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Inhibitory effect of *Gastrodia elata* Blume extract on alpha-melanocyte stimulating hormone-induced melanogenesis in murine B16F10 melanoma

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BACKGROUND/OBJECTIVES: *Gastrodia elata* Blume (GEB), a traditional herbal medicine, has been used to treat a wide range of neurological disorders (*e.g.*, paralysis and stroke) and skin problems (*e.g.*, atopic dermatitis and eczema) in oriental medicine. This study was designed to investigate whether GEB extract inhibits melanogenesis activity in murine B16F10 melanoma.
MATERIALS/METHOD: Murine B16F10 cells were treated with 0-5 mg/mL of GEB extract or 400 μg/mL arbutin (a positive control) for 72 h after treatment with/without 200 nM alpha-melanocyte stimulating hormone (α-MSH) for 24 h. Melanin concentration, tyrosinase activity, mRNA levels, and protein expression of microphthalmia-associated transcription factor (MITF), tyrosinase, tyrosinase-related protein (*Trp*)1, and *Trp2* were analyzed in α-MSH-untreated and α-MSH-treated B16F10 cells.
RESULTS: Treatment with 200 nM α-MSH induced almost 2-fold melanin synthesis and tyrosinase activity along with increased mRNA levels and protein expression of MITF, tyrosinase, *Trp1* and *Trp2*. Irrespective of α-MSH stimulation, GEB extract at doses of 0.5-5 mg/mL inhibited all these markers for skin whitening in a dose-dependent manner. While lower doses (0.5-1 mg/mL) of GEB extract generally had a tendency to decrease melanogenesis, tyrosinase activity, and mRNA levels and protein expression of MITF, tyrosinase, *Trp1*, and *Trp2*, higher doses (2-5 mg/mL) significantly inhibited all these markers in α-MSH-treated B16F10 cells in a dose-dependent manner. These inhibitory effects of the GEB extract at higher concentrations were similar to those of 400 μg/mL arbutin, a well-known depigmenting agent.
CONCLUSIONS: These results suggest that GEB displays dose-dependent inhibition of melanin synthesis through the suppression of tyrosinase activity as well as molecular levels of MITF, tyrosinase, *Trp1*, and *Trp2* in murine B16F10 melanoma. Therefore, GEB may be an effective and natural skin-whitening agent for application in the cosmetic industry.

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INTRODUCTION

Human skin color is affected by genetics and many factors, mainly by amounts and types of melanin. Melanin is produced in the melanosomes of melanocytes and transferred from melanocytes to keratinocytes in the basal layer of the epidermis in response to the exposure of skin to sunlight, stress, and melanin-stimulating factors [*e.g.*, alpha-melanocyte stimulating hormone (α-MSH), nitric oxide, and endothelin-1] [1,2]. For example, α-MSH promotes melanogenesis through microphthalmia-associated transcription factor (MITF) induction, which is a strong inducer of the melanocyte-specific enzymes such as tyrosinase, tyrosinase-related protein (*Trp)1*, and *Trp2* [1]. Melanin production is primarily regulated by tyrosinase, the rate-liming enzyme for melanogenesis, which catalyzes the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) by monophenolase and the oxidation of L-DOPA to dopaquinone by diphenolase, which is a common step for both eumelanin and pheomelanin biosynthesis. The subsequent steps are involved in two additional melanocyte-specific enzymes, *Trp*1 and *Trp2* (also known as dopachrome tautomerase), to produce eumelanin (brown/black pigment) and the cysteine/glutathione-associated non-enzymatic step to produce pheomelanin (red/yellow pigment), respectively. Tyrosinase inhibitors such as hydroquinone [3,4], kojic acid [5], ascorbic acid [6], and arbutin [7] have been used to treat hyperpigmentation disorders but they have recently been reported regarding several side effects in humans [1,2]. Consequently, much attention is paid to finding safer and more effective melanogenic inhibitors from natural sources that can induce depigmentation without side effects.

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Gastrodia elata Blume (GEB), an orchid plant devoid of chlorophyll, requires the symbiotic fungi, *Mycena osmundicola* and *Armillaria mellea* for its germination and growth/maturation, respectively [8,9]. GEB has been used as an oriental medicinal remedy to treat neurodegenerative disorders (*e.g.*, paralysis, vertigo, stroke, epilepsy, and dementia) [9,10]. A variety of bioactive compounds have been isolated from GEB, the most important ones are the phenolic compounds such as gastrodin, 4-hydroxybenzyl alcohol, 4-hydroxybenzaldehyde, vanillin, vanillyl alcohol, and vanillic acid [8-12]. It has been recently reported that bioactive compounds of GEB have an inhibitory potency for tyrosinase activity [13-16]. Moreover, recent studies have identified ergothioneine (Ergo) [8,11] and diosgenin [17] in GEB. Ergo is mainly found in mushrooms [18,19], while diosgenin in wild yam [20]. Both of them have been listed in the top 10 botanical ingredients present on anti-aging creams in 2010 owing to their strong antioxidant activity [21], suggesting that GEB may be used as a functional cosmetic agent as well as in medicinal food.

Although there are reports on the composition of freeze-dried and steam-dried GEB [22], its chemical constituents [11,12,23], and antioxidant [12], anti-platelet and anti-thrombotic [24] effects, little is known about the inhibitory effects of GEB extract on melanin synthesis and its relevant enzymes in B16F10 melanoma. Since GEB has been used as a traditional remedy for neurological disorders and skin problems because of its bioactive compounds and antioxidant properties, we analyzed the melanin content and tyrosinase activity as well as molecular levels of transcription factor and regulatory enzymes in murine B16F10 melanoma.

MATERIALS AND METHODS

Preparation of Gastrodia elata Blume (GEB) extract

GEB were obtained from the Hyewonchunma Farm (Chuncheon, Korea). The dried GEB (42°C for 16 h) were ground and then GEB powder (10 g) was extracted in 200 mL of de-ionized H_2O at 90°C for 3 h, followed by centrifugation at 4,500 rpm. The supernatant was filtered using filter paper with 6 μm pore size (No. 3; Whatman, Little Chalfont, England), and concentrated under vacuum using a rotary evaporator (Rotavapor R-100; Buchi, Flawil, Switzerland). The frozen extract was evaporated to a freeze-dried powder for 72 h using a laboratory freeze dryer (Freezone plus 6; Labconco, Kansas City, MO, USA). The GEB extract powder was stored at -20°C before use.

Cell culture

Murine B16F10 melanoma line (Korean Cell Line Bank, Seoul, Korea) was cultured in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco) in a 5% CO_2 atmosphere. B16F10 cells were cultured until 100% confluent for all experiments.

Cell viability assay

B16F10 cells were plated at a density of 4×10^4 cells/well in a 24-well plate. The 100% confluent cells were pretreated with 0-25 mg/mL of GEB extract for 24 h after treatment with/without 200 nM α-MSH (Sigma-Aldrich Co., St. Louis, MO, USA) for 24 h. The medium was replaced with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Amresco, Solon, OH, USA) dye solution [5 mg/mL in phosphate- buffered saline (PBS)] at 37°C for 3 h. The supernatant was decanted and the formazan salts were dissolved in 500 μL isopropanol. Absorbance was measured at 570 nm using a spectrophotometer (Gen5.2; Biotek, Winooski, VT, USA).

Melanin content analysis

B16F10 cells were treated with 0-5 mg/mL of GEB extract or 400 μg/mL arbutin (a positive control; Sigma-Aldrich) for 72 h after treatment with/without 200 nM α-MSH for 24 h. The cells were washed twice with 1X PBS, followed by lysis in radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Scientific, Rockford, IL, USA) and centrifugation at 14,000 rpm for 10 min at 25°C. The pellet (approximately 10^5 cells) was solubilized in 1 mL of 1N NaOH with 10% dimethyl sulfoxide for 2 h at 80°C. The absorbance of the supernatant was measured at 470 nm. The melanin concentration in the samples was derived from a synthetic melanin (Sigma-Aldrich) standard curve. Each sample was normalized to a total cellular protein content using the Pierce® BCA method (Thermo Scientific).

Measurement of tyrosinase activity

B16F10 cells were treated with 0-5 mg/mL of GEB extract or 400 μg/mL arbutin (a positive control) for 72 h after treatment with/without 200 nM α-MSH for 24 h. The cells were washed twice with 1X PBS, followed by lysis in RIPA lysis buffer (Thermo Scientific) and centrifugation for 15,000 rpm for 20 min at 4°C. The supernatant (100 μL) was placed in a 96-well plate and mixed with 100 μL of 5 mM L-DOPA and incubated at 37°C for 2 h. The absorbance was measured at 420 nm. Each sample was normalized to a total cellular protein content using the Pierce® BCA method (Thermo Scientific).

Analysis of real time-polymerase chain reaction (real time-PCR)

B16F10 cells were treated with 0-5 mg/mL of GEB extract or 400 μg/mL arbutin (a positive control) for 72 h after treatment with/without 200 nM α-MSH for 24 h. Total RNA of the cells was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and an RNA extraction kit (RNase Mini kit; Qiagen, Hilden, Germany). Total RNA (2 μg) was reverse-transcribed using a reverse transcription master premix (ELPIS, Daejeon, Korea). Real time-PCR (LightCycler 96; Roche, Indianapolis, IN, USA) was performed using the primers: murine MITF forward 5'- GGCCAA GGCAGAGCAACTT-3', backward 5'-GCCCATGGTGGCAAGCT-3'; tyrosinase forward 5′-ATAGGTGCATTGGCTTCTGG-3′, backward 5′-CCAACGATCCCATTTTTCTT-3′; *Trp1* forward 5′-GAGTGACATCC TGTGGCTCA-3′, backward 5′-CGATACCCTGGGAACACTTT-3′; *Trp 2* forward 5′-GCATCTGTGGAAGGGTTGTT-3′, backward 5′-ACTCC TTCCTGAATGGGACC-3′ ; glyceraldehyde 3-phosphate dehydrog enase (GAPDH) forward 5′-TCAATGAAGGGGTCGTTGAT-3′, back ward 5′-CGTCCCGTAGACAAAATGGT-3′. The expression levels were normalized to that of GAPDH.

Western blot analysis

B16F10 cells were treated with 0-5 mg/mL of GEB extract or

400 μg/mL arbutin (a positive control) for 72 h after treatment with 200 nM α-MSH for 24 h. Whole cell lysates (20 μg protein each) were resolved by 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Roche), and probed with primary antibodies specific to MITF, tyrosinase, *Trp1*, and *Trp2* or β-actin (Santa Cruz Biotechnology, Dallas, TX, USA). After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz). Immunodetection was performed using a chemiluminescence method (SuperSignal; Pierce Biotechnology, Rockford, IL, USA) and then normalized with β-actin.

Statistical analysis

Statistical analysis was performed using IBM SPSS software (Ver. 23; IBM-SPSS, Armonk, NY, USA). A one-way analysis of variance (ANOVA) followed by a post-hoc analysis with Tukey's test were used to detect differences between experimental groups. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Effects of Gastrodia elata Blume (GEB) on cell viability in B16F10 cells

To examine the potential toxicity of GEB extract, we first evaluated GEB cytotoxicity by incubating B16F10 cells with various concentrations (0-25 mg/mL) of GEB extract after treatment with/without 200 nM α-MSH for 24 h. Irrespective of α-MSH stimulation, cell viability was not inhibited by low GEB concentrations (0.5-5 mg/mL), whereas higher concentrations (10-25 mg/mL) significantly induced toxicity (Fig. 1). Cell viability at 10 and 25 mg/mL of GEB was 85.9% and 68.7% of the untreated control, respectively. Cell viability at 10 and 25 mg/mL of GEB after α-MSH stimulation was 89.9% and 65.3% of the untreated control, respectively. Therefore, a concentration of 0-5 mg/mL was used in further experiments.

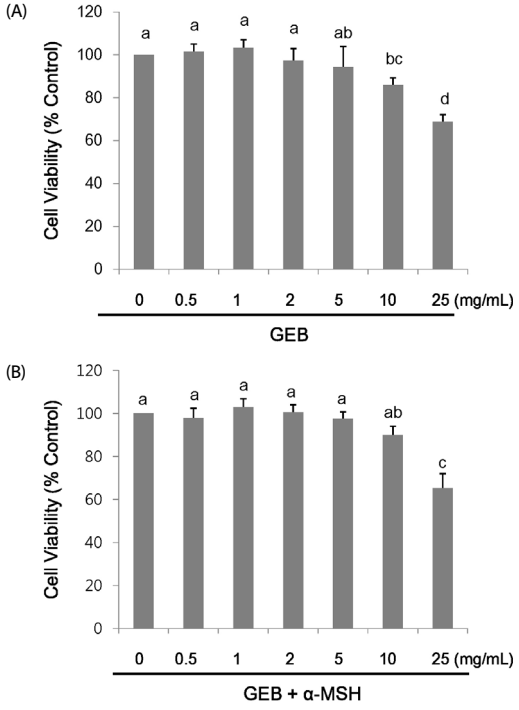

Fig. 1. Effect of *Gastrodia elata* Blume (GEB) extract on cell viability in B16F10 cells. The cells were plated at 4×10^4 cells/well and incubated in media containing 0-25 mg/mL concentrations of GEB for 24 h after treatment with (panel B)/without (panel A) 200 nM alpha-melanocyte stimulating hormone (α-MSH) for 24 h. Each bar represents the mean ± SD (n = 5). Different letters indicate a significant difference according to the ANOVA (P< 0.05).

Effect of Gastrodia elata Blume (GEB) on melanin synthesis and tyrosinase activity in B16F10 cells

To assess the melanogenesis activity of GEB extract, we measured melanin content in both α-MSH-untreated and α-MSH-treated B16F10 cells. The cells were pretreated with/without α-MSH for 24 h, followed by treatment with GEB extract at doses of 0-5 mg/mL or 400 μg/mL arbutin for 72 h. GEB treatment significantly decreased the cellular melanin content in a dose-dependent manner in higher doses (2-5 mg/mL) in the α-MSH-treated groups and all of the α-MSH-untreated groups. α-MSH alone increased melanin content by 180% compared to the control, and GEB treatment markedly inhibited melanin synthesis in the α-MSH-induced hyperpigmentation state in a dose-dependent manner compared to α-MSH-untreated groups (Fig. 2).

Since tyrosinase is the rate-limiting enzyme of melanin synthesis, we determined the inhibitory effect of GEB extract on cellular tyrosinase activity in B16F10 cells. α-MSH induced tyrosinase activity by 171.2% in B16F10 cells. Irrespective of α-MSH treatment, GEB extract significantly inhibited tyrosinase activity. Arbutin is well known as an inhibitor of melanin synthesis via tyrosinase pathway [7], which was significantly inhibited by 35-40% compared to untreated control in both the α-MSH-untreated and α-MSH-treated groups (Fig. 3). The melanin content and tyrosinase activities of GEB extract at 2 and 5 mg/mL were similar to those of 400 μg /mL arbutin, suggesting that GEB displays a dose-dependent anti-melano-

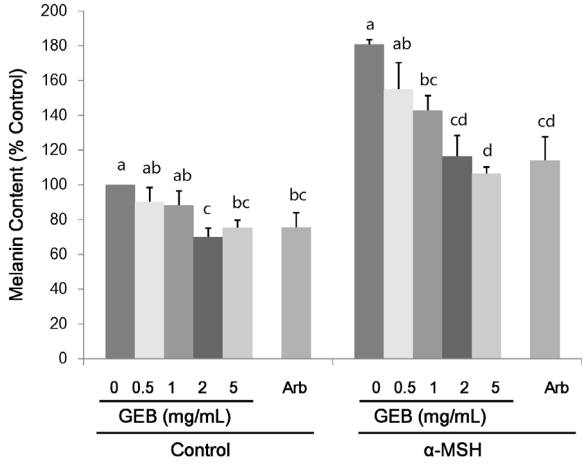

Fig. 2. Effect of *Gastrodia elata* Blume (GEB) extract on melanin content in B16F10 cells. The cells were treated with 0-5 mg/mL of GEB extract or 400 μg/mL arbutin (Arb, a positive control) for 72 h after treatment with/without 200 nM α-MSH for 24 h. The melanin concentration in the samples was derived from a synthetic melanin standard curve and each sample was normalized to a total cellular protein. Each bar represents the mean ± SD (n = 4). Different letters indicate a significant difference according to the ANOVA (P< 0.05).

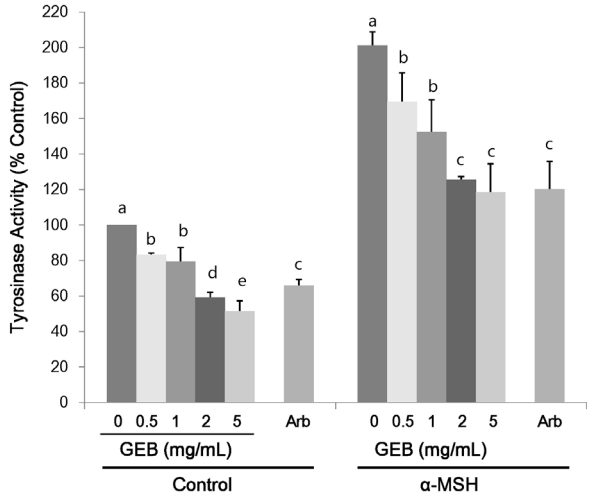

Fig. 3. Effect of *Gastrodia elata* Blume (GEB) extract on tyrosinase activity in B16F10 cells. The cells were treated with 0-5 mg/mL of GEB extract or 400 μg/mL arbutin (Arb, a positive control) for 72 h after treatment with/without 200 nM α-MSH for 24 h. The supernatant lysed in RIPA buffer was incubated with 5 mM L-DOPA for 2 h and each sample was normalized to total cellular protein. Each bar represents the mean ± SD (n = 4). Different letters indicate a significant difference according to the ANOVA ($P < 0.05$).

genesis activity to the level of a positive control and that this melanogenesis inhibition by GEB extract is related to decreased cellular tyrosinase activity (Fig. 3).

Effects of Gastrodia elata Blume (GEB) on mRNA levels and protein expression of MITF, tyrosinase, Trp1, and Trp2 in B16F10 cells

To determine whether the inhibition of melanin synthesis by GEB extract was associated with melanogenesis-related gene expression, we performed real time-PCR to examine the mRNA levels of MITF, tyrosinase, *Trp1*, and *Trp2*. mRNA levels of all these genes tended to be decreased by GEB extract in a dose-dependent manner in both the α-MSH-untreated and α-MSH-treated groups, but they were negligible in α-MSH-untreated cells. Molecular levels of MITF, tyrosinase, *Trp1*, and *Trp2* were inhibited at low GEB concentrations (0.5-1 mg/mL) and were markedly but not significantly inhibited at higher concentrations (2-5 mg/mL) in α-MSH-untreated cells. α-MSH markedly induced these genes' mRNA levels but GEB extract reduced it. Even in α-MSH-treated cells, lower doses of GEB extract had a tendency to decrease MITF, tyrosinase, *Trp1*, and *Trp2* mRNA levels, whereas higher doses of GEB extract significantly inhibited mRNA levels of all these genes. The inhibition levels of GEB extract at higher doses were similar to arbutin, which is a well-known skin whitening ingredient in cosmetics (Fig. 4).

Furthermore, we evaluated the protein expression of these transcription factor and melanogenic enzymes by western blot

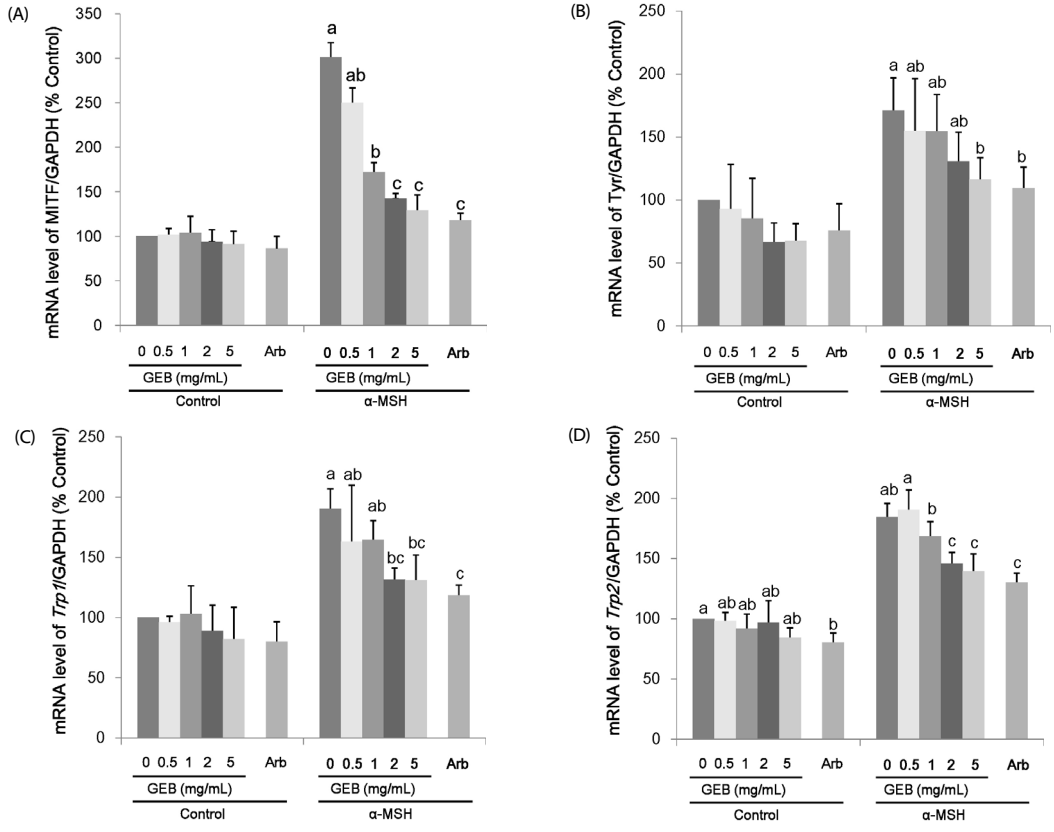

Fig. 4. Effects of *Gastrodia elata* Blume (GEB) extract on mRNA levels of (A) MITF (B) tyrosinase (Tyr), (C) *Trp1*, and (D) *Trp2* in B16F10 cells. The cells were treated with 0-5 mg/mL of GEB extract or 400 μg/mL arbutin (Arb, a positive control) for 72 h after treatment with/without 200 nM α-MSH for 24 h. Real time-PCR was performed using gene specific primers for MITF, tyrosinase, *Trp1*, and *Trp2*. The expression levels were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Each bar represents the mean ± SD (n = 3). Different letters indicate a significant difference according to the ANOVA ($P < 0.05$).

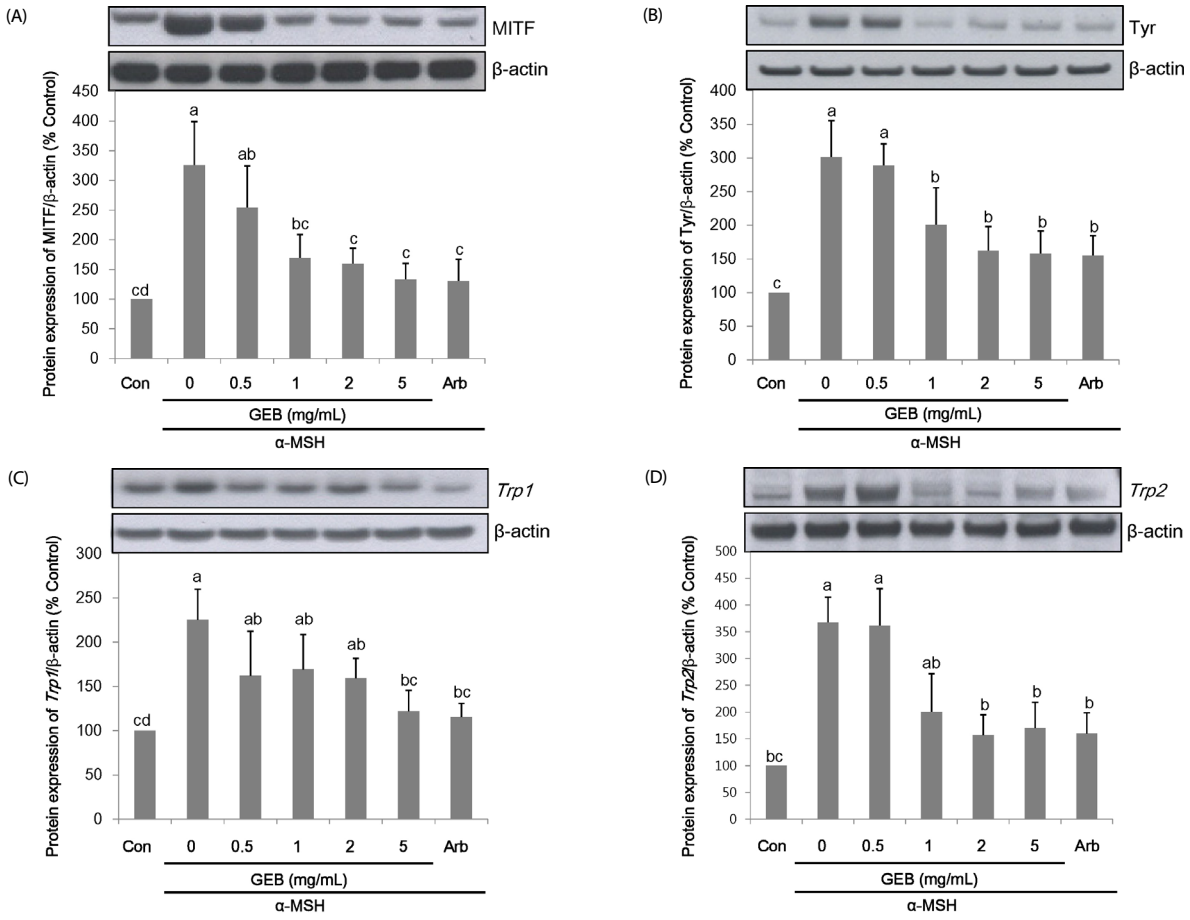

Fig. 5. Effects of *Gastrodia elata* Blume (GEB) extract on protein expression of (A) MITF (B) tyrosinase (Tyr), (C) *Trp1*, and (D) *Trp2* in B16F10 cells. The cells were treated with 0-5 mg/mL of GEB extract or 400 μg/mL arbutin (Arb, a positive control) for 72 h after treatment with/without 200 nM α-MSH for 24 h. Immunoblotting was performed with specific antibodies for MITF, tyrosinase, *Trp1*, and *Trp2*. The expression levels were normalized to that of β-actin. Each bar represents the mean ± SD (n = 3). Different letters indicate a significant difference according to the ANOVA ($P < 0.05$).

analysis. α-MSH treatment significantly induced protein expression as well as mRNA levels of all these genes, which were recovered in a dose-dependent manner. While lower doses of GEB extract had a tendency to decrease α-MSH-induced protein expression of MITF, tyrosinase, *Trp1*, and *Trp2*, higher doses significantly inhibited all these markers in α-MSH-treated B16F10 cells in a dose-dependent manner. The inhibition levels of GEB extract at higher doses were as effective as arbutin (Fig. 5).

DISCUSSION

In this study, we have found that GEB extract have inhibitory activity against melanogenesis and the activity and molecular expression of its relevant enzymes such as MITF, tyrosinase, *Trp1*, and *Trp2* in murine B16F10 melanoma cells. GEB extract at doses of 0.5-5 mg/mL inhibited melanogenesis-related markers in a dose-dependent manner. While lower doses (0.5-1 mg/mL) of GEB extract had a tendency to decrease melanogenesis, tyrosinase activity and mRNA levels and protein expression of MITF, tyrosinase, *Trp1*, and *Trp2*, whereas higher doses (2-5 mg/mL) of GEB extract significantly inhibited all these markers cells in a dose-dependent manner in both α-MSH-untreated and α-MSH-treated B16F10. These inhibitory effects of GEB extract at higher concentrations were similar to those of 400 μg/mL arbutin.

Melanin is formed from L-tyrosine in the melanosomes and it determines the characteristic color of the skin, hair, and eyes [1]. Although melanin plays protective role against ultraviolet (UV) light and stress, the over-production and accumulation of the melanin pigment results in hyperpigmentation and several skin problems such as freckles, age spots, and melasma in mammals [25]. Therefore, melanogenesis inhibition has been a matter of concern for medicinal and cosmetic treatments of skin depigmentation and lightening. The binding of α-MSH to the melanocortin-1 receptor (MC1R) increases cyclic adenosine monophosphate (cAMP) and subsequently activates the MITF. MIFT effectively up-regulates the expression of melanogenic enzymes, including tyrosinase, *Trp1*, and *Trp2*, which structurally share a 40-45% similar identity [1,2]. Most of all, tyrosinase inhibition is the most common approach to achieve skin hypopigmentation because this enzyme catalyzes the rate-limiting step of pigmentation. Both *Trp1* and *Trp2* are involved in consequent biosynthesis of melanin [1,2].

Tyrosinase, also known as monophenol monooxygenase or

polyphenol oxidase, is a multifunctional copper-containing enzyme [1]. Tyrosinase has two distinct catalytic activities: monophenolase and diphenolase. Tyrosinase contains two copper ions, that coordinate with histidine residues in the active site and they are critical for the catalytic activity [1]. It has been reported that tyrosinase is inhibited by hydroquinone [3,4], kojic acid [5], ascorbic acid [6], and arbutin [7]. However, these agents have been reported to cause skin irritation, poor skin penetration, low stability, and even potential carcinogenicity. On this matter, it is necessary to develop natural products in the cosmetics industry.

Lee *et al*. [26] screened 100 plant extracts for cosmetic use by measuring the inhibitory activities of tyrosinase and DOPA auto-oxidation and reported that many plant extracts such as *Gastrodia elata*, *Chaenomeles speciosa*, *Dryopteris crassirrhizoma*, *Glycyrrhizaglabra*, *Morusalba*, *Myristicafragrans*, *Rheum palmatum*, *and Sophorajaponica* showed an inhibition of mushroom tyrosinase activity. Chen *et al*. [2] also screened 78 medicinal herbal plants and found out that the rhizome of GEB has a potential to inhibit tyrosinase activity. Although GEB has been used as an oriental medicinal remedy to treat neurodegenerative disorders, there is high possibility to use GEB as a tyrosinase inhibitor from these screening studies.

A recent study [9] reported 64 GEB components using various chromatography techniques. Among them, the most important phenolic compounds are gastrodin [4-(β-d-glucopyranosyloxy) benzyl alcohol], 4-hydroxybenzyl alcohol (4-HBA), 4-hydroxy-benzaldehyde, vanillin (4-hydroxy-3-methoxybenzaldehyde), vanillyl alcohol (4-(hydroxymethyl)-2-methoxyphenol), and vanillic acid (4-hydroxy-3-methoxybenzoic acid) [8-12]. New natural compounds have been recently found in GEB; bis (4-hydroxybenzyl) sulfide [2], Ergo [8], and diosgenin [17]. The structure of hydroquinone and major phenolic compounds of GEB are quite similar [27]. Hydroxyl group (-OH) of hydroquinone is substituted with a hydroxymethyl group (-CH_2OH) in 4-HBA [27] and carbonyl group (-CHO) in 4-hydroxybenzaldehyde. A methoxy group (-OCH_3) is added to hydroquinone to form vanillyl alcohol and to 4-hydroxybenzaldehyde to form vanillin, respectively. Vanillic acid is an oxidized form of vanillin.

Recent studies reported that most of these compounds exert inhibitory activity against mushroom tyrosinase and melanogenesis in B16 [14,28] and HM3KO melanoma cells [13], normal human foreskin fibroblasts [29], and *in vivo* zebrafish models [2,29]. For example, the isolated phenolic fraction from butanol-extracted GEB [14] exerted inhibitory effects on melanogenesis by decreasing the activity of tyrosinase, *Trp1*, and *Trp2* as well as mRNA and protein expression of these enzymes. Gastrodin [15] and 4-HBA [16] suppressed melanogenesis by inhibiting tyrosinase activity in B16 melanoma cells. The hydroxyl groups of gastrodin and 4-HBA interacted primarily with histidine residues in the active site of tyrosinase, which led to inhibition of tyrosinase activity [30]. The oxygen from the phenolic OH of 4-HBA affects the catalytic activity of tyrosinase by a the nucleophilic attack [31]. Hydroxy and methoxy groups of vanillic acid and vanillyl alcohol act as inhibitors of the diphenenolase activity of tyrosinase [32] and especially the conversion of CH_2OH in vanillyl alcohol to -COOH (vanillic acid), which leads to a marked increase in tyrosinase inhibition [32]. Vanillic acid isolated from *Origanum vulgare* reduced cellular tyrosinase activity, DOPA oxidase and melanin contents, as well as down-regulating the expression of MC1R, MITF, *Trp1*, and *Trp2* in B16F10 cells stimulated with α-MSH. Vanillin did not express inhibition of tyrosinase activity [29]. Recently, a natural product isolated from GEB, bis(4-hydroxybenzyl)sulfide, acted as a strong competitive tyrosinase inhibitor to block melanin synthesis in human epidermal melanocytes and zebrafish models by showing coordinating the sulfur atom of bis (4-hydroxybenzyl) sulfide with copper ions in the active site of tyrosinase through computational molecular modeling [2]. These results suggest that a variety of phenolic compounds of GEB may act as tyrosinase inhibitors by interacting with copper ions and/or histidine residues in the active site of tyrosinase.

Ergo and diosgenin were selected as one of top 10 botanical ingredients in the 2010 anti-aging creams [21]. Ergo is a natural water-soluble thiol amino acid [18,19], recently found in GEB [8]. At physiological pH, Ergo exists mainly in the thione (= S) rather than the thiol (-SH) form [19]. Its slow degradation, resistance to disulfide formation, and auto-oxidization make it more stable than glutathione [18,19]. The presence of the sulfur substituted imidazole ring in Ergo inhibited mushroom tyrosinase activity in a dose-dependent manner [33]. Another compound, diosgenin from wild yam (*Dioscorea villosa*) extract has a depigmenting effect and can therefore be used in melasma, melanodermatitis, and sun lentigo [34]. A study carried out on melanoma cells has shown that the depigmenting effect is related to the activation of the cellular phosphatidylinositol-4,5-bisphosphate 3-kinase pathway, suggesting that diosgenin may be an effective inhibitor of hyperpigmentation [34]. In addition, it has anti-inflammatory and anti-aging properties by inducing anti-collagenase activity [35].

There are at least three possible signal pathways involved in the regulation of melanogenesis; cAMP-dependent, Wnt, and extracellular signal-regulated kinase signaling pathways [1]. All these signal pathways regulate melanogenesis-related enzymes, *i.e.*, tyrosinase, *Trp1*, and *Trp2* via MITF expression and/or activity [1]. The potential involvement of these signal pathways in the suppression of melanogenesis by GEB remains to be investigated in the future.

In our previous study, we demonstrated that GEB extract ameliorated skin-photoaging signs by promoting antioxidant activity with sufficient amounts of polyphenols, flavonoid, and ergo and by altering expression/activity of procollagen type I, matrix metalloproteinase-1, and elastase-1 in UVA- irradiated human dermal fibroblasts [36]. In this study, GEB inhibited melanin synthesis through the suppression of tyrosinase activity and mRNA levels and protein expression of MITF, tyrosinase, *Trp1*, and *Trp2* in a dose-dependent manner in murine B16F10 melanoma. Therefore, these studies suggest that GEB extracts may be used as effective and natural skin- whitening as well as anti-aging agents for cosmeceutical applications.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interests.

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