis

Experimental values were expressed as mean ± SD. The significance of differences was determined by Student''s t-test and expressed as a probability value. Mean differences were considered to be significant when $P<0.05$ and $P<0.01$.

Results

1. Cell viability

The MTT assay was used to assess the effect hexane extract of *Kaempferia galanga L.* (HKG) on B16F10 melanoma cells. The cells were treated with various concentration of HKG (6.25, 12.5, 25, 50, 100 μg/ml) for 2 and 3 days, and then MTT assay was performed. As shown Fig. 1, the extract had no significantly cytotoxic effect on B16F10 cells at the concentration used.

2. Tyrosinase activity

In this study, HKG displayed no inhibitory effect on mushroom tyrosinase activity (data not shown), suggesting that HKG dose not directly influence on the tyrosinase activity. Thus, to examine the inhibitory effect of the HKG on the cellular tyrosinase activity, the cells were pretreated with HKG 12.5, 25 μg/ml for 1 h and then cultured in the presence or absence of α-MSH for 3 days, respectively. As shown in Fig. 2, HKG treatment significantly decreased the cellular tyrosinase activity in a dose-dependent manner compared to that in the control group.

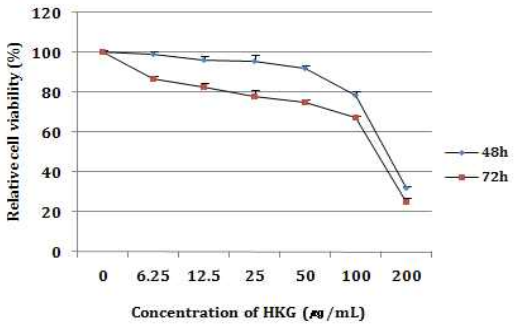

Fig. 1. Effect of HKG on B16F10 melanoma cells viability. Cell were treated with various concentration of HKG for 48h and 72h. The proportion of survival cells were measured by MTT assay. Results are the mean ± S.D. from three separated experiments. *p<0.05, **p<0.01 versus vehicle control treated cells.

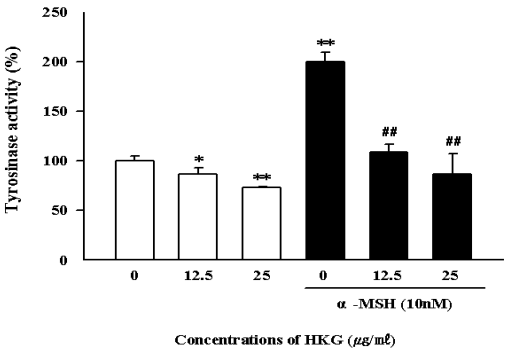

Fig. 2. Effect of cellular tyrosinase activity of HKG in B16F10 melanoma cells. (A) Cells were incubated with HKG at 12.5, 25 μg/ml for 3 days. (B) Cells were incubated with HKG at 12.5, 25 μg/ml and then stimulated with 100 nM of α-MSH for 3 days. The tyrosinase activity was estimated as described in Materials and methods. Results are the mean ± S.D. from three separate experiments. *p<0.05, **p<0.01 compared with the control and ##P<0.01 compared with only α-MSH.

3. Effects of HKG on the Melanin contents in B16F10 melanoma cells

Next, we examined whether HKG inhibited cellular melanin synthesis in B16F10 melanoma cells. The melanin contents in B16F10 melanoma cells in the presence or absence of α-MSH were measured at concentration of 12.5, 25 μg/ml after 3 days of treatment. As shown in Fig. 3A, HKG reduced the cellular melanin synthesis and attenuated α-MSH-induced melanin synthesis in B16F10 melanoma cells.

4. Down-regulation of tyrosinase, TRP-1, TRP-2 expressions by HKG in B16F10 melanoma cells

Above data showed that HKG treatment down-regulated cellular tyrosinase activity and melanin contents in a dose-dependent manner. Next, we investigated whether HKG can influence melanogenic enzyme expressions including tyrosinase, TRP-1 and TRP-2, western blot analysis was carried out using the B16F10 melanoma cells treated with HKG and α-MSH. As shown Fig. 4A, tyrosinase protein expression level was significantly decreased by HKG after 3 days of treatment. Also, TRP-1 was slightly decreased but TRP-2 was not modified. As shown Fig. 4B, upon exposure to α-MSH alone, a significantly increase in tyrosinase protein was observed, and TRP-1, TRP-2 expression also increased. HKG attenuated α-MSH-induced tyrosinase, TRP-1 and TRP-2 protein expression levels in a dose-dependent manner. These results suggested that HKG inhibits melanogenesis by suppressing the tyrosinase, TRP-2 and TRP-2 protein expressions.

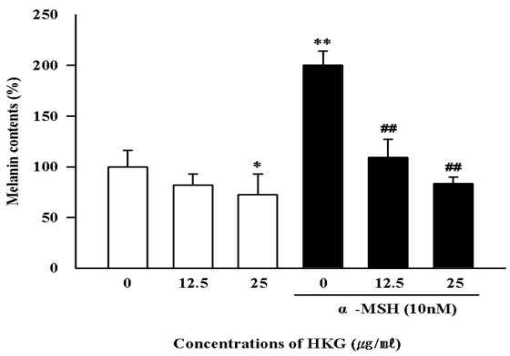

Fig. 3. Effect of HKG treatment on melanin contents of B16F10 melanoma cells. Cells were incubated with HKG at 12.5, 25 μg/ml and then cultured in the absence or presence of α-MSH (100 nM) for 3 days. The decrease in melanin production was estimated as described in Materials and methods. Results are the mean ± S.D. from three separated experiments. *p<0.05, *p<0.01 compared with the control and ##P<0.01 compared with only α-MSH.

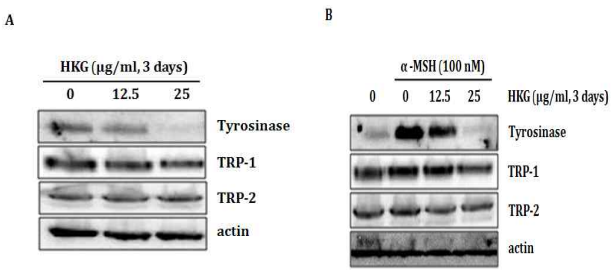

Fig. 4. Effect of HKG on melanogenic enzyme expressions in B16F10 melanoma cells. (A) Cells were incubated with 12.5, 25 μg/ml of HKG for 3 days. (B) Cells were pretreated with 12.5, 25 μg/ml of HKG for 1 h, and then stimulated with 100 nM of α-MSH for 3 days. After 3 days, whole cell lysates were then subjected to Western blot analysis using antibodies against tyrosinase, TRP-1 and TRP-2 as described in materials & methods.

5. Effects of HKG on the signal transduction pathways in B16F10 melanoma cells stimulated by α-MSH

We examined whether effects of HKG on the signal transduction pathways in B16F10 melanoma cells. Melanogenesis inhibition signaling pathway in B16F10 melanoma cells in the presence or absence of α-MSH were measured at concentration of 12.5, 25 μg/ml after 24 h of treatment. As shown Fig. 5, In melanin production by α -MSH stimulation conditions, melanin production inhibitory effect by HKG could be seen that the MAPKs and PI3K / Akt signaling pathway is associated with it. Also, We confirmed that α-MSH induced an increase in phosphorylation of p38 and JNK, and a decrease in phosphorylation of Akt.

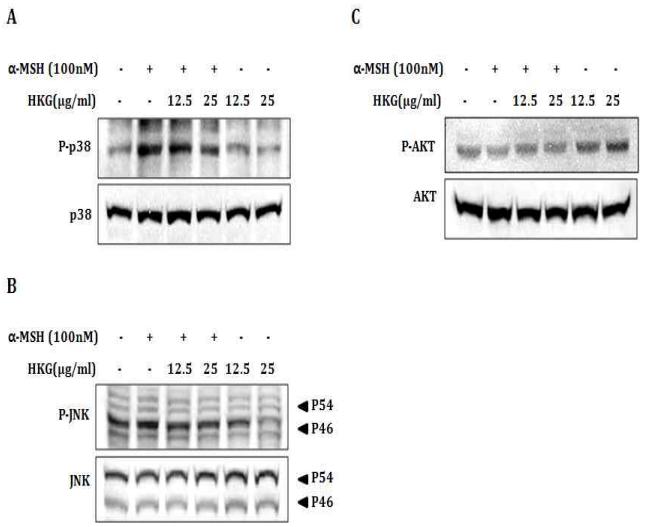

Fig. 5. Effects of HKG on the signal transduction pathways in B16F10 melanoma cells induced by α-MSH. Cells were stimulated with 100 nM of α-MSH for 1 h, and then treated with 12.5, 25 μg/ml of HKG for 24 h. After 24 h, whole cell lysates were harvested. The levels of phosphorylation of p38, JNK and Akt were analyzed by Western blot. Western blots are from one of three independent experiments. A: Phosphorylated p38 and total p38. B: Phosphorylated JNK and total JNK. C: Phosphorylated Akt and total Akt.

Discussion

Kaempferia galanga L. (Zingiberaceae), one of the perennial traditional aroma medicinal herbs, is mainly distributed in tropics and subtropics of Asia including Southern China, Indochina, Malaysia and India[23]. In China, it is specifically used in food as a spice and medicinal industry. Previous study has demonstrated that promising application of hexane extract of *Kaempferia galanga L.* and its constituents have a sedative effects in aromatherapy[24]. However, hexane extract of *Kaempferia galanga L.* on melanogenesis in B16F10 melanoma cells has not yet been well elucidated.

In this study, we investigated to determine whether hexane extract of *Kaempferia galanga L.* has a hypopigmentation effect in B16F10 melanoma cells, and found that it significantly inhibited cellural tyrosinase activity and melanin synthesis and melanogenesis-related proteins expression. However, the hexane fraction was less cytotoxic, having no significant direct killing effect on the B16F10 cells at the dose used (25 μg/ml). And it was also discovered to inhibit melanogenesis in α-MSH-stimulated B16F10 melanoma cells. Melanin is the most important chromophore in human skin and a ubiquitous pigment in animals, plants. It is the main component determining the color of skin in the innermost layer of the epidermis and melanin synthesis occurs predominantly in the melanosomes[25,26]. Melanogenesis is the physiological process by which cells produce the pigment melanin to protect against ultraviolet radiation and is known to be regulated by the tyrosinase gene family, such as tyrosinase, TRP-1 and TRP-2[27]. Tyrosinase is the rate-limiting enzyme in melanin biosynthesis in specialized organelles termed melanosomes. Tyrosinase catalyzes the oxidation of the amino acid tyrosine into DOPA and subsequently DOPAquinone[28] and thus melanin production is correlated with the expression level and the catalytic activity of tyrosinase. To investigate the mechanism of hexane extract of *Kaempferia galanga L.* in inhibiting melanogenesis, Firstly, we evaluated whether there existed potential cytotoxicity of HKG on B16F10 melanoma cells. As shown in Fig. 1, HKG had less cytotoxic effect at concentration of 25 μg/ml. However, the decrease in cellular tyrosinase activity at this dose of hexane fraction cannot be attributed to the cell cytotoxic effect because assays were normalised to use the same quantity of protein. To investigate a mechanism of the depigmenting efficacy shown by HKG, cellular tyrosinase assay and melanin contens was carried out with α-MSH-stimulated B16F10 malanoma cells. HKG signigicantly inhibited tyrosinase activity and melanin contents in the absence or presence of α-MSH with dose-dependent manner(Fig. 2, 3). These results suggested that HKG down-regulated tyrosinase activity and melanin synthesis in B16F10 melanoma cells. To clearly elucidate the molecular mechanisms of HKG induced down-regulating action, melanogenesis-related proteins was examined by western blot. As tyrosinase protein is key enzyme required melanin synthesis and plays an important role in melanogenesis. Tyrosinase activity and expression was drastically increased while DHICA oxidase and DOPAchrome tautomerase activities were constant, as was TRP-1 and TRP-2 expression[29]. Thus tyrosinase with TRP-1, TRP-2 is an important melanogenic determinant. The expression of tyrosinase, TRP-1, TRP-2 treated by 12.5, 25 μg/ml HKG in α-MSH-stimulated B16F10 melanoma cells at 3 days was examined. As shown in Fig. 4, HKG significantly decreased tyrosinase protein expression level at 3 days in α-MSH-stimulated and unstimulated B16F10 melanoma cells, and decreased TRP-1, TRP-2 protein expression level in α-MSH-stimulated.

Other several studies, melanogenic stimuli such as α-melanocyte specific hormone (α-MSH), UV irradiation, or lipopolysaccharide promote a sustained increase of phospho-p38 MAP Kinase[30]. Also Several reports indicated that Akt phosporylation regulate melanogenesis[9]. In our study, melanogenesis was regulated not only Tyrosinase, TRP1 and TRP2 but also MAPKs and Akt signaling pathway. In melanin production by α-MSH stimulation conditions, phosphorylation of Akt was increased by HKG and phosphorylation of p38 and JNK were decreased in dose-dependent manners significantly(Fig. 5). Therefore, we were confirmed that MAPKs (p38, JNK) and Akt may be related to inhibit the melanogenesis by HKG in B16F10 melanoma cells.

In conclusion, hexane extract of *Kaempferia galanga L.* inhibited cellular tyrosinase activity and melanin synthesis in α-MSH-stimulated B16F10 melanoma cells. It was also down-regulated melanogenesis-related protein (tyrosiase, TRP-1, TRP-2) expression level in a dose-dependent manner. HKG suppressed the expression of tyrosinase via controlling of p38, JNK MAPK and Akt signaling pathway. These results indicate that the hexane extract of *Kaempferia galanga L.* can be further developed and formulated into a therapeutic treatment for skin hyperpigmentation disorders.

Acknowledgements

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