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Mixture of Mastic Gum and Peppermint Extracts Promotes Hair Growth and Health in Vitro and in C57BL/6 Mice

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Purpose: Hair disorders, which are often attributed to conditions associated with a shortened anagen growth phase, oxidative stress, and hormonal dysregulation, especially during aging, have profound psychological implications. Currently, only minoxidil has been approved as a topical hair growth solution; thus, alternative therapies for treating hair loss and promoting hair health are urgently needed. Herein, we aimed to develop and assess a novel method to promote hair growth and health using mastic (Pistacia lentiscus) gum and peppermint (Mentha piperita L.) extracts.

Materials and Methods: After determining the optimal ratio of mastic gum and peppermint extracts, we performed in vitro and in vivo experiments to verify the efficacy of the 7:3 mastic gum-peppermint mixture (MP73; FHH-MG) for enhancing hair growth and health.

Results: Mastic gum significantly promoted cell proliferation and demonstrated synergistic benefits when combined with peppermint extract. In vitro, FHH-MG increased human dermal follicle papilla cell proliferation and demonstrated anti-inflammatory and antioxidant effects. In vivo, treatment with FHH-MG dose-dependently enhanced hair growth and gloss and increased the expression of vascular endothelial growth factor, epidermal growth factor, β-catenin, and insulin-like growth factor-1 in C57BL/6 mice compared to the negative control.

Conclusion: The novel mixture exhibited hair growth-promoting effects in C57BL/6 mice; thus, FHH-MG may serve as a botanical alternative for hair growth and health promotion.

Key Words: Mastic gum, peppermint, hair growth, hair health, anti-inflammatory, antioxidant

INTRODUCTION

In contemporary society, hair profoundly influences self-perception, extending beyond its biological function of protection. Hence, the implications of hair disorders are not constrained by physical concerns and can affect psychological well-being.[1-3] Hair loss can stem from various medical, natural, or nutritional conditions, frequently associated with the shortening of the anagen phase of the hair growth cycle.[4] Oxidative stress and hormonal dysregulation contribute to hair loss and thinning, particularly with aging.[5,6] With the growing prevalence of hair loss and thinning with age, the demand for products designed to foster hair growth is progressively growing and mirrors the global trend of increased life expectancy.[6] Currently, minoxidil is the only topical treatment approved by the United States Food and Drug Administration for hair loss. Minoxidil pro-

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motes hair growth through indirect mechanisms, such as increasing vasodilation or inducing local irritation.[7-9] However, minoxidil has been associated with diverse adverse side effects, including weight gain, edema, scalp itching, scaling, respiratory challenges, and mild tachycardia.[7]

Considering the inherent limitations of minoxidil, the potential of traditional plant extracts as alternative hair-loss remedies has been explored.[10] For example, mastic (Pistacia lentiscus) gum, known to exert antioxidant and anti-inflammatory properties, is a botanical derivative that has received substantial attention.[11-13] Its antioxidative effects can be attributed to its ability to suppress the production of superoxide and hydrogen peroxide by nicotinamide-adenine dinucleotide phosphate oxidases in a protein kinase C-dependent manner and by chelating metals, thereby functioning as both a primary and secondary antioxidant.[11,12] Moreover, this antioxidant activity has been associated with mastic-mediated anti-inflammatory effects, including reduced edema and myeloperoxidase activity in murine in vivo studies.[12,14] Inhibition of nuclear factor kappa B activation is another mechanism speculated to contribute to the anti-inflammatory effects of mastic gum.[11] In clinical studies, high doses of mastic gum have been found to be well tolerated.[13] Free oxygen radicals and microinflammation are well-known to accelerate hair loss, and antioxidants can increase human dermal papillae cell proliferation.[15,16] However, despite its historical application as a remedy and its apparent alignment with the causal factors of hair loss, scientific evidence documenting the efficacy of mastic gum in reducing hair loss remains insufficient.[13]

Peppermint (Mentha piperita) extract has also garnered interest owing to its ability to induce hair growth via its main constituent, menthol.[17,18] In vivo mouse studies have revealed that peppermint can stimulate hair growth by rapidly inducing the anagen phase of the hair growth cycle, which is characterized by an increase in hair follicle number, follicle depth, and dermal thickness.[18-20] Peppermint reportedly increases the expression of various growth factors, including epidermal growth factor (EGF), a known regulator of the hair growth cycle, with androgens known to regulate EGF expression.[21,22] EGF activates the Wnt/β-catenin signaling pathway, which is pivotal in promoting anagen induction and duration.[18,21,23] Wnt/β-catenin signaling activation also leads to increased expression and production of vascular endothelial growth factor (VEGF), ensuring adequate vascularization of hair dermal papillae for the follicles in the anagen phase.[24] Moreover, menthol functions as a vasodilator that preserves vascularization, allowing further antioxidant and anti-inflammatory activities to promote hair growth.[25] However, although these mechanisms may affect the cellular processes essential for hair growth, their impact on hair growth remains relatively unexplored.

The growing interest in developing formulations that comprise diverse herbal extracts is fueled by their potential to leverage synergistic effects while simultaneously reducing the need for high-dose individual components, thereby addressing concerns related to potential toxicity.[19,20] In the current study, we aimed to determine whether a combination of mastic gum and peppermint could enhance hair growth and health. We present findings on the hair-growing effects of a novel formula containing mastic gum and peppermint, highlighting antioxidant and anti-inflammatory activities, as well as its capacity to promote hair health.

MATERIALS AND METHODS

Materials for in vitro assessments

Mastic gum and peppermint extracts were obtained from Frombio Co. Ltd. (Yongin, Republic of Korea). Mastic gum was prepared from mastic gum powder, and peppermint extract powder was prepared from dried peppermint, which was extracted with 50% ethanol, ethanol at 50°C for 8 hours, evaporated, and freeze-dried.

To determine the optimal ratio of mastic gum and peppermint, cells were treated with the following ratios of test substances: mastic gum, mastic gum 7: peppermint 3 (MP73); mastic gum 5, peppermint 5 (MP55); mastic gum 3, peppermint 7 (MP37); and peppermint. Additionally, we established suitable controls: negative control, vehicle (distilled water); and positive control, 2.5 μM minoxidil (M4145; Sigma, St. Louis, MO, United States).

To assess the dose-dependent effects of the MP73 (FHH-MG) 7:3 mastic gum-peppermint mixture, cells were treated with the following different doses of the FHH-MG: 300 ppm mastic gum +700 ppm peppermint (1000 ppm FHH-MG); 150 ppm mastic gum +350 ppm peppermint (500 ppm FHH-MG); 75 ppm mastic gum +175 ppm peppermint (250 ppm FHH-MG); 37.5 ppm mastic gum +87.5 ppm peppermint (125 ppm FHH-MG); and 18.75 ppm mastic gum +43.75 ppm peppermint (62.5 ppm FHH-MG). Additionally, cells treated with the respective controls, negative (distilled water) and positive (2.5 μM minoxidil or 0.5 μM dexamethasone), were established.

Cell culture

Human follicle dermal papilla cells (HFDPCs) were purchased from PromoCell (C-12071; Heidelberg, Germany) and cultured in ready-to-use follicle dermal papilla cell growth medium (C-26501; PromoCell) supplemented with growth medium supplement mix (C-39625; PromoCell). RAW 264.7 cells were purchased from American Type Culture Collection (TIB-71; Manassas, VA, United States) and cultured in Dulbecco's modified Eagle's serum (12-604Q; Lonza, Basel, Switzerland) supplemented with 100 mg/mL fetal bovine serum (16000-044; Gibco, Grand Island, New York, MT, United States) and 100 U/mL penicillin-streptavidin (15140122; Gibco). Cells were incubated at 37°C with 5% carbon dioxide (CO_2).

Experimental animals and design

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine (IACUC No. 2023-0017). Experiments were performed following the guidelines of the Yonsei University Avison Biomedical Research Center for the use and care of laboratory animals.

Five-week-old male C57BL/6 mice were obtained from the ORIENT BIO Animal Center (Seongnam, Republic of Korea) and acclimatized for 1 week. Animals were housed in cages under the following conditions: temperature of 24±0.5°C; relative humidity of 55%–65%; and an automatic 12-h light and dark cycle. Food and water were provided ad libitum. After acclimatization, mice were anesthetized with isoflurane, and their dorsal surface was shaved using animal clippers. Shaving cream was applied to the shaved areas and left for 3 min to remove any residual fine hair. After a 24-h skin stabilization period post-shaving, mice with telogen phase hair were weighed and randomly divided into five groups (n=12 per group): negative control, vehicle (distilled water); 5 mg/kg/day minoxidil (M4145; Sigma); FHH-MG 100 mg/kg/day (FHH-MG 100); FHH-MG 150 mg/kg/day (FHH-MG 150); and FHH-MG 200 mg/kg/day (FHH-MG 200). Mice were administered test substances (100 μL) by oral gavage five times per week at regular intervals. One or 3 weeks after initiating the experiment, mice were anesthetized with CO_2 and euthanized. RNA and protein were extracted from skin tissues to evaluate gene expression and protein secretion and for visual and histological analyses.

Cell counting kit-8 assay

In brief, HFDPCs (5×10^3 cells/well) and RAW 264.7 (5×10^3 cells/well) cells were seeded into 96-well cell culture plates and incubated at 37°C under 5% CO_2. At 80% confluency, HFDPCs and RAW 264.7 cells were treated with various concentrations (20, 200, and 2000 ppm) of different test substance ratios for 24 h or with 200 ppm of different test substance ratios for 24, 48, or 72 h. Thereafter, cells were incubated with cell counting kit-8 (CCK-8) (CK04; Dojindo, Tokyo, Japan) solution at 37°C for 2 h. Absorbance was measured at 450 nm using a Varioskan LUX Multimode Microplate Reader (VL0000D0; Thermo Fisher Scientific, Waltham, MA, USA). Cell viability and proliferation were calculated by measuring the difference between the optical densities (ODs) of the experimental and control groups:

$$\text{Cell viability or proliferation (\%)}=\frac{OD_{experimental}-OD_{blank}}{OD_{control}-OD_{blank}}\times100.$$

Nitric oxide assay

In brief, RAW 264.7 (1×10^6 cells/well) cells were seeded into 6-well plates and incubated at 37°C under 5% CO_2. After 24 h, cells were treated with 1 μg/mL lipopolysaccharide (LPS), followed by 200 ppm of different ratios of MP73 with phenol red-free media. After 24 h, the culture medium was collected and centrifuged at 2000×g for 10 min. The supernatant was collected and mixed with Griess reagent (G4410; Sigma-Aldrich, San Luis, MI, United States) at a 1:1 ratio at a temperature of 20–25°C. The absorbance was measured at 540 nm using a Varioskan LUX Multimode Microplate Reader (VL0000D0; Thermo Fisher Scientific), and the level of nitric oxide (NO) produced was calculated from a standard curve constructed using a 1:1 mixture of Griess reagent and $NaNO_2$ using the measured OD.

Enzyme-linked immunosorbent assay

In brief, HFDPCs (5×10^4 cells/well) or RAW 264.7 (1×10^6 cells/well) cells were seeded into 6-well plates and incubated at 37°C under 5% CO_2. HFDPCs at 80% confluency were treated with 200 ppm of different ratios of test substances. RAW 264.7 cells at 80% confluency were treated with 1 μg/mL LPS, followed by treatment with 200 ppm of different ratios of test substances in phenol red free media. After 24 h, the culture media were collected and centrifuged at 2000×g for 10 min. Mouse enzyme-linked immunosorbent assay kits were used to measure the levels of tumor necrosis factor (TNF)-α (ab208348; Abcam, Cambridge, United Kingdom) and interleukin (IL)-6 (M6000B; R&D systems, Minneapolis, MN, United States), following the manufacturer's guidelines for the cell type. Absorbance was measured using a Varioskan LUX Multimode Microplate Reader (VL0000D0; Thermo Fisher Scientific), and the concentration of each protein was calculated from a standard curve using the measured OD.

Antioxidant activity assessment

For in vitro assessments, HFDPCs (5×10^4 cells/well) were seeded into 6-well cell culture plates and incubated at 37°C under 5% CO_2. At 80% confluency, cells were treated with 400 μM H_2O_2, followed by treatment with 200 ppm of different ratios of test substances. After 24 h, the culture media were collected and centrifuged at 2000×g for 10 min, and supernatants were collected to exclude cell debris. For in vivo assessments, dorsal skin tissue was isolated from mice treated with the test substances for 3 weeks. Protein was extracted from the skin tissue using a sucrose buffer (pH 7.4) and centrifuged at 10000×g for 60 min at 4°C. The supernatants were collected using a superoxide dismutase (SOD) assay kit (BO-SOD-250; Biomax Ltd., Guri, Republic of Korea) or a catalase (CAT) kit (BO-CAT-400; Biomax Ltd.) according to the manufacturer's guidelines. Absorbance was measured at 450 nm for the SOD assay and 570 nm for the CAT assay using a Varioskan LUX Multimode Microplate Reader (VL0000D0; Thermo Fisher Scientific). SOD and CAT activities were calculated following the manufacturer's guidelines for their respective assay kits using the measured OD.

Reverse transcription-quantitative polymerase chain reaction

For in vivo assessments, dorsal skin tissues were harvested from mice treated with the test substances for 3 weeks. Total RNA was extracted from cells and tissues using an RNeasy RNA ex-

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traction kit (74104; Qiagen Inc., Valencia, CA, USA) and quantified using a Nanodrop 2000 spectrophotometer (ND2000; Thermo Fisher Scientific). cDNA was synthesized using the RNA-to-cDNA EcoDry Premix kit (639541; Clontech, Mountain View, CA, United States) following the manufacturer's guidelines. cDNA was amplified using Taqman Fast Advanced Master Mix (4444556; Applied Biosystems, Waltham, MA, United States) and Taqman primers according to the cell type [VEGF: Mm00437306_m1, insulin-like growth factor-1 (IGF-1): Mm00439560_m1, EGF: Mm00438696_m1, β-catenin: Mm00483039_m1; Applied Biosystems] using reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and the relative expression of each gene was normalized to a housekeeping gene, GAPDH (Hs02786624_g1 or Mm99999915_g1; Applied Biosystems). The relative mRNA expression was calculated using the $2^{-\Delta\Delta C_T}$ method.

Visual analysis of hair growth improvement in vivo

Photographs of experimental animals were captured weekly during the treatment period using an Aura X Pro scanner (CZUR, Shenzhen, China). The hair growth effect was evaluated by blinded investigators who visually scored the degree of hair regrowth expressed as a hair growth index score, following a numerical rating scale: 0–19% (1–1.5 point), 20–39% (2–2.5 points), 40–59% (3–3.5 points), 60–79% (4–4.5 point), and 80–100% (5 points). Subsequently, average values of the assessment scores were calculated.

Histological analysis

Dorsal skin tissues from mice treated with the test substances for 1 week were excised and fixed in 10% formalin for 24 h, followed by the preparation of paraffin blocks and paraffin-embedded slides. Deparaffinized tissue specimens were stained with a hematoxylin (104302; Merck, Kenilworth, NJ, United States) solution that stained the nucleus, followed by washing under running water. The cytoplasm was stained using eosin (230251; Sigma-Aldrich). After rinsing, tissues were dehydrated and fixed using a mounting solution. An optical microscope (Olympus BX43; Olympus Co., Tokyo, Japan) was used to measure the number of hair follicles in the dermis and subcutaneous layers at 100× magnification, which was expressed as the number of hair follicles per area (mm^2). The ratio of hair follicles in the anagen phase relative to the entire follicular population was expressed as a percentage.

Hair gloss evaluation

For all mice treated with the test substances for 3 weeks, the entire dorsal skin area with newly grown hair was excised. Photographs of the hair were obtained for all animals at uniform distances and under lighting conditions using an EOS 800D camera (Canon, Tokyo, Japan) for subsequent evaluation. Using these photographs, hair gloss was evaluated by blinded investigators or by using Image-Pro software (I-MAXPLUS, Seoul, Republic of Korea). Blinded investigators visually scored the area and degree of hair gloss using the following numerical rating scale: normal (1 point), a bit shiny (2 points), shiny (3 points), very shiny (4 points), or extremely shiny (5 points). The average value of the assessment scores was calculated. Image-Pro software was used to measure pixel values according to the degree and area of hair gloss, and the average pixel value of the entire image was calculated.

Measurement of hair thickness

Hair was collected from the dorsal surface of mice treated with the test substances for 3 weeks and fixed on a glass slide. An optical microscope (Olympus BX43; Olympus Co.) was used to measure the hair thickness at various points along the hair strand at 400× magnification. An average of 10 randomly selected hair strands was measured for each animal.

Statistical analysis

All experimental data are presented as a mean±standard deviation or percentages, and all experiments were conducted in triplicate (n≥3). Statistical analyses were performed using SPSS Statistics (version 25.0; IBM Corp., Armonk, NY, United States). Statistical significance was calculated using an independent sample t-test. Statistical significance was considered at $p<0.05$, $p<0.01$, and $p<0.001$, indicated by #, ##, and ### or by *, **, and ***, respectively, depending on the comparison group.

RESULTS

Determination of combinational ratio of mastic gum and peppermint in vitro

Assessment of cell viability and proliferation

To assess the impact of the combined formulation on cell viability in vitro, we performed CCK-8 assays using HFDPCs and RAW 264.7 cells treated with different ratios of mastic gum and peppermint for 24 h. Treatment with 2000 ppm peppermint significantly reduced HFDPC viability (5.408%, $^{**}p<0.01$) compared to treatment with the negative control (Fig. 1A). All mixtures of mastic gum and peppermint did not reduce cell viability up to 200 ppm; however, cell viability gradually decreased as the relative ratio of peppermint increased when treated with 2000 ppm of MP37 (96.790%, ns), MP55 (88.272%, $^{*}p<0.05$), and MP73 (65.714%, $^{**}p<0.01$) (Supplementary Fig. 1, only online). For both HFDPCs and RAW 264.7 cells, test substance concentrations with cell viabilities >80% were confirmed to be nontoxic and selected for subsequent experiments.

To assess the effect of the combined formulation on cell proliferation in vitro, we performed the cell proliferation assay using HFDPCs treated with 200 ppm of different ratios of mastic gum and peppermint for 24, 48, and 72 h. All test groups showed

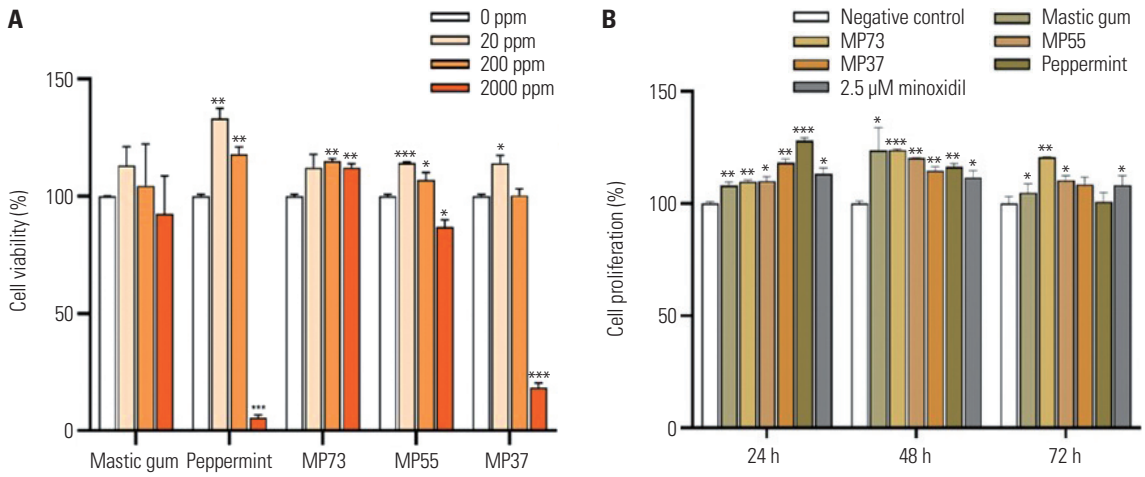

Fig. 1. Effects of mastic gum and peppermint extracts mixed at different ratios on the viability and proliferation of HFDPCs, as determined using the CCK-8 assay. (A) Cell viability was evaluated in HFDPCs treated with different concentrations of mastic gum and peppermint (0–2000 ppm) for 24 h. (B) Cell proliferation was evaluated in HFDPCs treated with 200 ppm mastic gum and peppermint ratios for 24, 48, and 72 h. Data are presented as the mean±SD (n=3). *p<0.05, **p<0.01, and ***p<0.001 compared to the negative control. MP73, mastic gum:peppermint=7:3; MP55, mastic gum:peppermint=5:5, MP37, mastic gum:peppermint=3:7. HDFPCs, human dermal follicle papilla cells; SD, standard deviation; CCK-8, cell counting kit-8.

increased cell proliferation at 24 and 48 h. However, at 72 h, increased cell proliferation was observed only in cells treated with mastic gum, MP73, MP55, and minoxidil, in contrast to the peppermint group, which failed to demonstrate any such an increase, compared to the negative control group (Fig. 1B). Notably, the MP73 mixture displayed the most significant increase in proliferation at both 48 and 72 h (123.834%, ***p<0.001 and 120.554%, **p<0.01, respectively). Additionally, treatment with MP73 exhibited the highest increase in VEGF expression, a key hair growth factor, compared to treatment with the negative control (Supplementary Fig. 2, only online).

In the cell viability experiment, all treatment groups exhibited cell viability of >80% at 200 ppm. Notably, MP73 was nontoxic at all tested concentrations (0–2000 ppm). In cell proliferation experiments, MP73 displayed the highest increase in cell proliferation at 48 and 72 h. Therefore, MP73 (FHH-MG) was selected for subsequent experiments.

Efficacy evaluation by dosage of selected mixture: FHH-MG (MP73) in vitro

Cell proliferation of FHH-MG (MP73) mixture

To evaluate the impact of the FHH-MG on cell proliferation, we first confirmed that FHH-MG did not alter the morphology of HFDPCs after 24 h of culture (Fig. 2A). To further assess cell proliferation, we conducted CCK-8 assays on HFDPCs treated with various concentrations of MP73 (62.5–1000 ppm) at 24, 48, and 72 h. Cells treated with all MP73 concentrations exhibited increased proliferation when compared to those treated with the negative control (*p<0.05, **p<0.01, and ***p<0.001) (Fig. 2B).

Antioxidant activity of FHH-MG mixture

We conducted SOD and CAT assays to measure the activities of enzymes that scavenge reactive oxygen species, as well as to assess the antioxidant effects of FHH-MG treatment in vitro. HFDPCs were initially treated with H_2O_2 to induce oxidative stress, followed by the addition of FHH-MG at concentrations ranging from 62.5 to 250 ppm. Oxidative stress was confirmed by a decrease in SOD activity in the H_2O_2-only treatment group (11.440%, #p<0.05) compared to the negative control (22.261%). All FHH-MG-treated groups exhibited a dose-dependent increase in SOD activity when compared to the H_2O_2-only treatment group (*p<0.05, **p<0.01) (Fig. 3A). Similarly, CAT activity was lower in the H_2O_2-only treatment group (1071.343 mU/mL, #p<0.05) than in the negative control (1157.387 mU/mL). However, a dose-dependent increase in CAT activity was observed in all FHH-MG-treated groups when compared to the H_2O_2-only treatment group *p<0.05, **p<0.01 (Fig. 3B).

Anti-inflammatory activity of FHH-MG mixture

To evaluate the anti-inflammatory activity of the FHH-MG mixture, RAW 264.7 cells were treated with 62.5–250 ppm FHH-MG, and relative NO levels were measured to assess the anti-inflammatory activity of prepared mixtures. Application of LPS induced inflammation, as confirmed by the increased NO levels, compared to application of the negative control (191.586 μM, ###p<0.001). All FHH-MG treated groups showed a dose-dependent decrease in NO levels compared to the LPS-only treatment group (**p<0.01, ***p<0.001) (Fig. 4A).

We also measured the protein levels of secreted TNF-α and IL-6 in the culture media of RAW 264.7 cells treated with 62.5–250 ppm FHH-MG. All FHH-MG-treated groups showed a dose-dependent reduction in TNF-α and IL-6 secretion compared to the LPS-only treatment group. In particular, the FHH-MG 250 ppm group exhibited significantly decreased TNF-α (1350.759 pg/mL, ***p<0.001) and IL-6 (2847.245 pg/mL, ***p<0.001) ex-

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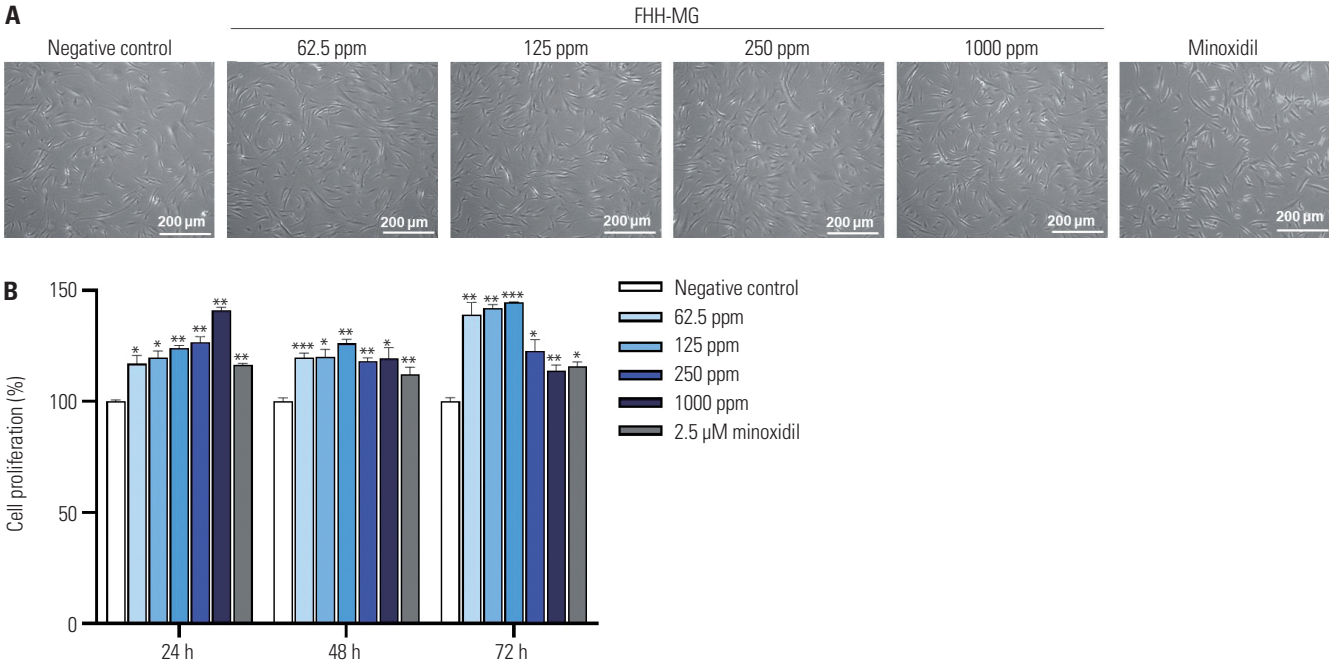

Fig. 2. Effects of FHH-MG treatment on HFDPC morphology and evaluation using the CCK-8 assay. (A) Examination of HFDPC morphology after FHH-MG treatment. (B) HFDPC proliferation after treatment with FHH-MG 62.5–1000 ppm for 24, 48, and 72 h measured using the CCK-8 assay. *p<0.05, **p<0.01, and ***p<0.001 compared to the negative control. HDFPCs, human dermal follicle papilla cells; CCK-8, cell counting kit-8.

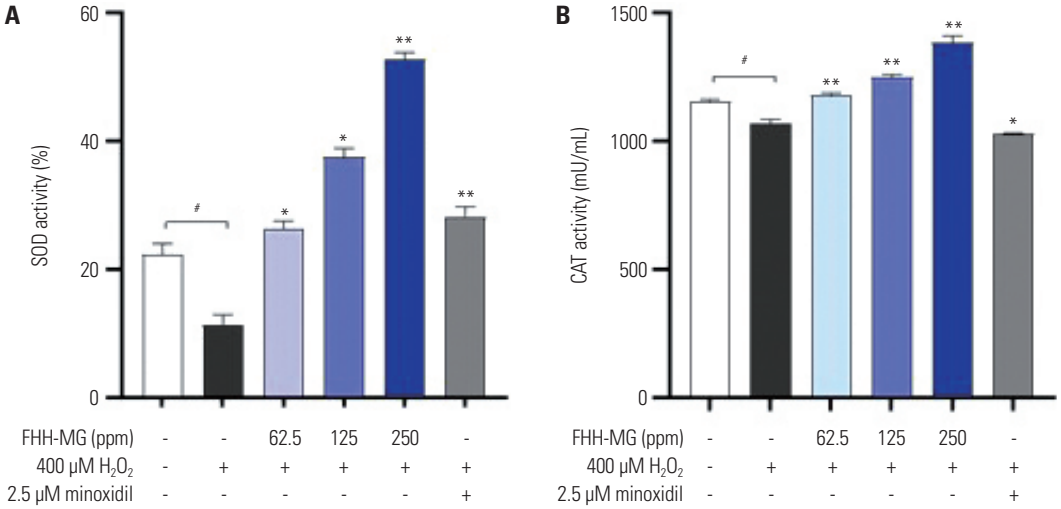

Fig. 3. Antioxidant effect of FHH-MG mixture determined using SOD and CAT assays. HFDPCs were treated with 400 μM H_2O_2, followed by immediate treatment with 200 ppm FHH-MG mixtures. All FHH-MG mixture groups exhibited a dose-dependent increase in SOD (A) and CAT (B) activities. Data are presented as the mean±SD (n=3). #p<0.05 compared to the negative control; *p<0.05 and **p<0.01 compared to the H_2O_2-only treatment groups. +, present; –, absent; CAT, catalase; HDFPCs, human dermal follicle papilla cells; SD, standard deviation; SOD, superoxide dismutase.

pression compared to the LPS-only treatment group, whereas the 0.5 μM dexamethasone group expressed 1457.000 pg/mL TNF-α and 3330.562 pg/mL IL-6 (Fig. 4B and C).

In vivo efficacy evaluation following oral administration of FHH-MG mixtures

Hair growth-promoting effect of the FHH-MG in C57BL/6 mice

To assess whether oral consumption of FHH-MG promotes hair growth in vivo, we administered the test substance orally to 60 of the C57BL/6 mice five times per week for either 1 or 3 weeks. Based on weekly body weight measurements, treatment with FHH-MG did not affect the body weight of mice (Supplementary Table 1, only online). After 3 weeks of treatment, visual evaluations of hair growth in C57BL/6 mice revealed that FHH-MG significantly accelerated murine hair growth (Fig. 5A). According to assessments by blinded independent investigators, the improvement in hair growth was significantly greater in the

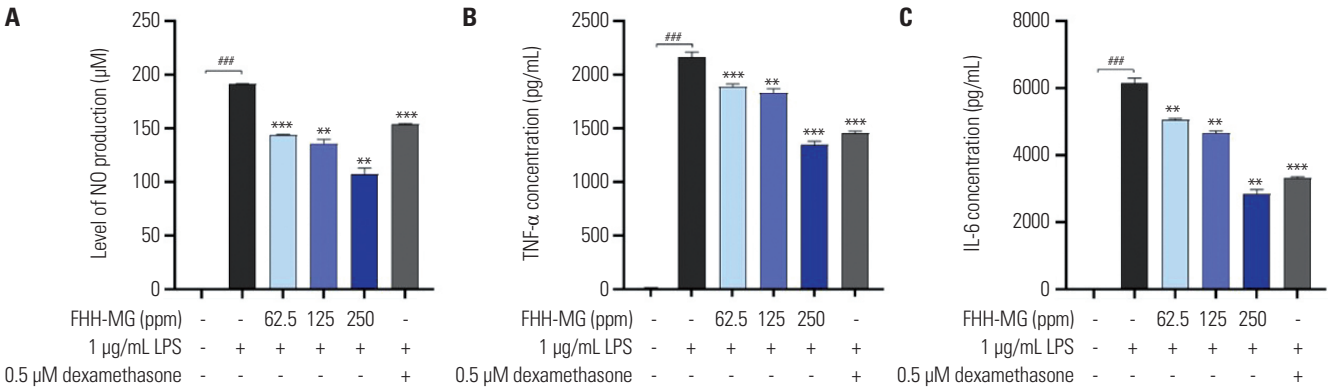

Fig. 4. Effects of different ratios of FHH-MG mixture on the expression of inflammatory cytokines in the RAW 264.7 cells treated with 1 μg/mL LPS. (A) The FHH-MG-treated groups showed significant reduction in NO production levels compared to the LPS-only treatment group. Similarly, protein levels of TNF-α (B) and IL-6 (C) decreased in a dose-dependent manner in the FHH-MG-treated groups. Data are presented as the mean±SD (n=3). ###p<0.001 compared to the negative control. **p<0.01 and ***p<0.001 compared to the LPS-only treatment group. +, present; –, absent; IL-6, interleukin-6; LPS, lipopolysaccharide; NO, nitric oxide; SD, standard deviation; TNF-α, tumor necrosis factor-α.

FHH-MG 100, FHH-MG 150, and FHH-MG 200 groups on days 7, 11, and 14 than in the negative control group, with a dose-dependent increase in improvement scores noted on day 14 (**p<0.01) (Fig. 5B and Supplementary Table 2, only online).

Histological analysis after 7 days of treatment with FHH-MG revealed that both hair follicle density and the anagen ratio were significantly higher in all FHH-MG-treated groups compared to the negative control group (*p<0.05 and **p<0.01, respectively) (Fig. 5C and D). Furthermore, based on the visual assessment scores assigned by blinded investigators, the FHH-MG-treated groups showed a significant, dose-dependent increase in hair gloss when compared to the negative control group on day 21 (*p<0.05 and **p<0.01) (Fig. 5E and Supplementary Fig. 3, only online). Hair thickness was also significantly greater in all FHH-MG-treated groups than in the negative control group (***p<0.001) (Fig. 5F).

Antioxidant activity of FHH-MG in C57BL/6 mice

To assess the antioxidant effects of orally administered FHH-MG in vivo, we performed SOD assays using proteins extracted from the dorsal skin tissue of C57BL/6 mice after a 3-week treatment regimen. Antioxidant activity was significantly increased in groups treated with FHH-MG 100, 150, and 200, revealing values of 37.316, 41.357, and 39.275% (*p<0.05, ***p<0.001, and **p<0.01), respectively, whereas the minoxidil group exhibited a 41% increase (***p<0.001) compared to the negative control (Fig. 6).

Effect of FHH-MG on gene expression of growth factors in C57BL/6 mice

We performed RT-qPCR to determine the effect of oral FHH-MG on the gene expression of growth factors using RNA extracted from the dorsal skin tissue of C57BL/6 mice after a 3-week treatment regimen. We measured the expression levels of VEGF, IGF-1, EGF, and β-catenin. The FHH-MG-treated groups exhibited a significant, dose-dependent upregulation of VEGF and β-catenin compared to the negative control (*p<0.05, **p<0.01) (Fig. 7A and B). Moreover, IGF-1 expression was significantly increased in all FHH-MG-treated groups compared to that in the negative control (**p<0.01) (Fig. 7C). Lastly, EGF expression was significantly increased in the FHH-MG 150 (**p<0.01) and FHH-MG 200 (*p<0.05) groups compared to that in the negative control (Fig. 7D). To further validate the effect of FHH-MG on the secretion of growth factors, we treated HDFPCs with 250 ppm of FHH-MG and performed ELISA to measure the protein expression levels of VEGF, IGF-1, and β-catenin (Supplementary Fig. 4, only online). When treated with 250 ppm of FHH-MG, we observed significant increases in the protein expression levels of VEGF, IGF-1, and β-catenin compared to the negative control.

DISCUSSION

Although minoxidil has been widely used to promote hair growth, its precise mechanism and molecular targets remain poorly characterized and may mediate its action indirectly by inducing irritation and vasodilation.[7-9] Considering these limitations, there is a growing interest in alternative approaches to facilitate hair growth and improve hair quality. Peppermint extract is known to enhance hair growth by inducing anagen phase, while mastic gum exerts antioxidative and anti-inflammatory activities, making both compounds attractive botanical alternatives to minoxidil.[11,12,18] Although mastic gum and peppermint are known for their antioxidant and anti-inflammatory properties, their combined effect on hair growth remains unexplored.[13,18]

Therefore, we conducted a pilot study to assess the combined effects of mastic gum and peppermint extracts (FHH-MG) on hair growth and quality, utilizing both in vitro and in vivo approaches. Our findings reveal that the MP73 formulation, comprising a 7:3 ratio of mastic gum to peppermint, significantly enhances cell proliferation in HFDPCs. This synergistic effect

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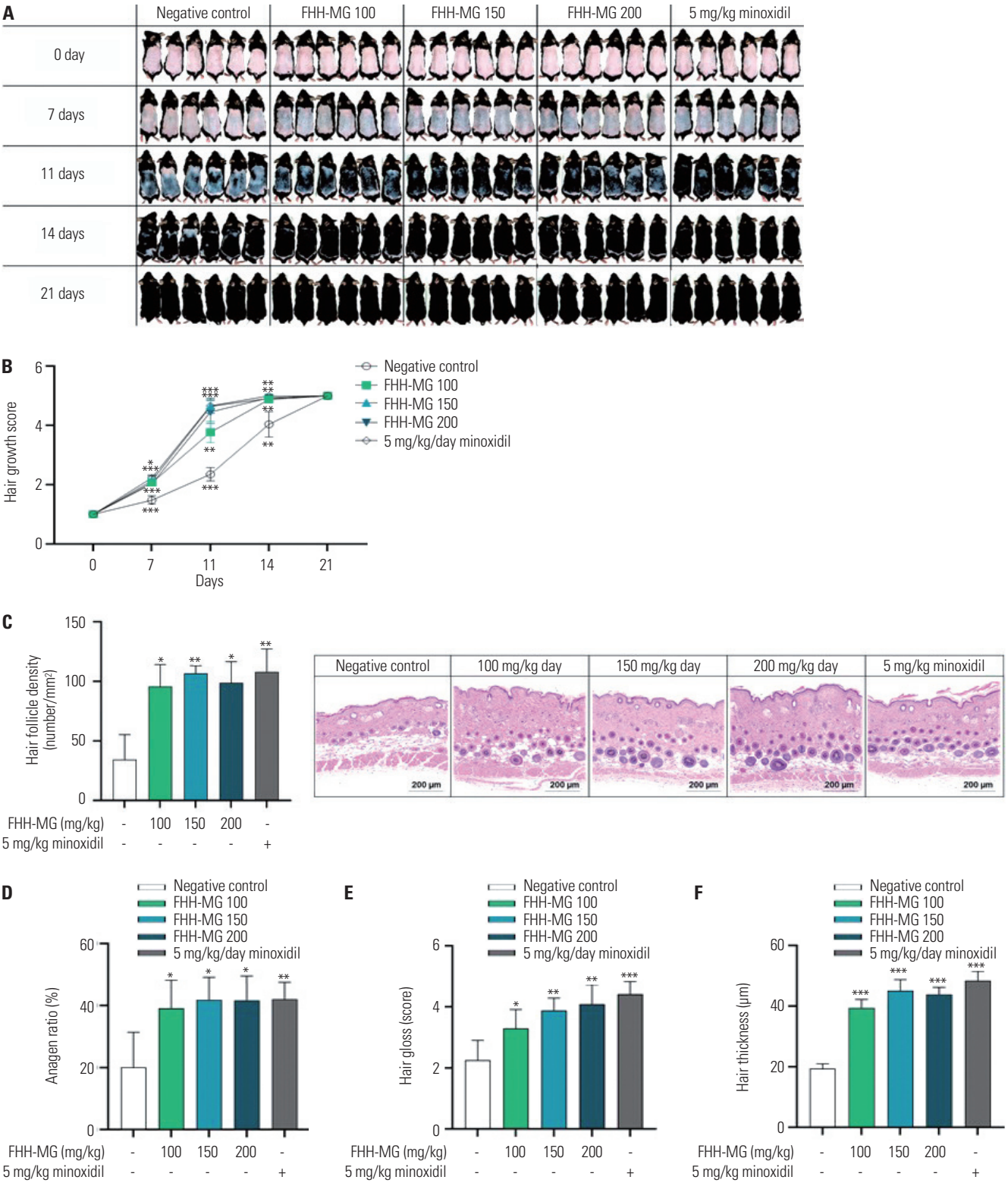

Fig. 5. Hair growth-promoting effect of different dosages of the FHH-MG mixture in C57BL/6 mice. After shaving, the FHH-MG mixtures were orally administered to mice five times per week. (A) Photographs of animals on days 0, 7, 11, 14, and 21. (B) Hair growth scores were calculated as follows: 0–19% (1 score), 20–39% (2–2.5 score), 60–79% (4–4.5 score), and 80–100% (5 score). Histological analysis of the dorsal skin for hair follicle density (C) and anagen ratio (D) was performed on day 7 after administering the FHH-MG mixture for 7 days. (E) Hair gloss was scored as normal (1 point), a bit shiny (2 points), shiny (3 points), very shiny (4 points), or extremely shiny (5 points). (F) Hair thickness was measured from 10 randomly selected strands of hair after 21 days of oral treatments with FHH-MG mixtures or minoxidil. Hair gloss and hair thickness were measured after 3 weeks of FHH-MG administration. Data are presented as the mean±SD (n=12). *p<0.05, **p<0.01, and ***p<0.001 compared to the negative control. Negative control, vehicle only; FHH-MG 100, FHH-MG 100 mg/kg/day; FHH-MG 150, FHH-MG 150 mg/kg/day ppm; FHH-MG 200, FHH-MG 200 mg/kg/day; SD, standard deviation.

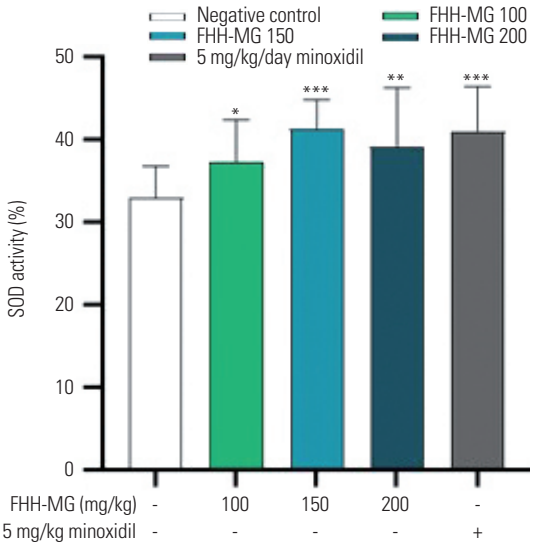

Fig. 6. The antioxidant activity of different doses of FHH-MG and minoxidil in C57BL/6 mice was measured after 3 weeks of administration. Data are presented as the mean±SD (n=12). *p<0.05, **p<0.01, and ***p<0.001 compared to the negative control. Negative control, vehicle only; FHH-MG 100, FHH-MG 100 mg/kg/day; FHH-MG 150, FHH-MG 150 mg/kg/day ppm; FHH-MG 200, FHH-MG 200 mg/kg/day; SD, standard deviation.

underscores the benefits of using combined botanical extracts over single-ingredient formulations. Integrating the anti-inflammatory and antioxidant properties of mastic gum with the role of peppermint in initiating the anagen phase provides a comprehensive strategy for improving hair health and promoting growth. Further, our in vitro and in vivo studies indicate that MP73 elicits a potent protective mechanism against oxidative stress and inflammation, both of which are critical for sustaining cellular proliferation and subsequent hair growth. Oxidative stress can damage hair follicle cells, while inflammation may disrupt the normal hair growth cycle.[26-28] By counteracting these detrimental factors, MP73 addresses two essential aspects of hair health: it potentially reduces hair loss and creates an environment conducive to hair growth.

In vivo studies further corroborate the hair growth-promoting effects of the MP73 mixture. C57BL/6 mice treated with MP73 demonstrated significant improvements in hair growth, gloss, and thickness. These improvements were supported by a dose-dependent increase in the expression of VEGFA, EGF, β-catenin, and IGF-1, all vital mediators of hair follicle development and growth. As a growth factor that promotes angiogenesis, VEGF is

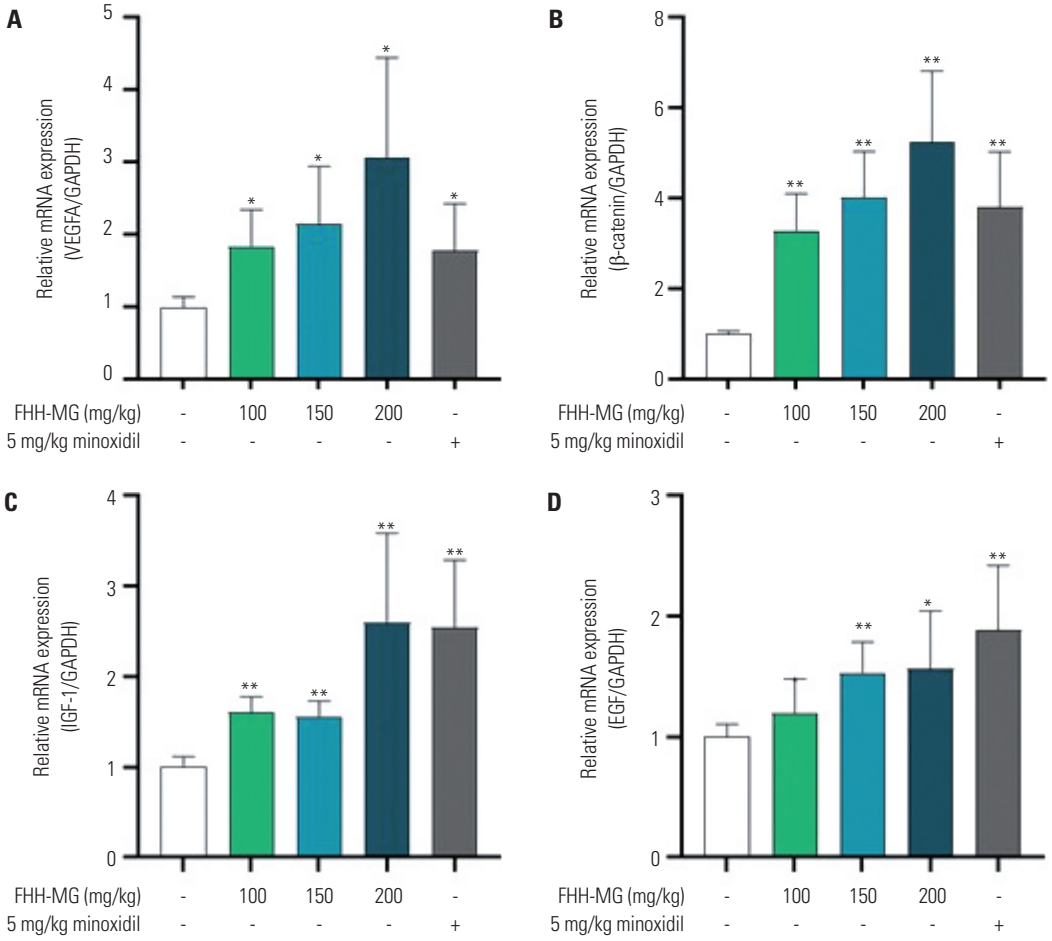

Fig. 7. The effect of FHH-MG treatment on the expression of VEGF (A), β-catenin (B), IGF-1 (C), and EGF (D) in C57BL/6 mice after 3 weeks of administration. Data are presented as the mean±SD (n=12). *p<0.05 and **p<0.01 compared to the negative control. Negative control, vehicle only; FHH-MG 100, FHH-MG 100 mg/kg/day; FHH-MG 150, FHH-MG 150 mg/kg/day ppm; FHH-MG 200, FHH-MG 200 mg/kg/day; SD, standard deviation; VEGFA, vascular endothelial growth factor A; IGF-1, insulin-like growth factor-1; EGF, endothelial growth factor.

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essential for transitioning hair follicles into the anagen phase and enhancing their vascularization. This facilitates nutrient delivery, accelerates hair growth, and stimulates cell proliferation.[24,29] EGF and IGF-1, also essential for β-catenin activation, play notable roles during the induction and maintenance of the anagen phase, with deficiencies in these factors potentially leading to hair loss.[30-34]

Despite these promising findings, this study had several limitations that warrant consideration. The use of HFDPCs and C57BL/6 mice may fail to comprehensively capture the complexity of human scalp conditions, necessitating cautious interpretation of these findings in the context of human application. The absence of clinical trials involving humans is an important limitation, given that trials are essential to confirm the efficacy, safety, and optimal dosage for human use. Moreover, the investigation did not differentiate among various types of hair loss conditions, and the efficacy of MP73 in treating specific alopecia types or other hair disorders is an area for future research. The potential for side effects in humans has not been thoroughly evaluated, especially considering the topical application of hair growth products. Although this study compares MP73 with minoxidil, it lacks a comprehensive comparison with other existing hair growth treatments, both synthetic and botanical.

In conclusion, our findings reveal that the combination of mastic gum and peppermint extract can induce HDFPC proliferation, thereby suggesting that mastic gum and peppermint extract may be a potential therapeutic botanical product to promote hair growth and health. The ability of the formulated mixture to enhance cell proliferation, exhibit antioxidant and anti-inflammatory properties, and stimulate key growth factors presents a promising alternative to current hair growth treatments. Future studies focusing on clinical trials and mechanism elucidation will be crucial to further validate the efficacy and safety of the MP73 mixture for human use.

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AUTHOR CONTRIBUTIONS

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