Running title : Anti-Melanogenesis Effect of *Glechoma hederacea* L. Extract.

Anti-Melanogenesis Effect of *Glechoma hederacea* L. Extract on B16 Murine Melanoma Cells

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Glechoma hederacea L. (Labiatae) has been used in folk medicine to treat various ailments for centuries. We investigated the effects of *G. hederacea* extract on melanogenesis in B16 melanoma cells. It significantly reduced both the cellular melanin content and tyrosinase activity in a concentration-dependent manner. An MTT assay did not reveal any obvious cytotoxicity. Furthermore, we found that *G. hederacea* extract decreased tyrosinase and microphthalmia-associated transcription factor protein expression, but did not inhibit tyrosinase-related protein-1 and tyrosinase-related protein-2 expression. RT-PCR analysis indicated that the antimelanogenic effect of *G. hederacea* extract might be due to inhibition of tyrosinase gene transcription. Moreover, this effect is regulated *via* suppression of microphthalmia-associated transcription factor protein expression. Our data indicate that *G. hederacea* extract inhibits melanin synthesis in B16 melanoma cells but is not cytotoxic. Hence it might prove a useful therapeutic agent for treating hyperpigmentation and an effective component of whitening cosmetics.

Key words: melanogenesis; *Glechoma hederacea*; tyrosinase; tyrosinase-related protein; microphthalmia-associated transcription factor

In mammals, skin pigmentation is due to the synthesis and distribution of melanin. Melanin is a mixture of pigmented biopolymers synthesized by melanocytes.[1] Melanin-containing granules are known as melanosomes, and are exported from melanocytes to adjacent keratinocytes.[2] Melanocytes produce two types of melanin: black-to-brown eumelanin and red-to-yellow pheomelanin.[3] Melanin synthesis, called melanogenesis, is a complicated physiological process that is regulated by melanogenic enzymes. Three melanocyte-specific enzymes, tyrosinase, tyrosinase-related protein-1 (TRP-1), and dopachrome tautomerase, also known as TRP-2, are involved in melanogenesis.[4] Tyrosinase, the rate-limiting enzyme in melanogenesis, catalyzes the two initial steps of this process, the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to DOPAquinone.[5] TRP-2 catalyzes the conversion of DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA),[6,7] and TRP-1 catalyses the oxidation of DHICA to indole-5,6-quinone-2-carboxylic acid.[8,9]

Melanin production plays an important role in preventing UV irradiation. Various signaling pathways mediate melanin production, and a cAMP-dependent pathway appears to be the major pathway in UV-dependent stimulation of melanin production.[10] When keratinocytes in the skin are exposed to UV irradiation, α-melanocyte-stimulating hormone (α-MSH), a peptide hormone, is produced, and is released as a paracrine factor.[11] Secreted α-MSH binds to the melanocortin 1 receptor, which activates adenylate cyclase through G proteins to increase the levels of intracellular cAMP.[12] cAMP activates protein kinase A, which phosphorylates the transcription factor cAMP response element-binding protein at Ser133 and increases the expression of microphthalmia-associated transcription factor (MITF). MITF, characterized by an essential basic helix-loop-helix leucine zipper structure, is believed to regulate melanocyte pigmentation, proliferation, and survival.[13] It mediates the transcriptional activation of pigmentation genes, such as tyrosinase, TRP-1, and TRP-2, by binding with the M-box in the promoter regions of these genes.[14,15] Although melanin production plays an important role in protecting the skin against damage due to UV irradiation, excessive melanin biosynthesis causes several skin disorders, including melasma,

lentigines, freckles, nevus, and age spots.[16] Inhibition of tyrosinase activity is related to anti-melanogenesis, and many tyrosinase inhibitors are used in the treatment of hyperpigmentation, including kojic acid,[17] arbutin,[18] and linoleic acid.[19] However, many of the currently used skin-whitening agents exhibit toxicity toward melanocytes and produce adverse side effects. Thus, there is a need for the development of depigmenting agents.

Glechoma hederacea L. (Labiatae), commonly known as ground ivy, creeping Charlie, or gill-over-the-ground, is a perennial hairy herb with a creeping stem. It is distributed widely in Asia, Europe, and America, where it grows in shady areas, on waste ground, in dry ditches, near fences and hedges, and on the sides of moist meadows.[20] It has been used as a folk medicine for centuries in the treatment of various ailments, e.g., asthma, bronchitis, colds, diabetes, and inflammation.[21] In addition, it has been reported that *G. hederacea* extract has anti-oxidant activity,[22] but no report on melanin biosynthesis or tyrosinase activity has been demonstrated. Hence we focused on the whitening effect of this plant and evaluated its biological effect on melanogenesis as a new activity. In this study, we investigated the inhibitory effect of *G. hederacea* on melanin biosynthesis and the molecular mechanism of that effect.

Materials and Methods

Preparation of G. hederacea *extract. Glechoma hederacea* was collected at growth phase early in the morning, while it was watery. Weeds were removed from it. Soils and sands that adhered to the roots were removed, and it was washed under running tap water 4 or 5 times. The whole plant was cut to about 2-cm pieces and blended in a blender with running tap water (45 g, 100 mL) at room temperature. Then the supernatant was collected and the dregs were discarded. The supernatant was stirred slowly until it separated. When it became clear, it was filtered with cotton bags and samples were stored at 4℃. Debris was removed by centrifugation at 5000 g for 10 min before use. The samples were concentrated using a rotary evaporator (70℃, 3 h) (EYELA, Tokyo, Japan) to measure the concentration. The concentrated samples were dissolved with medium (DMEM without phenol red, Sigma, St. Louis, MO) completely for use.

Cell culture. B16F10 melanoma cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen Crop., Carlsbad, CA), 100 U/mL of penicillin, 0.1 mg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (antibiotic-antimycotic; Invitrogen) in a humidified atmosphere containing 5% CO_2 in air at 37℃. The cell passage number in the dish was 12-15, and the culture medium was changed every 2 d.

Melanin quantification. B16F10 melanoma cells were seeded at a density of 5×10^4 cells per well in 6-well culture plates and incubated for 24 h. They were then incubated with and without *G. hederacea* extract (0.1, 0.2, 0.5, 1 mg/mL) in the medium (DMEM without phenol red, Sigma) for 72 h. The melanin content was measured as described previously,[23] with some modifications. After treatment, the cells were detached by incubation in trypsin/EDTA. They were collected in a test tube and washed twice with PBS. The melanin was then extracted using 2 m NaOH at 100℃ for 30 min. The melanin content was measured by measuring the

absorbance at 405 nm (Infinite 200, TECAN, Kanagawa, Japan).

Cell viability assay. Cell viability was determined by a modification of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. B16F10 melanoma cells (1×10^5 cells/mL) were cultured in 96-well microplates with various concentrations of *G. hederacea* extract for 72 h. After treatment, the cells were treated with 10 μL of 5.5 mg/mL MTT and incubated at 37 ℃ for 4 h. A 90-μL aliquot of the extraction solution (40% *N*,*N*-dimethylformamide, 2% CH_3COOH, 20% sodium dodecyl sulfate, and 0.03 m HCl) was added to each well and mixed thoroughly by overnight agitation at room temperature. Cell viability was determined by measuring the optical density at 570 nm using a Model 550 microplate reader (Bio-Rad, Hercules, CA).

Mushroom tyrosinase activity assay. Mushroom tyrosinase activity assay was performed in the 96-well microplates. The L-DOPA oxidation activity of tyrosinase was measured by spectrophotometry as described previously,[24] with some modifications. Briefly, 160 μL of 5 mM L-DOPA (in 100 mM sodium phosphate buffer pH 6.8) and 20 μL of the same buffer with and without the test sample were placed in the wells of a 96-well microplate, and then 20 μL of mushroom tyrosinase (200 units/mL) were mixed into each well at 37 ℃ over 30 min. The absorbance at 475 nm was measured (Infinite 200, TECAN, Kanagawa, Japan).

Cellular tyrosinase activity assay. Tyrosinase activity was analyzed by spectrophotometry following the oxidation of DOPA to DOPAchrome. B16F10 melanoma cells (5×10^4 cells/well) were plated in 60-mm diameter dishes and incubated at 24 h. They were then treated with various concentrations of *G. hederacea* extract for 72 h. They were washed with PBS twice and lysed with 100 mM sodium phosphate buffer (pH 6.8) containing 1% Triton X-100 and 0.1 mM phenylmethylsulfonyl fluoride. They were then disrupted, and lysates were clarified by centrifugation at 13,000 rpm for 20 min at 4 ℃. After protein quantification and

adjustment of the protein concentration with lysis buffer, 100 μL of each lysate (each containing 100 μg the protein) was aliquoted into a 96-well plate, and 100 μL of 5 mm DOPA was then added to each well. Following incubation at 37 ℃ for 2 h, the absorbance was measured at 475 nm (Infinite 200, TECAN, Kanagawa, Japan).

Western blotting analysis. B16F10 melanoma cells were cultured in 60-mm diameter dishes with and without various concentrations of *G. Hederacea* extract (0.1, 0.2, 0.5, 1 mg/mL) for 72 h. The cells were then lysed in a buffer (1% NP40, 0.5% deoxycholic acid sodium salt, 10% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL of leupeptin, and 1 μg/mL of pepstatin A). Proteins (50 μg) were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to polyvinylidene difluoride membranes. The membranes were blocked overnight in 5% skim milk in TBST (20 mM Tris-HCl pH 7.4, 100 mM NaCl, and 0.1% Tween 20) buffer at 4 ℃. After a brief wash in TBST buffer, they were then incubated for 3 h with a primary antibody: goat anti-tyrosinase antibody (1:500), anti-TRP-1 antibody (1:500), anti-MITF antibody (1:500), or rabbit anti-TRP-2 antibody (1:500) (Santa Cruz Biotechnology, CA). After incubation, the membranes were rinsed 5 times with TBST buffer and incubated for 2 h with a secondary antibody: anti-goat IgG (1:50,000) or anti-rabbit IgG (1:2000) (Sigma). Then they were rinsed 5 times with TBST buffer. The loading control was measured using anti-β-actin antibody. Bound antibodies were detected using the ECL or ECL Prime Western Blotting Detection System (GE Healthcare, London, London). Bands were scanned by GS-800, and band intensities were quantified by measuring optical densities with Quantity One software (Bio-Rad).

Reverse transcription-polymerase chain reaction (RT-PCR). B16F10 melanoma cells were incubated with and without *G. Hederacea* extract (0.1, 0.2, 0.5, or 1 mg/mL) for 72 h. After treatment, total RNA was extracted using RNAiso Plus (TaKaRa, Otsu, Japan). cDNA was synthesized using 1 μg of total RNA with SuperScript III First-Strand Synthesis SuperMix (Invitrogen). cDNA amplification was performed

using the following primers: tyrosinase, 5′-CCA GAA GCC AAT GCA CCT AT-3′ (forward) and 5′-ATA ACA GCT CCC ACC AGT GC-3′ (reverse); TRP-1, 5′-GCT GCA GGA GCC TTC TTT CTC-3′ (forward) and 5′-AAG ACG CTG CAG TGC TGG TCT-3′ (reverse); TRP-2, 5′-GGA TGA CCG TGA GCA ATG GCC-3′ (forward) and 5′-CGG TTG TGA CCA ATG GGT GCC-3′ (reverse); MITF, 5′-CCC GTC TCT GGA AAC TTG ATC G-3′ (forward) and 5′-CTG TAC TCT GAG CAG CAG GTG-3′ (reverse); GAPDH, 5′-ACCACA GTC CAT GCC ATC AC-3′ (forward) and 5′-TCC ACC ACC CTG TTG CTG T-3′ (reverse). Preparatory experiments were performed to determine the appropriate number of PCR cycles. The reaction consisted of 28 (tyrosinase and GAPDH) or 32 cycles (TRP-1, TRP-2, and MITF) of 30 s at 94 ℃, 30 s at 55 ℃, and 1 min at 72 ℃. The PCR products were separated on 1.5% agarose gels and stained with ethidium bromide. The bands were analyzed using Quantity One software (Bio-Rad).

Statistical analysis. Values were expressed as mean ± standard deviation (SD) of three different experiments. To check for quantitative differences between the groups, analysis of variance (ANOVA) and the Tukey-Kramer Multiple Comparison tests were performed. $p < 0.05$ was considered as statistically significant. All analysis were performed using JMP 8 (SAS Institute, Cary, NC).

Results

Effect of G. hederacea *extract on melanin production and cell proliferation*

In this study, we used murine B16F10 melanoma cells as the cell model in assaying the inhibitory effect of *G. hederacea* extract on melanogenesis. To investigate the effect of *G. hederacea* extract on melanin production, the melanin content of *G. hederacea* extract-treated B16F10 melanoma cells was quantified. As shown in Fig. 1A, melanin levels were reduced in a concentration-dependent manner by *G. hederacea* extract treatment. Then we investigated the effect of the *G. hederacea* extract on B16F10 melanoma cell proliferation by MTT assay. As shown in Fig. 1B, the extract had no significant cytotoxic effect on B16F10 cells at the concentrations used. These results indicate that *G. hederacea* extract exerts antimelanogenic effects on B16F10 melanoma cells without inducing cytotoxicity.

Fig. 1.

Effect of G. hederacea *extract on mushroom tyrosinase activity*

To determine whether *G. hederacea* extract affects tyrosinase activity directly, we performed a mushroom tyrosinase assay using L-DOPA as substrate and mushroom tyrosinase as enzyme source. Tyrosinase is a key enzyme catalyzing the rate-limiting step in melanin biosynthesis. Kojic acid, a well-known inhibitor of tyrosinase, inhibited tyrosinase activity by 11%, 49%, 69%, and 83% at concentrations of 0.1, 0.3, 1, and 3 mm, respectively. These results confirmed that kojic acid inhibits mushroom tyrosinase in a concentration-dependent manner as positive control (Fig. 2A). However, as shown in Fig. 2B, *G. hederacea* extract did not exert a significant inhibitory effect on L-DOPA oxidation by mushroom tyrosinase. These results suggest that kojic acid displayed inhibitory effects on mushroom tyrosinase activity, while *G. hederacea* extract did not directly inhibit mushroom tyrosinase activity *in vitro*.

Fig. 2.

Inhibitory effect of G. hederacea *extract on B16F10 melanoma cellular tyrosinase activity*

Based on these results, *G. hederacea* extract displayed no inhibitory effect on mushroom tyrosinase activity, suggesting that it does not directly affect tyrosinase activity. Hence, to examine the possible mechanism of the anti-melanogenesis effect of *G. hederacea* extract, we measured cellular tyrosinase activity. Cells were treated with various concentrations of *G. hederacea* extract for 3 days, and lysates of the cells treated with *G. hederacea* extract were used as enzyme source. As shown in Fig. 3, tyrosinase activity in the cultured B16F10 melanoma cells was suppressed in a concentration-dependent manner by the *G. hederacea* extract.

Fig. 3.

Western blotting of the expression of tyrosinase and related proteins in G. hederacea *extract-treated cells*

To determine whether the antimelanogenic effect of *G. hederacea* extract was related to melanogenic proteins such as tyrosinase and related proteins TRP-1 and TRP-2, we performed Western blotting using the cell lysate of B16F10 melanoma cells treated with *G. hederacea* extract. As shown in Fig. 4A and B, tyrosinase protein levels were significantly decreased by *G. hederacea* extract treatment, in a concentration-dependent manner. In contrast, the protein levels of TRP-1 and TRP-2 exhibited no significant changes in the *G. hederacea* extract-treated B16F10 melanoma cells (Fig. 4A, C, and D). β-Actin was used as internal control, and the levels of it were not changed by *G. hederacea* extract treatment. These results indicate that the *G. hederacea* extract decreased melanogenesis by downregulating tyrosinase protein expression.

Fig. 4.

Effect of G. hederacea *extract on the gene expression of tyrosinase and related proteins*

Furthermore, in view of the data, we performed RT-PCR to examine the effect of the *G. hederacea* extract on the gene transcription of tyrosinase and related proteins. As shown in Fig. 5A and B, tyrosinase mRNA levels significantly decreased, in a concentration-dependent manner, but TRP-1 and TRP-2 gene expression did not change in this experiment (Fig. 5A, C, and D). In addition, the gene expression of

GAPDH, which was used as internal control, exhibited no change. In accordance with its effect on protein expression, the *G. hederacea* extract also reduced the mRNA expression levels of tyrosinase in B16F10 melanoma cells. **Fig. 5.**

Effect of G. hederacea *extract on MITF expression*

MITF has been reported to bind to the M-box within the tyrosinase promoter and in this way to regulate tyrosinase gene expression.[14, 15] Since MITF controls tyrosinase protein levels, we investigated the effect of the *G. hederacea* extract on MITF protein levels by Western blotting. As shown in Fig. 6A and B, MITF protein levels decreased in a concentration-dependent manner. The diminished levels of MITF protein were perhaps caused by decreased MITF gene expression. Hence we examined to determine whether the *G. hederacea* extract would have an effect on MITF transcription. RT-PCR was performed using primer specific for MITF. As shown in Fig. 6C and D, we did not observe any significant change in levels of MITF PCR fragments in the *G. hederacea* extract-treated cells, whereas the levels of MITF protein decreased significantly. **Fig. 6.**

Discussion

In this study, we investigated to determine whether *G. hederacea* extract has a hypopigmentation effect in B16F10 melanoma cells, and found that it strongly downregulated melanin synthesis without showing significant cytotoxicity. Melanin production occurs predominantly in the melanosomes, which are lysosome-like structures of melanocytes. Melanin is packaged and delivered to the keratinocytes by melanosomes. The two types of melanin, pheomelanin and eumelanin, are different not only in color but also in the size, shape, and the packing of their granules.[25] Both pheomelanin and eumelanin are produced from the same precursor, tyrosine, in a common tyrosinase-dependent pathway. Melanogenesis is known to be regulated by the tyrosinase gene family, which includes tyrosinase, TRP-1, and TRP-2.[4] Tyrosinase controls two key steps in the melanin synthesis pathway by catalyzing the hydroxylation of tyrosine to DOPA, followed by the oxidation of DOPA to DOPAquinone,[5] and thus melanin production is correlated with the expression level and the catalytic activity of tyrosinase. To investigate the mechanism of *G. hederacea* extract in inhibiting melanogenesis, first we investigated to determine whether *G. hederacea* extract inhibits tyrosinase activity directly in cell-free assay systems. We used mushroom tyrosinase as enzyme source. In this assay, kojic acid was used as positive control. It has been reported to affect melanin synthesis in melanocytes and melanoma cells.[26] We observed that the *G. hederacea* extract did not inhibit the L-DOPA oxidation activity of mushroom tyrosinase, suggesting that the antimelanogenic effect of the *G. hederacea* extract was not due to direct inhibition of tyrosinase enzyme activity. In contrast, we performed a cellular tyrosinase activity assay using *G. hederacea* extract-treated cell lysates as enzyme source, and cellular tyrosinase activity was inhibited in a concentration-dependent manner. These results indicate that the *G. hederacea* extract probably downregulated tyrosinase expression, but had no effect on tyrosinase catalytic activity.

To investigate further the anti-melanogenesis mechanism of the *G. hederacea* extract in B16F10 melanoma cells, Western blotting was done. The results indicate that the *G. hederacea* extract inhibited tyrosinase protein expression in a

concentration-dependent manner, but the *G. hederacea* extract had no influence on TRP-1 or TRP-2 expression. Next, we evaluated the effects of *G. hederacea* extract on tyrosinase, TRP-1, and TRP-2 mRNA expression. In accordance with the changes at the protein level, the *G. hederacea* extract inhibited tyrosinase mRNA expression in a concentration-dependent manner, but did not alter TRP-1 and TRP-2 mRNA levels. Thus, the present study clearly indicates that the *G. hederacea* extract inhibited tyrosinase expression at the transcription level, which resulted in anti-melanogenesis in the B16F10 melanoma cells.

MITF is believed to be a major regulator gene in melanocyte development and differentiation. The MITF gene, encoding a basic-helix-loop-helix-leucine zipper transcription factor, is expressed in melanocytes, the retinal pigment epithelium, mast cells, osteoclasts, and melanoma cells.[27-29] The MITF protein binds to specific consensus DNA sequences in the promoter regions of various target genes, regulating several processes, including differentiation, proliferation, migration, invasion, and tumorigenesis.[30, 31] Moreover, MITF is known to be a transcription factor of the melanogenic enzymes tyrosinase, TRP-1, and TRP-2. Next we investigated the effect of the *G. hederacea* extract on the mRNA and protein levels of MITF. The results indicated that the *G. hederacea* extract decreased MITF protein levels but did not decrease MITF mRNA levels. Since MITF binds to a highly conserved motif to activate tyrosinase gene transcription, reduced expression of tyrosinase must have been responsible for the inhibition of MITF protein expression in the *G. hederacea* extract-treated cells. The reason MITF downregulation did not reduce TRP-1 and TRP-2 expression remains unclear. Our results are in accordance with several reports. Pyrroloquinoline quinone inhibited tyrosinase expression, but not TRP-1 or TRP-2 expression, in α-MSH-stimulated B16 melanoma cells.[32] Fermented *Viola mandshurica* has also been reported to decrease MITF and tyrosinase protein expression without affecting the expression of TRP-1 or TRP-2.[33]

Many signal transduction pathways have been found to balance melanin production. Mitogen-activated protein kinase pathways, particularly the extracellular signal-regulated kinase (ERK) 1/2 pathway, are perhaps involved in MITF regulation.[34,

[35] Significant MITF degradation occurs after phosphorylation of it at serine 73 by ERK, leading to ubiquitin-dependent proteasomal degradation.[36] In the present study, we found that the *G. hederacea* extract reduced MITF protein expression but had no effect on transcription. The mechanism might be related to ERK phosphorylation. Future studies should be conducted to elucidate this mechanism.

Here we report a depigmenting effect of *G.hederacea*, but we did not investigate the active constituent. Several studies have reported that the chemical composition of *G. hederacea* includes alkaloids, sesquiterpenoids, triterpenoids, flavonoid glycosides, and rosmarinic acid,[20-22, 37-39] but there is no report on the active constituent of *G.hederacea* as to anti-melanogensis. Further work is needed to define it.

Medicinal tea and liquor made with *G. hederacea* have been used recently. In that *G. hederacea* has high water solubility, perhaps it can be used as an active ingredient of skin whitening agents for external use, such as milky lotion, cream, and skin lotion. We investigated the effect of *G. hederacea* on anti-melanogensis in this study, but clinical application as a whitening agent remains to be investigated.

In conclusion, the present study found that *G. hederacea* extract inhibited cellular melanin biosynthesis and tyrosinase activity in B16 murine melanoma cells by suppressing MITF, leading to tyrosinase downregulation at the transcriptional level. These results indicate that the *G. hederacea* extract is an effective inhibitor of melanogenesis, and be useful as a therapeutic treatment for skin hyperpigmentation disorders.

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Figure legends

Fig. 1. Effect of the *G. hederacea* (GH) Extract on Melanin Production and Cell Viability in B16F10 Melanoma Cells.

(A) B16F10 melanoma cells were incubated without (control) and with 0.1, 0.2, 0.5, or 1 mg/mL of *G. hederacea* extract for 72 h. Melanin content was measured as described in "Materials and Methods." Results were expressed as percentages relative to control, and are presented as mean ± SD for three separate experiments. ANOVA determined that there were significant differences among groups ($p < 0.001$). Values not sharing the same letter are significantly different from each other ($p < 0.05$, Tukey-Kramer multiple comparisons test). (B) After incubation of B16F10 melanoma cells with various concentrations of *G. hederacea* extract in a 96-well plate for 72 h, cell viability was determined by MTT assay. Percentage values in the treated cells were compared with respect to that in the control cells. Data are expressed as mean ± SD for three independent experiments.

Fig. 2. Effect of the *G. hederacea* (GH) Extract on Mushroom Tyrosinase Activity as to the Oxidation of L-DOPA Compared with That of Kojic Acid.

Various concentrations of kojic acid (A) and the *G. hederacea* extract (B) were incubated with mushroom tyrosinase and L-DOPA at 37℃. Mushroom tyrosinase activity was measured by the change in absorption at 475 nm. Results are expressed as percentages of control. Data are presented as mean ± SD for independent triplicate experiments. ANOVA determined that there were significant differences among the groups Fig. 2A ($p < 0.001$). Values not sharing the same letter are significantly different from each other ($p < 0.05$, Tukey-Kramer multiple comparisons test).

Fig. 3. Effect of the *G. hederacea* (GH) Extract on Cellular Tyrosinase Activity.

After incubation of B16F10 melanoma cells with various concentrations of the *G. hederacea* extract for 72 h, cellular tyrosinase activity was assessed as described in "Materials and Methods." Results are expressed as percentages of control. Data are presented as mean ± SD for independent triplicate experiments. ANOVA determined that there were significant differences among groups ($p < 0.001$). Values not sharing the same letter are significantly different from each other ($p < 0.05$, Tukey-Kramer multiple comparisons test).

Fig. 4. Effect of the *G. hederacea* (GH) Extract on Tyrosinase, TRP-1, and TRP-2 Protein Expression in B16F10 Melanoma Cells.

(A) B16F10 melanoma cells were treated with the indicated concentrations (0, 0.1, 0.2, 0.5, and 1 mg/mL) of the *G. hederacea* extract for 72 h. Cell lysates were subjected to Western blotting using antibodies against tyrosinase, TRP-1, and TRP-2. Western blotting was performed using 50 μg of each sample. The loading control was assessed using β-actin antibody. The relative intensities of tyrosinase (B), TRP-1 (C), and TRP-2 (D) expression compared with the β-actin expression were determined using Quantity One software. Values represent the mean ± SD for three independent experiments. ANOVA determined that there were significant differences among the groups Fig. 4B ($p < 0.001$). Values not sharing the same letter are significantly different from each other ($p < 0.05$, Tukey-Kramer multiple comparisons test).

Fig. 5. Effect of the *G. hederacea* (GH) Extract on Tyrosinase, TRP-1, and TRP-2 mRNA Levels in B16F10 Melanoma Cells.

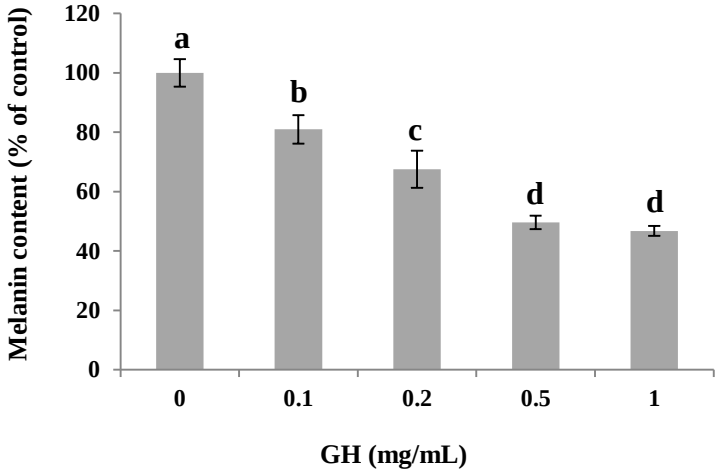
(A) B16F10 melanoma cells were treated with the *G. hederacea* extract (0, 0.1, 0.2, 0.5, and 1 mg/mL) for 72 h. Total RNA was extracted and cDNA was prepared. Equivalent amounts of cDNA were amplified using primers specific for tyrosinase, TRP-1, TRP-2, and GAPDH. GAPDH mRNA levels were determined as control. The relative intensities of tyrosinase (B), TRP-1 (C), and TRP-2 (D) expression compared with the total GAPDH expression were determined using Quantity One software. Values represent the mean ± SD for three independent experiments. ANOVA determined that there were significant differences among the groups Fig. 5B ($p < 0.001$). Values not sharing the same letter are significantly different from each other ($p < 0.05$, Tukey-Kramer multiple comparisons test).

Fig. 6. Effect of the *G. hederacea* (GH) Extract on MITF Protein and mRNA Levels in B16F10 Melanoma Cells.

(A) Effect of *G. hederacea* extract on the MITF protein expression in B16F10 melanoma cells. The MITF protein expression in B16F10 cells after 72 h of treatment with *G. hederacea* extract was analyzed. Total cellular proteins (50 μg/lane) were subjected to 10% SDS-PAGE. Variation of loading was determined by blotting with a β-actin antibody. (B) The relative intensity of MITF compared with that of β-actin was determined using Quantity One software. Values represent mean ± SD for three independent experiments. ANOVA determined that there were significant differences among groups ($p < 0.001$). Values not sharing the same letter are significantly different from each other ($p < 0.05$, Tukey-Kramer multiple comparisons test). (C) Effect of the *G. hederacea* extract on mRNA expression of the MITF genes. B16F10

cells were incubated with various concentrations of *G. hederacea* extract for 72 h. The resulting cDNA was subjected to 32 cycles of PCR using specific MITF primers. (D) The relative intensity of MITF compared with that of total GAPDH was determined using Quantity One software. Values represent mean ± SD for three independent experiments.

A


Melanin content (% of control)
120
100
80
60
40
20
0
a
b
c
d
d
0
0.1
0.2
0.5
1
GH (mg/mL)

B

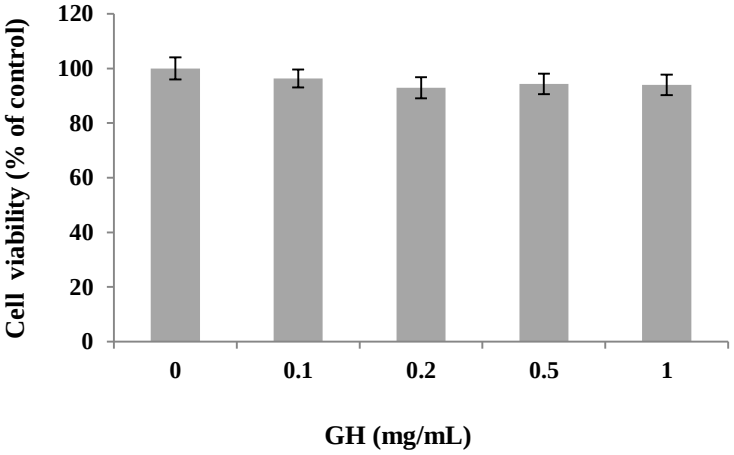
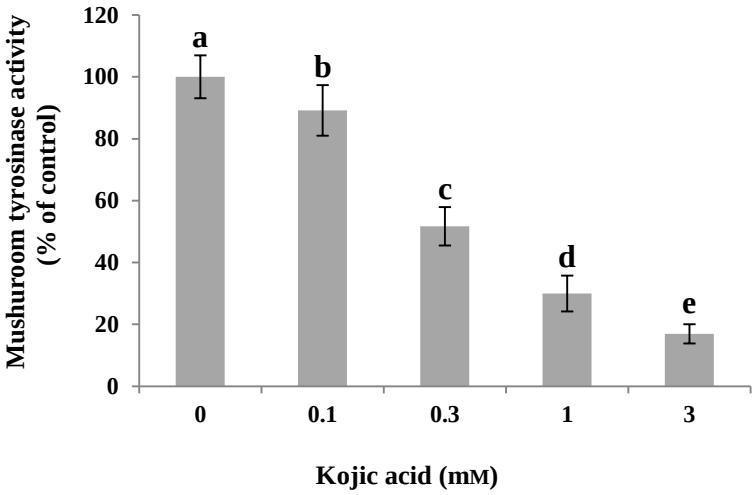

Cell viability (% of control)
120
100
80
60
40
20
0
0
0.1
0.2
0.5
1
GH (mg/mL)

Fig. 1

A


Mushuroom tyrosinase activity (% of control)
120
100
80
60
40
20
0
a
b
c
d
e
0
0.1
0.3
1
3
Kojic acid (mM)

B

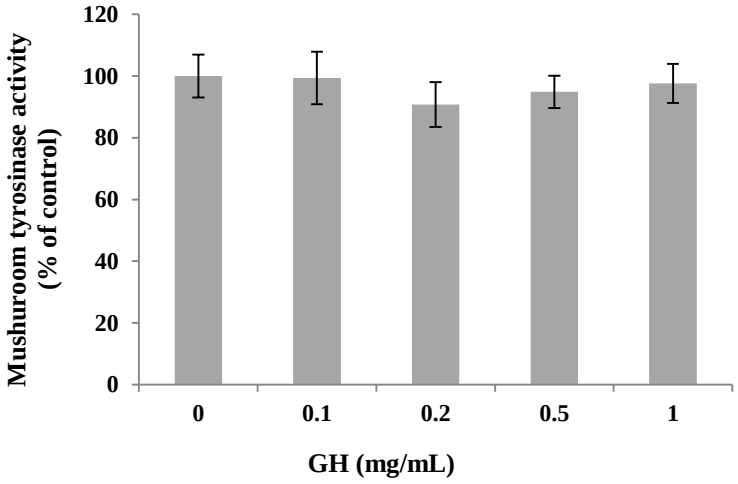

Mushuroom tyrosinase activity (% of control)
120
100
80
60
40
20
0
0
0.1
0.2
0.5
1
GH (mg/mL)

Fig. 2

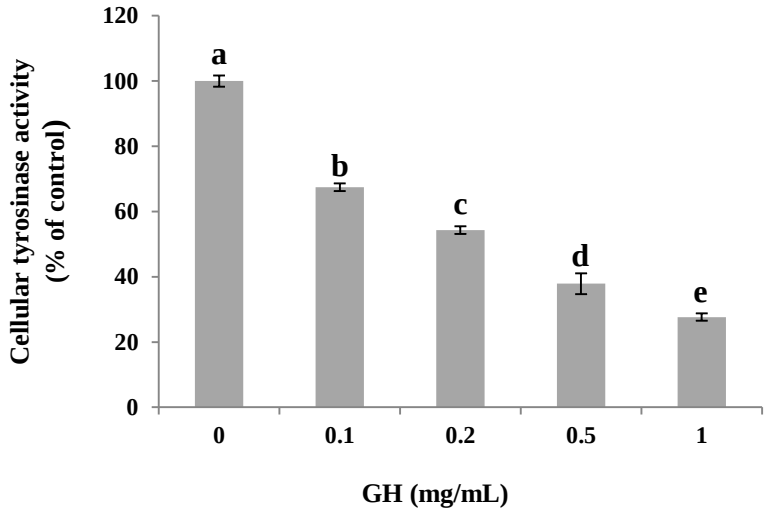

Cellular tyrosinase activity
(% of control)
120
100
80
60
40
20
0
a
b
c
d
e
0
0.1
0.2
0.5
1
GH (mg/mL)

Fig. 3

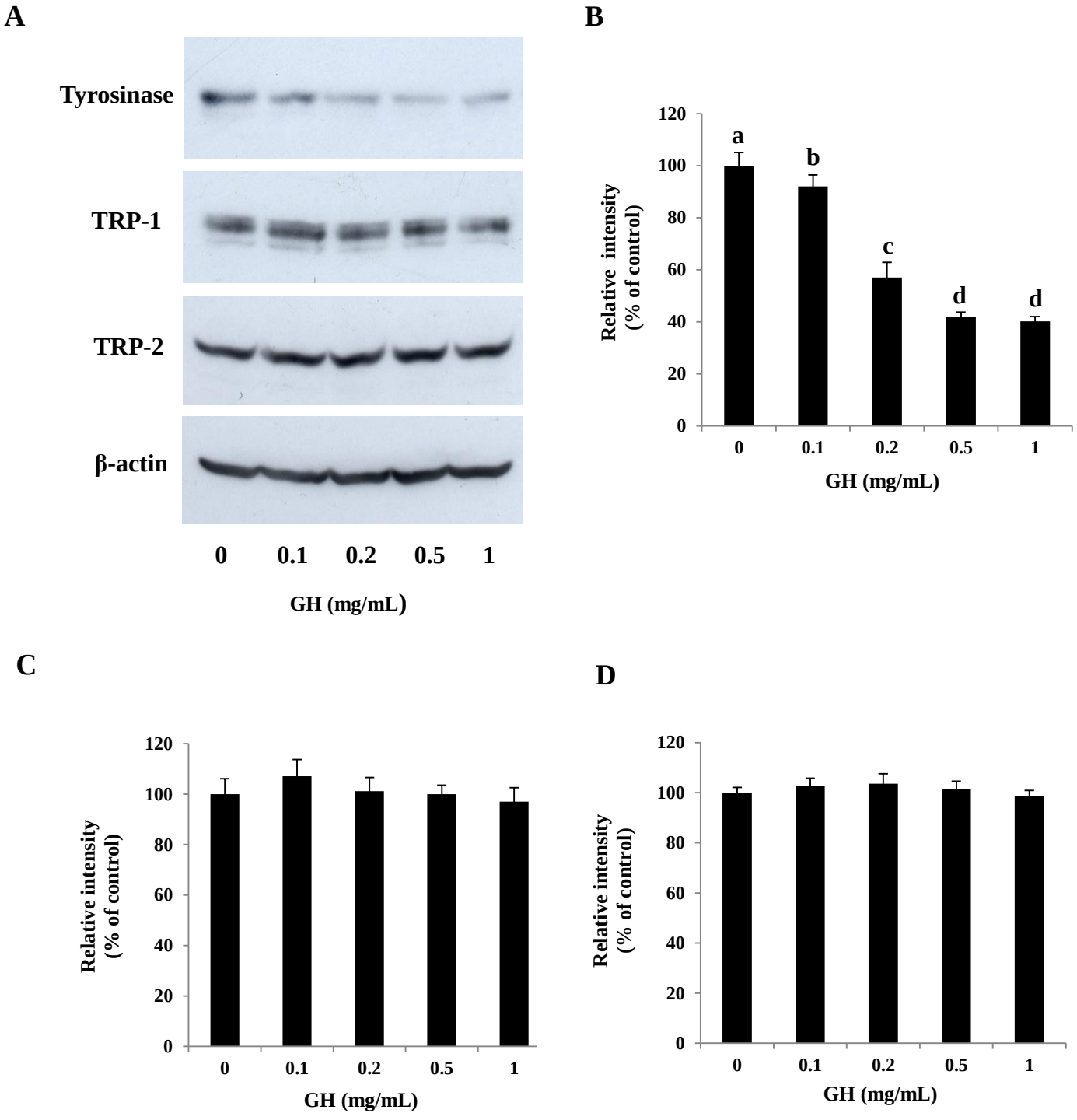

A
Tyrosinase
TRP-1
TRP-2
β-actin
0
0.1
0.2
0.5
1
GH (mg/mL)
B
Relative intensity (% of control)
120
100
80
60
40
20
0
a
b
c
d
d
0
0.1
0.2
0.5
1
GH (mg/mL)
C
Relative intensity (% of control)
120
100
80
60
40
20
0
0
0.1
0.2
0.5
1
GH (mg/mL)
D
Relative intensity (% of control)
120
100
80
60
40
20
0
0
0.1
0.2
0.5
1
GH (mg/mL)

Fig. 4

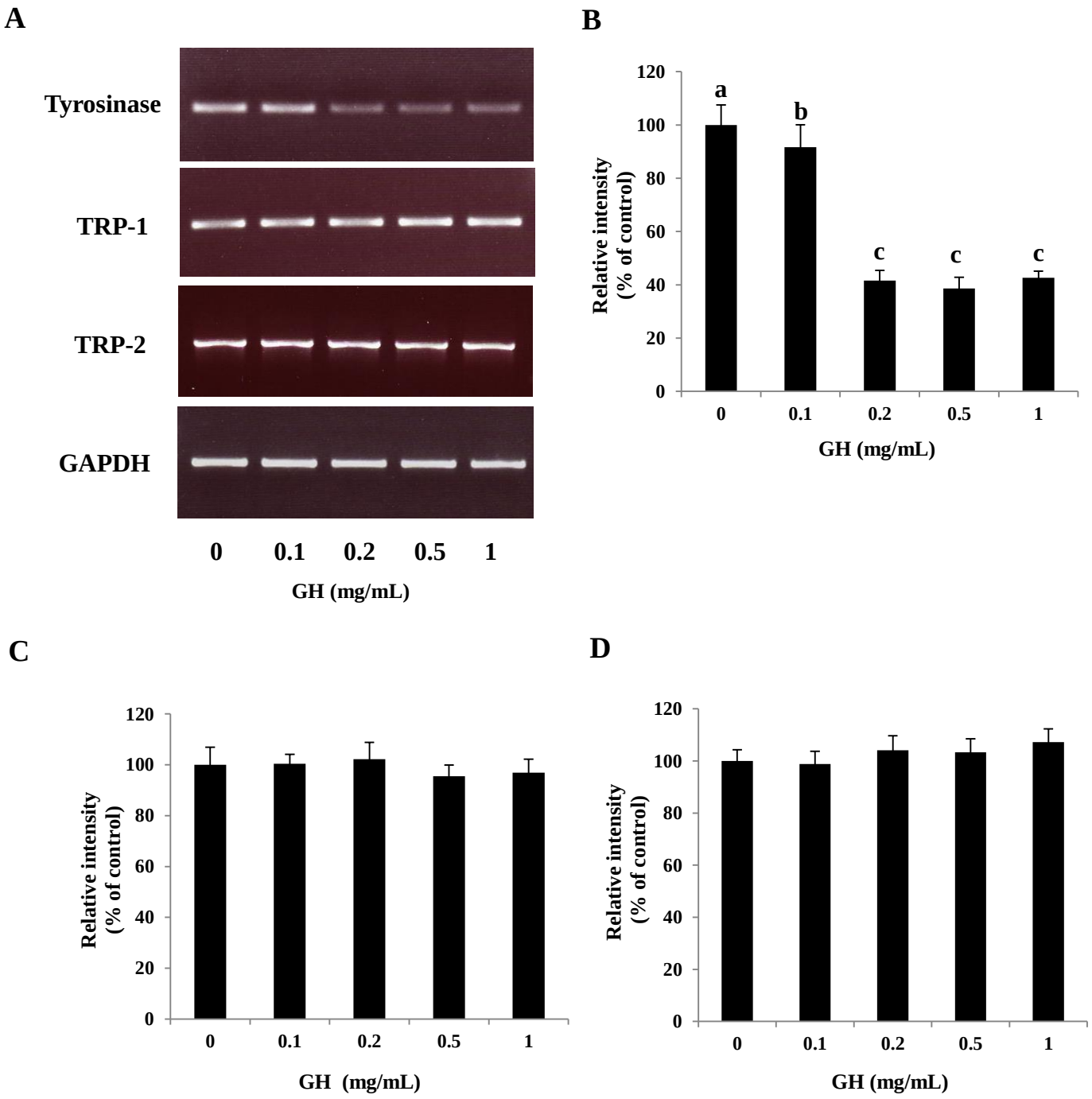

A
Tyrosinase
TRP-1
TRP-2
GAPDH
0 0.1 0.2 0.5 1
GH (mg/mL)
B
Relative intensity (% of control)
120
100
80
60
40
20
0
a
b
c
c
c
0 0.1 0.2 0.5 1
GH (mg/mL)
C
Relative intensity (% of control)
120
100
80
60
40
20
0
0 0.1 0.2 0.5 1
GH (mg/mL)
D
Relative intensity (% of control)
120
100
80
60
40
20
0
0 0.1 0.2 0.5 1
GH (mg/mL)

Fig. 5

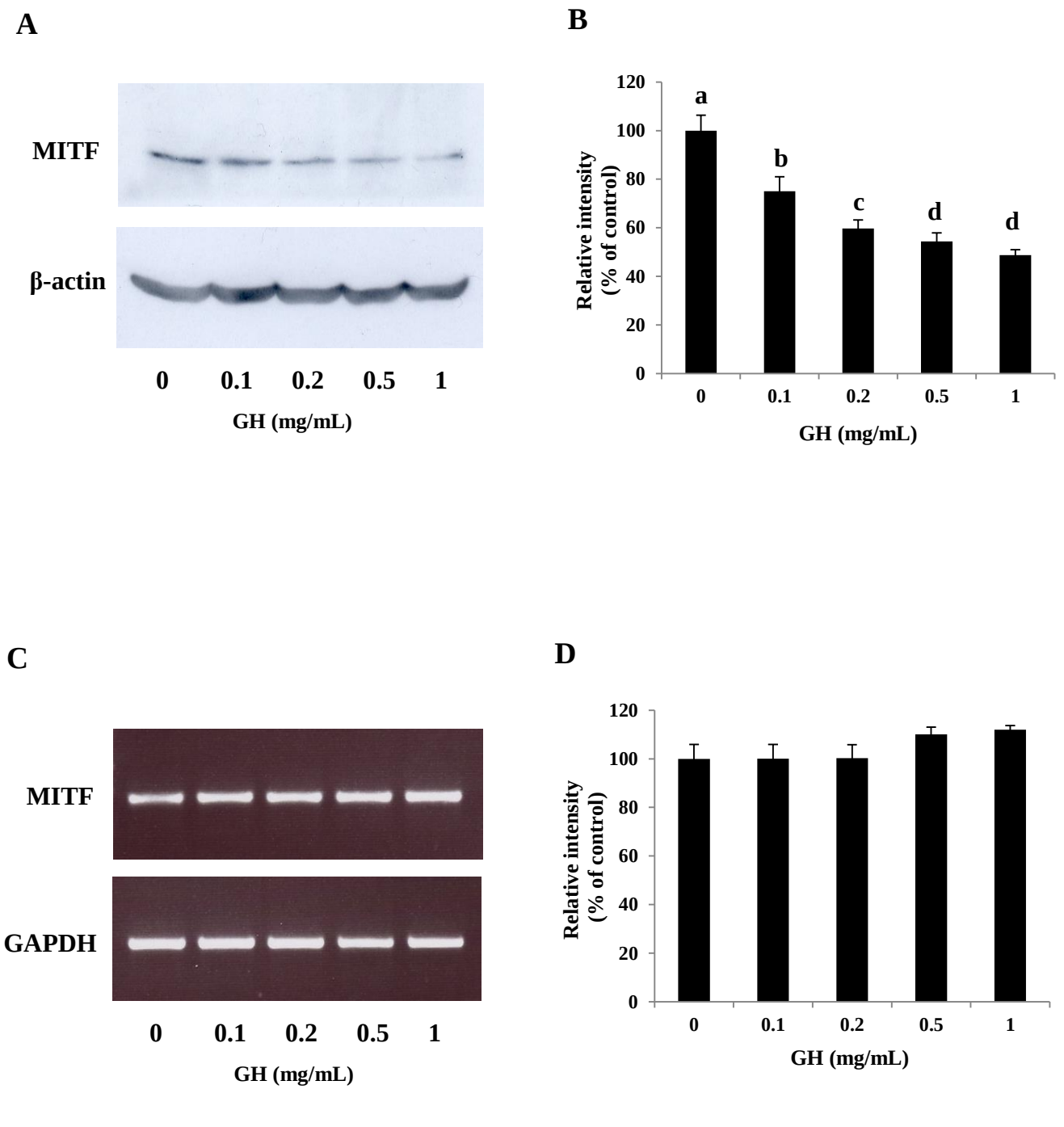

A
MITF
β-actin
0
0.1
0.2
0.5
1
GH (mg/mL)
B
Relative intensity (% of control)
120
100
80
60
40
20
0
a
b
c
d
d
0
0.1
0.2
0.5
1
GH (mg/mL)
C
MITF
GAPDH
0
0.1
0.2
0.5
1
GH (mg/mL)
D
Relative intensity (% of control)
120
100
80
60
40
20
0
0
0.1
0.2
0.5
1
GH (mg/mL)

Fig. 6