

Inhibitory effect of *Gastrodia elata* extract on melanogenesis in HM3KO melanoma cells

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Synopsis

Effective and safe antimelanogenic agents derived from natural products get interest continuously for both medical and cosmetic purposes. Melanin synthesis is regulated by melanogenic enzymes such as tyrosinase, tyrosinase-related protein 1 (TRP-1), and tyrosinase-related protein 2 (TRP-2) in mammals. *Gastrodia elata* (GE) has been shown to have multiple therapeutic actions related to antioxidation in many diseases. In this study, we investigate whether water extraction of GE has inhibitory effects on melanogenesis *in vitro* and on the expression of mRNA and protein of tyrosinase, TRP-1, and TRP-2 in HM3KO melanoma cells. To examine the inhibitory effect of GE on melanogenesis, mushroom tyrosinase inhibition assay; reverse transcription polymerase chain reaction; and western blotting of tyrosinase, TRP-1, and TRP-2 were performed using HM3KO melanoma cells.

In this study, the GE extract was found to significantly inhibit mushroom tyrosinase activity ($69.3 \pm 7.2\%$ of the control, $p < 0.05$), and the expression of mRNA and protein of tyrosinase, TRP-1, and TRP-2 was reduced significantly. These results suggest that the antimelanogenic effect of GE extract is mainly due to the decreased expression of mRNA and protein of tyrosinase, TRP-1, and TRP-2 in the process of melanin synthesis.

INTRODUCTION

Melanin, synthesized in the melanosome of melanocytes in mammals, is transferred to neighboring keratinocytes. The role of melanin is to protect the skin from ultraviolet (UV) damage by absorbing UV sunlight and removing reactive oxygen species (1). The color of skin and hair is determined by a number of factors, but most important is the degree and distribution of melanin pigmentation. However, its excessive accumulation

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causes hyperpigmentation induced by UV irradiation or medical conditions such as melasma, postinflammatory hyperpigmentation, and solar lentigo. Therefore, the control of melanogenesis is an important strategy in the treatment of abnormal skin pigmentation for cosmetic and medical purposes (2).

Most controllers of melanogenesis act not only by altering tyrosinase gene expression but also by altering the mechanisms responsible for the transfer of melanosomes to keratinocytes (3). The melanocyte-specific enzymes, tyrosinase and tyrosinase-related protein 1 and 2 (TRP-1 and TRP-2), are involved in converting tyrosine into melanin pigments (4,5). In particular, tyrosinase catalyzes two rate-limiting reactions involved in melanogenesis: the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), and the oxidation of DOPA, which results in the formation to dopaquinone (6). Since tyrosinase is a key enzyme in melanogenesis, it has been the target during the screening of new pigmentation inhibitors from natural products. Some examples of these products include kojic acid, arbutin, ascorbic acid derivatives, retinoic acid, azelaic acid, hydroquinone, catechins, aloesin, resveratrol, oxyresveratrol, and 4,4'-dihydroxybiphenyl (7–16).

Various plant extracts have recently been studied to find new natural antimelanogenic products. *Gastrodia elata* (GE), of the Orchidaceae family, is a traditional herbal medicine in East Asian countries. Zhao *et al.* (17) identified the components of GE Blume using the capillary zone electrophoresis, the five constituents—gastrodin (GA), 4-hydroxybenzyl alcohol (4-HBA), vanillyl alcohol, 4-hydroxybenzaldehyde (HD), and vanillin—in the extracts of GE Blume roots.

It has been used for centuries as an anticonvulsant, analgesic, and sedative for the medical treatment of headaches, epilepsy, dizziness, rheumatism, neuralgia, paralysis, hypertension, and other neuralgic disorders (18,19). A previous study showed that the anticonvulsive effect of GE was attributed to its antioxidant activity, which was due to the antioxidant actions of 4-HBA, one of several phenolic components of GE extract (20). Since GE extract has several phenolic components that have antioxidant activity, this plant has been suggested to also have a depigmenting effect.

Our group is currently trying to find new antimelanogenic substances from natural sources. In this regard, GE has long been used in the treatment of many diseases, especially skin diseases, and is well known for its antioxidant effects. In this study, we evaluate the antimelanogenic effects of GE in HM3KO melanoma cells by suppressing the expression of tyrosinase and TRP-1 mRNA and protein levels.

MATERIALS AND METHODS

GE EXTRACT

Plant extracts were purchased from the PURIMED Co., Ltd (Seoul, Korea). The pharmaceutical name was *Gastrodiae Rhizoma*, which is the tuberous root extract of the GE Blume. The dried herb recommended in the protocol provided by PURIMED Co. Ltd was purchased from a Chinese market (Hong Kong, China) and used in this study. For the extraction of water-soluble fraction, 200 mg of the plants were cut into small pieces and boiled in 200 ml of distilled water under reflux conditions. After 1 h, the mixture was filtered and the filtrate was collected. An additional 250 ml of water was added to the

plants residue and boiled for 1 h. The filtrate was collected using a 0.2- μ m syringe filter, combined with the previous filtrate sample, and allowed to cool at room temperature. The extracts were stored in the matrix tube and then sterilized using an autoclave.

TYROSINASE INHIBITION ASSAY

Tyrosinase activity was measured by its DOPA oxidase activity. The tyrosinase inhibition in a cell-free system was tested by mushroom tyrosinase (Sigma, St. Louis, MO). Preincubation was conducted with 0.05 ml of 2000 U/ml of mushroom tyrosinase in a 0.1 M phosphate buffer at pH 6.5, followed by incubation in 0.05 M phosphate buffer with the varied concentrations of GE extracts (100, 500, and 1000 μ g/ml) and 3 mM L-tyrosine for 10 min at 37°C. The tyrosinase activity was determined by optical density measured at 475 nm using an ELISA microplate reader (Molecular Device Ltd, Wokingham, United Kingdom) and is expressed as a percentage of the control value (100%); an arbutin was used as the reference.

CELL CULTURE

A pigmented melanoma cell line, HM3KO, established by Ohashi *et al.* (21) from metastatic melanoma cells of peritoneal fluids was kindly supplied by Yoko Funasaka (Kobe University School of Medicine, Kobe, Japan). Cells were grown in culture medium consisting of Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Gibco, Grand Island, NY) and 100 U/ml penicillin–streptomycin in a humidified incubator with 5% CO₂ at 37°C. The cells were regularly observed using an inverted microscope and seeded onto 60-mm dishes at a density of 1×10^5 cells per dish.

RNA EXTRACTION AND REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was extracted from HM3KO melanoma cells using TRIzol Reagent according to the manufacturer's protocol. The RNA samples (2 μ g per reaction) were reverse transcribed with reverse transcriptase (Promega, Madison, WI) in the presence of oligo-dT, and the RT product was used for amplification with *Taq* polymerase. The resulting cDNA was amplified using specific primers (Bioneer, Daejeon, Korea) (Table I). Specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were added as an internal standard for the same reverse transcriptase product. Amplification conditions were 94°C (30 s), 60°C (30 s), and 72°C (40 s) for 35 cycles (tyrosinase), 25 cycles (TRP-1, TRP-2), and 20 cycles (GAPDH), respectively. The PCR products were separated by electrophoresis on 1.2% agarose gels and stained with ethidium bromide.

WESTERN BLOT ANALYSIS

The HM3KO melanoma cells were seeded onto 60-mm dishes at a density of 1×10^5 cells per dish and cultivated by the method described above. After incubation for 24 h, the medium

Table I
Specific Primers for the Human Tyrosinase, TRP-1, TRP-2s, and GAPDH

Gene	Primer sequences
Tyrosinase	5'-TTGGCAGATTGTCTGTAGCC-3' 5'-AGGCATTGTGCATGCTGCTT-3'
TRP-1	5'-AGAGATGATCGCGAGGTCTG-3' 5'-CTGTGCCATGTGAGAAAAGC-3'
TRP-2	5'-AGAGATGATCGCGAGGTCTG-3' 5'-CTGTGCCATGTGAGAAAAGC-3'
GAPDH	5'-GGCCAGCTTTCAGGCAGAGGT-3' 5'-TGGTGCTTCATGGGCAAATC-3'

was replaced with medium containing 10 $\mu\text{g}/\text{ml}$ of GE extract and incubated for an additional 24 h. The medium was then removed, the cells were washed twice with phosphate-buffered saline, and the total protein was extracted using radioimmunoprecipitation assay (RIPA) buffer (Sigma, St Louis, MO) according to the manufacturer's instructions. The protein content was measured, using a Bio-Rad colorimetric protein assay kit (Bio-Rad, Hercules, CA). An aliquot (30 $\mu\text{g}/\mu\text{l}$) of protein was fractionated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 10% gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Postfach, Germany). The primary antibodies used were raised against tyrosinase, TRP-1, and TRP-2 (1:500; Sigma). The membranes were blocked with 5% nonfat dry milk and incubated with human tyrosinase, TRP-1, TRP-2, and β -actin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Band detection was performed using the enhanced chemiluminescence detection system (Amersham Biosciences, Uppsala, Sweden), and band intensity was normalized to the β -actin measured in the same sample.

STATISTICAL ANALYSIS

Statistical analyses were performed using the Statistical Package for Social Sciences version 11.0 (SPSS Inc., Chicago, IL). Student's two-tailed *t*-test was used to evaluate the differences between the study groups. *p*-values less than 0.05 were considered statistically significant.

RESULTS

EFFECTS OF GE EXTRACT ON TYROSINASE ACTIVITY

To confirm the inhibitory effect of GE extract, mushroom tyrosinase ELISA was performed *in vitro*. The tyrosinase inhibitory activities of the GE extract were $69.3 \pm 7.2\%$ of the control ($p < 0.05$) (Figure 1).

EFFECT OF GE EXTRACT ON THE EXPRESSION OF TYROSINASE, TRP-1, AND TRP-2 mRNA

As shown in Figure 2, treatment of GE extract on HM3KO melanoma cell significantly reduced tyrosinase mRNA expression compared to the control ($43.8 \pm 17.4\%$ of control

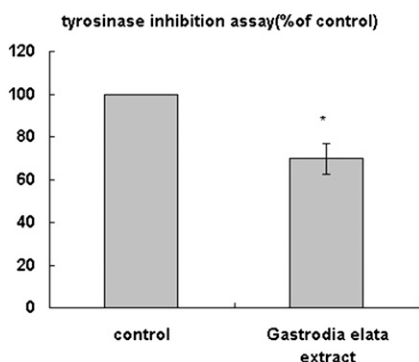


Figure 1. Mushroom tyrosinase activity was decreased significantly when treated with GE extract ($69.7 \pm 7.2\%$ of control value, $*p < 0.05$).

value, $*p < 0.05$). In addition, there was a reduction in mRNA from either TRP-1 ($45.2 \pm 12.3\%$ of control value, $*p < 0.05$) or TRP-2 ($38.6 \pm 15.9\%$ of control value, $*p < 0.05$) (Figure 2B) compared to the control (Table II).

EFFECT OF GE EXTRACT ON THE EXPRESSION OF TYROSINASE, TRP-1, AND TRP-2 PROTEINS

To confirm the inhibitory effect of GE extract on the expression of tyrosinase, TRP-1, and TRP-2 proteins, western blotting was performed to measure protein expression with β -actin as an internal control. Treatment of GE extract on HM3KO cell significantly reduced tyrosinase protein expression compared to the control ($26.1 \pm 10.1\%$ of control value, $*p < 0.05$). Moreover, the levels of both TRP-1 ($60.6 \pm 7.9\%$ of control value, $*p < 0.05$) and TRP-2 ($35.8 \pm 12.8\%$ of control value, $*p < 0.05$) proteins were significantly reduced compared to the control (Figures 3A and 3B). These results were in concordance with the mRNA expression results (Table II).

DISCUSSION

Mammalian pigmentation results from the synthesis and accumulation of photoprotective epidermal melanins that are formed from the amino acid precursor l-tyrosine within specialized cells (22). Melanin, which determines the color of the skin and hair, transfers from melanocytes to keratinocytes after synthesis in the melanosomes (23). Although melanin plays an important protective role against UV light, overproduction and accumulation of melanin pigment could create serious skin problems such as freckles, age spots, and melasma (24). The inhibition of melanogenesis has been a matter of concern in the development of medicinal and cosmetic treatments for skin depigmenting and lightening. The major mechanism of antimelanogenic action is direct inhibition of tyrosinase activity or inhibition of its gene expression. Other mechanisms include microphthalmia-associated transcription factor inhibition, downregulation of melanocortin receptor 1 activity, and interference with melanosome maturation and transfer. Another mechanism is increasing melanocyte loss via desquamation and chemical peeling. The inhibition of tyrosinase activity is the most common approach to achieve skin hypopigmentation, since

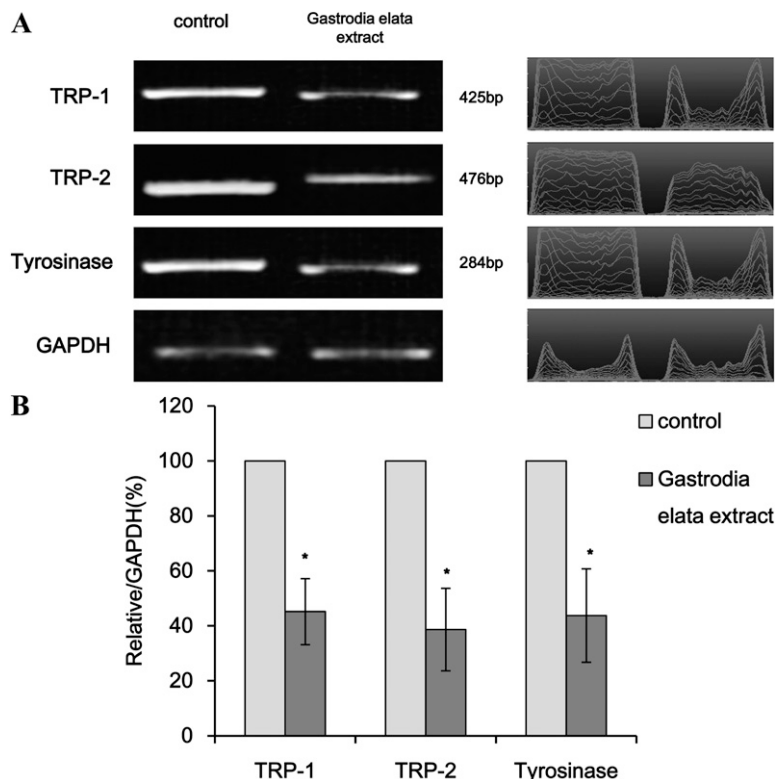


Figure 2. The inhibitory effect of GE extract on the expression of tyrosinase, TRP-1, and TRP-2 mRNA. (A) The tyrosinase gene family mRNA was measured by RT-PCR. (B) Treatment of HM3KO cells with GE extract significantly reduced tyrosinase mRNA expression compared to the control ($43.8 \pm 17.4\%$ of control value, $*p < 0.05$). Moreover, there was reduction in mRNA from either TRP-1 ($45.2 \pm 12.3\%$ of control value, $*p < 0.05$) or TRP-2 ($38.6 \pm 15.9\%$ of control value, $*p < 0.05$) compared to the control.

this enzyme catalyzes the rate-limiting step of pigmentation. Human melanocytes are known to express TRP-1 and TRP-2 as well as tyrosinase (25–27). These proteins constitute a specific family of membrane proteins that are structurally related, but with distinct enzyme functions (28). TRP-2 functions as a DOPochrome tautomerase and catalyzes the rearrangement of DOPochrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA), whereas TRP-1 oxidizes DHICA to a carboxylated indole-quinone. Both TRP enzymes

Table II
mRNA and Protein Expression of Tyrosinase, TRP-1, and TRP-2 in HM3KO Melanoma Cells Treated with GE Extract Compared to the Control

	mRNA expression by RT-PCR (% of control value)	Protein expression by western blotting (% of control value)
Tyrosinase	$43.8 \pm 17.4^*$	$26.1 \pm 10.1^*$
TRP-1	$45.2 \pm 12.3^*$	$60.6 \pm 7.9^*$
TRP-2	$38.6 \pm 15.9^*$	$35.8 \pm 12.8^*$

$*p < 0.05$.

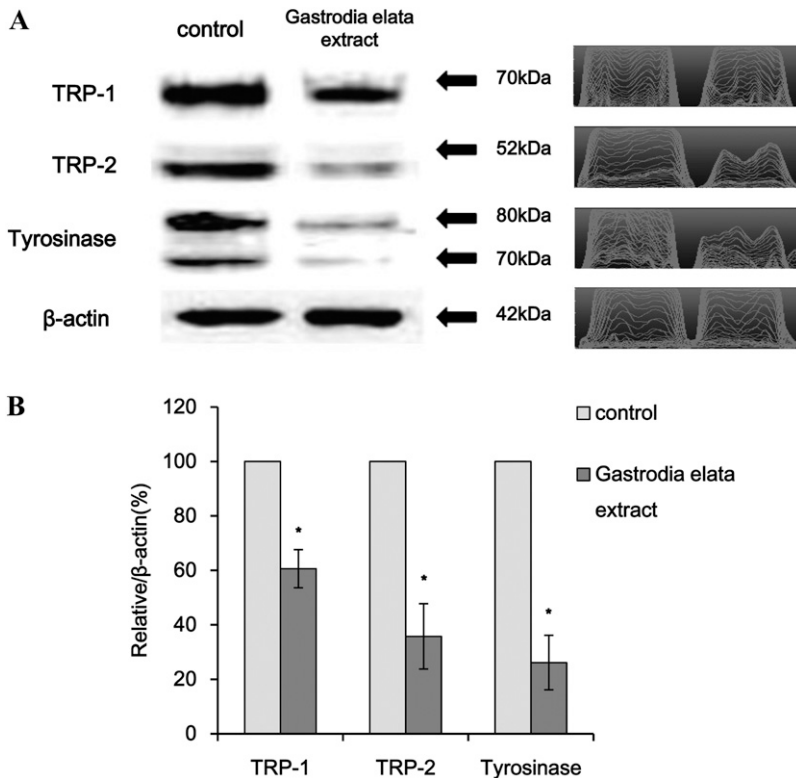


Figure 3. The inhibitory effect of GE extract on the expression of tyrosinase, TRP-1, and TRP-2 proteins. (A) Western blotting was performed to measure protein expression with β -actin as an internal control. (B) Treatment of HM3KO cells with GE extract significantly reduced tyrosinase protein expression compared to the control ($26.1 \pm 10.1\%$ of control value, $*p < 0.05$). Moreover, the levels of both TRP-1 ($60.6 \pm 7.9\%$ of control value, $*p < 0.05$) and TRP-2 ($35.8 \pm 12.8\%$ of control value, $*p < 0.05$) proteins were significantly different from the control.

are involved in the melanin biosynthetic pathway (29). A number of tyrosinase inhibitors have been reported from both natural and synthetic sources, but only a few of them are used as skin-whitening agents, primarily due to various safety concerns (30). Lee *et al.* reported the biological screening of 100 plant extracts for cosmetic use by the measurement of the inhibitory activities of tyrosinase and DOPA auto-oxidation. Many plant extracts such as *Chaenomeles speciosa*, *Dryopteris crassiribizoma*, *Gastrodia elata*, *Glycyrrhiza glabra*, *Morus alba*, *Myristica fragrans*, *Rheum palmatum*, and *Sophora japonica* have shown an inhibition of mushroom tyrosinase activity (31). Among these plants, GE of the Orchidaceae family was the agent that has been used as a medicine in Asia. In addition, it has been widely used in folk medicine and Korean traditional medicine (32) as an anticonvulsant, analgesic, and sedative agent. It has been used for the medical treatment of headaches, epilepsy, dizziness, rheumatism, neuralgia, paralysis, hypertension, and other neurologic disorders (33). In a previous study, GE extract was shown to consist of phenolic compounds and their derivatives such as 4-HBA, 4-HD, 4-hydroxy-3-methoxybenzaldehyde, and GA [4-(β -d-glucopyranosyl) benzyl alcohol] (34). Because of these phenolic compounds, it can be suggested this plant can also have a depigmenting effect.

Kim *et al.* (35) reported the extract of GE had inhibitory activity not only against mushroom tyrosinase but also against melanin synthesis in B16 melanoma cells *in vitro*. Liu *et al.* reported that 4-HBA, one of the phenolic compounds of GE extract, has an antimelanogenic effect. This inhibitory effect of 4-HBA is due to the direct inhibition of melanosomal tyrosinase activity rather than a decrease in tyrosinase gene expression (36). In our study, GE extract showed about 30% inhibition of mushroom tyrosinase at the concentration of 100 $\mu\text{g/ml}$ ($p < 0.05$). To examine the tyrosinase inhibitory mechanism of GE extract, we investigated the enzyme involved in melanogenesis and its gene expression. Our results showed that GE extract can reduce the expression of both mRNA and protein of enzymes associated with melanin biosynthesis. The mRNA and protein expression of tyrosinase in the cultured HM3KO melanoma cells was significantly reduced ($p < 0.05$). In addition, examination of melanogenic proteins showed that GE inhibited the expression of key proteins associated with melanin biosynthesis, such as tyrosinase, TRP-1, and TRP-2 in cultured HM3KO melanoma cells. These results suggest that the inhibitory effect of GE on melanogenesis is not only by inhibiting tyrosinase catalytic activity but also by inhibiting expression of melanogenic proteins at the transcriptional and translational levels. Further studies are required to elucidate the regulation of transcription factor involved in melanogenesis, to check the *in vivo* effects of GE, and to identify the active component(s) of GE which will be helpful for understanding the mechanism of antimelanogenesis. Moreover, cell study has its limitations so far and we suggest that these results should be replicated in normal melanocytes or human study.

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