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Full Length Research Paper

Evaluating the Hair Regrowth Potential of Botanical Ethanol Extracts Using Cellular and Mouse Models

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This study determined the effect of 30% ethanol extract of plant mixture (EPM: *Glycyrrhiza uralensis, Angelica gigas, Acorus calamus, Cnidium officinale, Panax ginseng, Camellia sinensis, Salvia miltiorrhiza, Zanthoxylum schinifolium, Carthamus tinctorius, Prunus persica* and *Scrophularia buergeriana*) on promotion of hair growth in human hair dermal papilla cells and C57BL/6J mice. EPM significantly increased the proliferation of human hair dermal papilla cells in a dose- and time-dependent manner ($p < 0.05$ and $p < 0.01$). EPM also enhanced mRNA and protein levels of growth factors such as IGF-1, VEGF, KGF and HGF. Moreover, photographical and histological observation showed that application of EPM resulted in the early onset and prolongation of the anagen phase of the hair cycle. In addition, the EPM was revealed to possess potent inhibitory effect on the 5α-reductase activity. Taken together, these results suggest that EPM has hair growth promoting potential and can be used for hair growing products.

Key words: Hair growth, extract of plant mixture, growth factors, steroid 5α-reductase.

INTRODUCTION

Hair growth is controlled by a unique repetitive cycle comprising a hair fiber production phase (anagen), a brief regression phase (catagen) and a resting period (telogen) (Stenn *et* al., 1996; Paus and Cotsarelis, 1999). The regulatory mechanism of the hair cycle has not yet been fully understood as several factors were implicated to exert their specific roles in hair growth control. Androgens are known to cause regression and balding on the scalp in genetically disposed individuals. Testosterone and dihydrotestosterone (DHT), which is formed from

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testosterone by the action of 5α-reductase, are two major androgens, and DHT has been considered more potent to trigger hair growth or hair loss (Russell and Wilson, 1994). The main role of 5α-reductase is to metabolize testosterone into a more potent potent androgen, DHT, which can bind firmly to androgen receptors with greater affinity and slower dissociation rate than testosterone. Molecular cloning studies have characterized two genes that encode two isoenzymes, 5αR1 and 5αR2. The former exists predominantly in the skin, whereas the latter exists in the prostate (Liu and Yamauchi, 2008). Human hair dermal papilla cells (HHDPCs) are a group of specialized fibroblasts within the hair follicle bulb; these cells play an essential role in the control of hair growth through the secretion of diffusible proteins such as insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) (Philpott and Kealey, 1994; Itami et al., 1995; Lachgar et al., 1996; Fujie et al., 2001).

Recently, the Food and Drug Administration (FDA) has approved two drugs- finasteride (inhibitor of 5α-reductase) and minoxidil (anti-hypertensive), for the treatment of hair loss in men. However, these drugs have limited therapeutic uses owing to their unsatisfactory cure rates (D'Amico and Roehrborn, 2007). Lately, several studies have shown that hair growth is affected by hair follicle inflammation, hair follicle oxidation and hair follicle keratinocyte apoptosis (Peters et al., 2006). There is little published data regarding the anti-inflammatory, anti-oxidative and anti-apoptotic properties, and hair growth effects of *Glycyrrhiza uralensis* (Shin et al., 2008), *Angelica gigas* (Lee et al., 2003), *Acorus calamus* (Sandeep and Nair, 2010), *Cnidium officinale* (Onishi et al., 1998), *Panax ginseng* (Matsuda et al., 2003), *Camellia sinensis* (Hsu, 2005), *Salvia miltiorrhiza* (Yagi and Takeo, 2003), *Zanthoxylum schinifolium* (Tsai et al., 2000), *Carthamus tinctorius* (Akihisa et al., 1996), *Prunus persica* (Valero et al., 2012) and *Scrophularia buergeriana* (Jeong et al., 2008). In the present study, whether EPM affects proliferation and up-regulation of HHDPCs growth factors was investigated. Furthermore, topical application of EPM promoted hair regeneration in C57BL/6J mice.

MATERIALS AND METHODS

All the plant mixtures were obtained from Deakwang, Korea. About 250 g of plant mixture [*G. uralensis* (root), *A. gigas* (root), *A. calamus* (root), *C. officinale* (root), *P. ginseng* (root), *C. sinensis* (leaf), *S. miltiorrhiza* (root), *Z. schinifolium* (bark), *C. tinctorius* (flower), *P. persica* (seed) and *S. buergeriana* (root) (1:1:1:1:1:1:1:1:1:1:2.5) were crushed and 2000 ml of 30% ethanol was mixed at 65°C for 120 min. The extract was then concentrated in a vacuum evaporator. The weight of the resulting residue was determined as 50 g, and it was then dissolved in 30% ethanol for subsequent treatment for *in vivo* study. EPM for *in vitro* study were then filtered to sterilize using 0.20 µm-filter units (ADVANTEC, Toyo Roshi Kaisha, Japan).

Preparation of rat microsomes

The method of preparation of rat microsomal suspension has been reported elsewhere (Liu et al., 2006). Two male Sprague-Dawley rats were killed. The livers were removed and rinsed with cold normal saline solution. Specimens were then minced with scissors and homogenized in a solution composed of Krebs-Ringer phosphate buffer (pH 7.2). The homogenate was then centrifuged three times at 900 x *g*, 4°C for 10 min each time. All the supernatants were collected. The resulting supernatants containing microsomal particles were tested for soluble protein by the Lowry method (Lowry et al., 1951) and kept at -80°C until use.

Measurement of steroid 5α-reductase inhibitory activity

5α-Reductase assay was performed according to the method of Matsuda et al. (2001) with some modifications. The 0.75 ml reaction solutions each contained 0.05 ml of various plant extracts in dimethyl sulfoxide (DMSO) solution, 0.05 ml of freshly prepared 500 µg/ml testosterone solution in 50% ethanol solution, 0.2 ml of microsomal suspension, 0.08 ml of 0.77 mg/ml NADPH in 0.01 M Tris-HCl buffer (pH 7.2), and 0.37 ml of 2 mM dithiothreitol water solution; the reaction solutions were then incubated at 37°C for 30 min. The reactions were then stopped by adding 0.82 ml dichloromethane, followed by 0.1 mg/ml p-hydroxybenzoic acid n-hexyl ester (as an internal standard for HPLC). Samples were shaken for 60 s, and then centrifuged at 400 xg for 10 min. The methylene chloride (MC) phase was decanted and evaporated till dry. The residue was redissolved in 500 µl methanol. A 20 µl aliquot was analyzed for remaining testosterone content using high pressure liquid chromatography (HPLC). Samples were injected into an analytical Hypersil®-Gold column (Thermo Scientific, USA, part No 25005-154630) 150 x 4.6 mm i.d. with 5 µm internal particle size, using testosterone (> 98% pure) as a standard. A isocratic elution mode was applied, in which the 65% methanol water solution (v/v) were used as the mobile phase with a flow rate of 1 ml/min and was detected under UV absorbance at 242 nm. The temperature of the column was maintained at 40°C. To determine inhibitory activity, two control reactions must be completed: firstly, a complete reaction (rxn) containing 0.2% by the volume of the 50% ethanol instead of the extract; secondly, an enzyme blank (control) in which 5.0 ml dichloromethane is added before the addition of NADPH, so that the conversion of testosterone into DHT does not occur due to the enzyme denaturation. The percent inhibition was calculated using peak area ratio (r) of testosterone/internal standard following the equation:

$$\text{Inhibition (\%)} = [(r_{sample} - r_{rxn})/(r_{ctrl} - r_{rxn})] \times 100$$

Cells culture and proliferation assay

HHDPCs were purchased from ScienCell (Calsbad, CA) as primary cells and grown in mesenchymal stem cell medium (MSCM) supplemented with 5% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% mesangial cell growth supplement (MSCGS) in humidified environment at 37°C in 5% CO_2. The cell proliferation assay were performed using MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] assay kit (Promega, Madison, USA). Briefly, HHDPCs were seeded into 96-well plates (1.5×10^4 cells/well) and treated with various concentrations of EPM (0, 0.05, 0.1, 0.2, 0.5 and 1 µg/ml) and minoxidil (1 µM) for 24, 48 and 72 h, respectively. After incubation, 20 µl/well of MTS solution was incubated for 1 h at 37°C in a humidified 5% CO_2 atmosphere. The optical density at 490 nm was measured three times using an ELISA reader (Bio-Tek, Vermont).

Table 1. Characteristics of specific primers used for RT-PCR analysis.

Gene	Forward primer (from 5'to 3')	Reverse primer (from 5'to 3')
IGF-1	TCAACAAGCCCACAGGGTAT	ACTCGTGCAGAGCAAAGGAT
VEGF	TCTTCAAGCCATCCTGTGTG	GCGAGTCTGTGTTTTTGCAG
KGF	GACATGGATCCTGCCAACTT	AATTCCAACTGCCACTGTCC
HGF	CGAGGCCATGGTGCTATACT	ACACCAGGGTGATTCAGACC
Actin	GCTCTTTTCCAGCCTTCCTT	TCTCCTTCTGCATCCTGTCA

Animals

All animal experiment procedures were as per the guidelines approved by the Institutional Animal Care and Use Committees (IACUC) of Hallym University (Hallym-2012-70-1). Five 5-week-old male C57BL/6J mice (n = 5) purchased from Central Lab Animal (SLC, Japan) were housed individually in stainless steel cages in a room with controlled temperature (22 ± 2°C), relative humidity (50 ± 10%), and 12 h light-dark cycle. Mice were maintained on a commercial pellet diet and fresh tap water. After acclimatization for 7 days, all mice were anesthetized with an intraperitoneal injection of zoletil (Virbac, Carros, France). Mice were randomly allocated into three experimental groups of five animals each and provided with one of the following treatments: 30% ethanol as vehicle for negative control group, 5% minoxidil for positive control group, and 1 mg/ml EPM for experimental group. About 200 µl sample was topically applied to all mice every day for 30 days.

Histological analysis

Individual back dermal tissue samples were fixed in 4% paraformaldehyde (Sigma-Aldrich, MO, USA), and the tissues were then dehydrated and embedded in paraffin using an automatic tissue processor (Leica ASP 300; Leica Microsystems, Wetzler, Germany), sectioned to 5 µm thickness with a microtome, stained with hematoxylin and eosin, and then examined under light microscope (Olympus CX 31; Olympus Tokyo, Japan) for studying histological morphology.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the HHDPCs and mouse back dermal tissue using the EASY-BLUE total RNA extraction kit (Intron Bio, Beverly, MA). cDNA synthesis was performed with 1 µg of total RNA, oligo $(15)_{dT}$ primers and reverse transcriptase in a total volume of 50 µl. PCR reactions were performed in a total volume of 20 µl comprising 2 µl of cDNA product, 0.2 mM of each dNTP, 20 pmol of each primer, and 0.8 unit of *Taq* polymerase. Oligonucleotide primer sequences used in the experiments are shown Table 1. The RT-PCR products were electrophoresed in 1% agarose gels under 100 V and stained with 0.5 µg/ml ethidium bromide. Scanning densitometry was performed using an i-MAX^{TM} Gel Image Analysis system (Core-Bio, Seoul, Korea).

Protein extraction and Western blot

HHDPCs and mouse back dermal tissue were homogenized in lysis buffer. Total proteins (30 µg) were separated using 8% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA), and then hybridized overnight at 4°C with 1:1000 diluted IGF-1, VEGF, KGF, HGF and Actin primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with 1:2000 diluted horseradish-peroxidase-conjugated goat anti-rabbit or donkey anti-rabbit immunoglobulin G secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature, immunoreactive proteins were visualized by an enhanced chemiluminescent solution (Amersham, Uppsala, Sweden) and quantified using a densitometric analysis.

Statistical analysis

The results are represented as the mean ± S.E. The findings of the control and treated groups were compared using ANOVA variance analysis and their significance was analyzed using Student's *t*-test. Differences of $p < 0.05$ were considered statistically significant.

RESULTS

EPM induction of HHDPCs proliferation

EPM increased the proliferation of HHDPCs in a dose and time-dependent manner (Figure 1). Compared with the control, in the HHDPCs, the proliferation rates were increased by 31% on adding 1 µg/ml of EPM and by 36% on adding 1 µM of minoxidil after 72 h. The result suggests that none of the EPM inhibited cell viability at concentrations shown to be effective with minoxidil.

EPM expression of growth factors in HHDPCs

Insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) have been previously identified as key mediators of hair growth and generation (Peus and Pittelkow, 1996; Yano et al., 2001; Peters et al., 2005). Therefore, the expression of these growth factors in the presence and absence of EPM were compared by using RT-PCR and Western blot analysis. As shown in Figure 2 EPM was observed to increase the expression of growth factors (IFG-1, VEGF, KGF and HGF) in HHDPCs. EPM treatment for 24 h with various concentrations increased the expression of growth factor mRNA (Figure 2A) and protein (Figure 2B).

Effect of EPM on 5α-reductase activity

Conversion of testosterone to DHT is an important factor

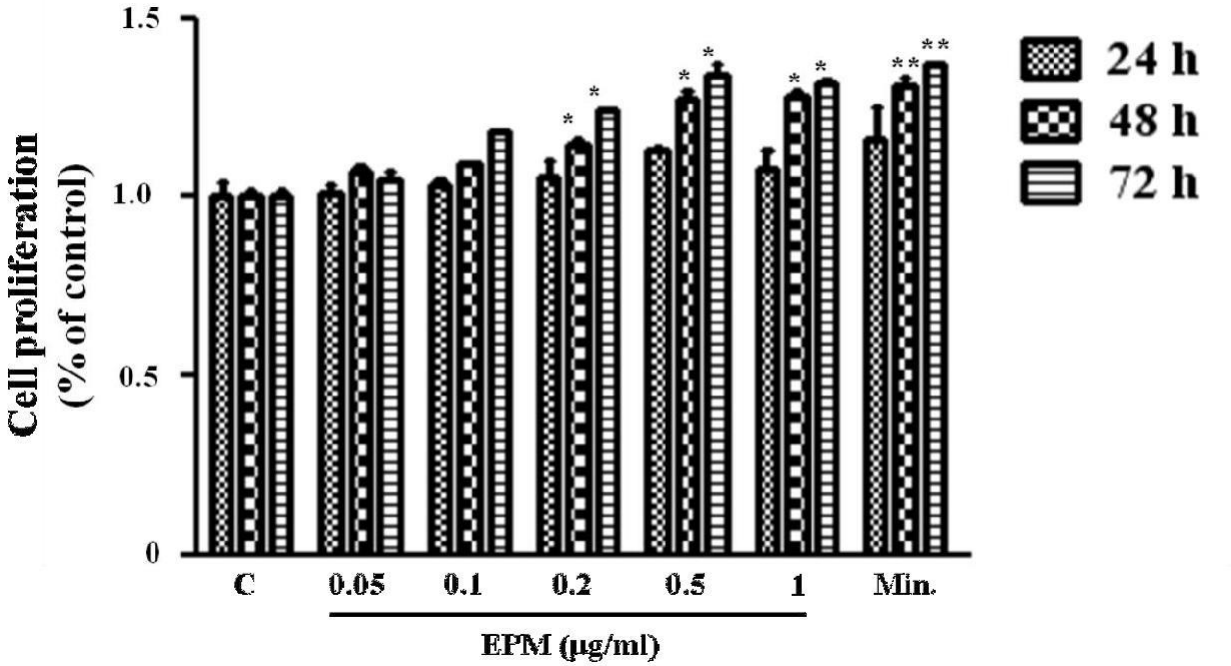

Figure 1. EPM induced the proliferation of HHDPCs as shown by MTS assay. HHDPCs were treated with various concentration of EPM (0.05 to 1 μg/ml) or minoxidil (min; 1 μM) for 24, 48 and 72 h. Values are expressed as mean ± S.E.; *p < 0.05, **p < 0.01 as compared to the vehicle-treated control.

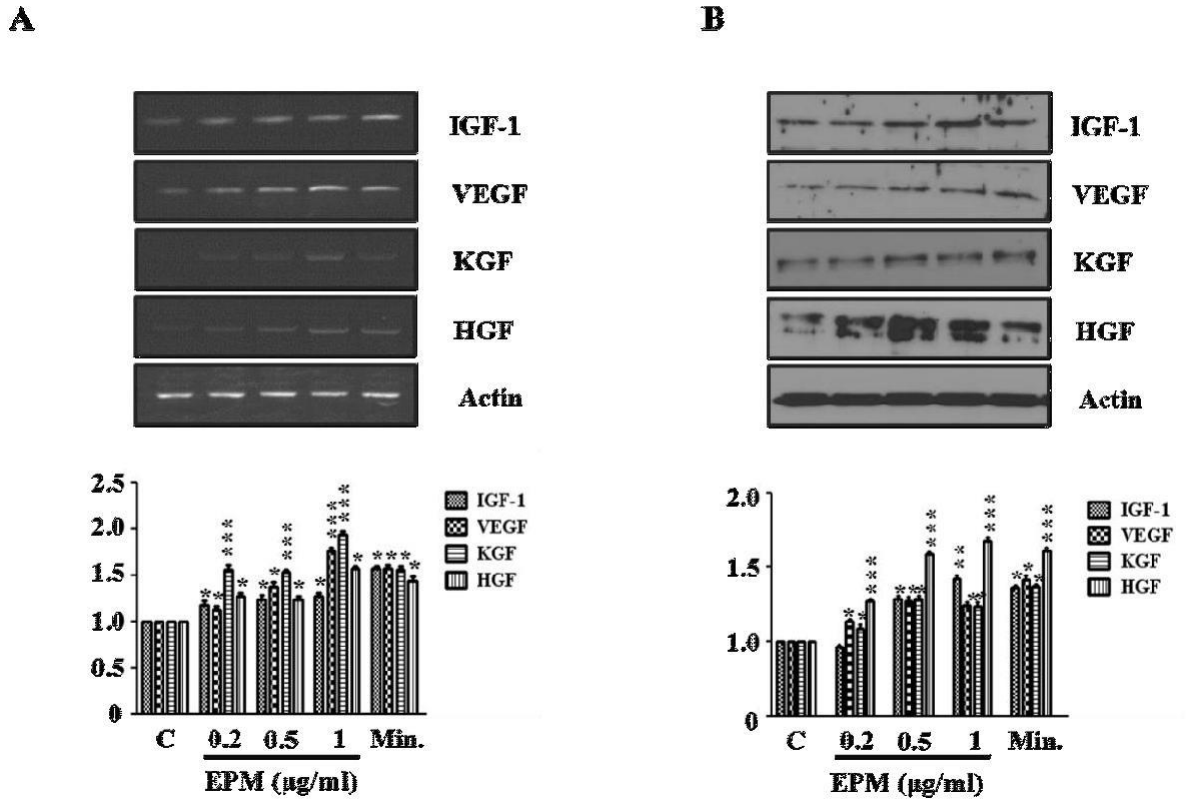

Figure 2. Effects of EPM on the levels of growth factor mRNAs and proteins in HHDPCs were determined by RT-PCR analysis (A) and Western blot (B). Values are expressed as mean ± S.E.; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared to the vehicle-treated control.

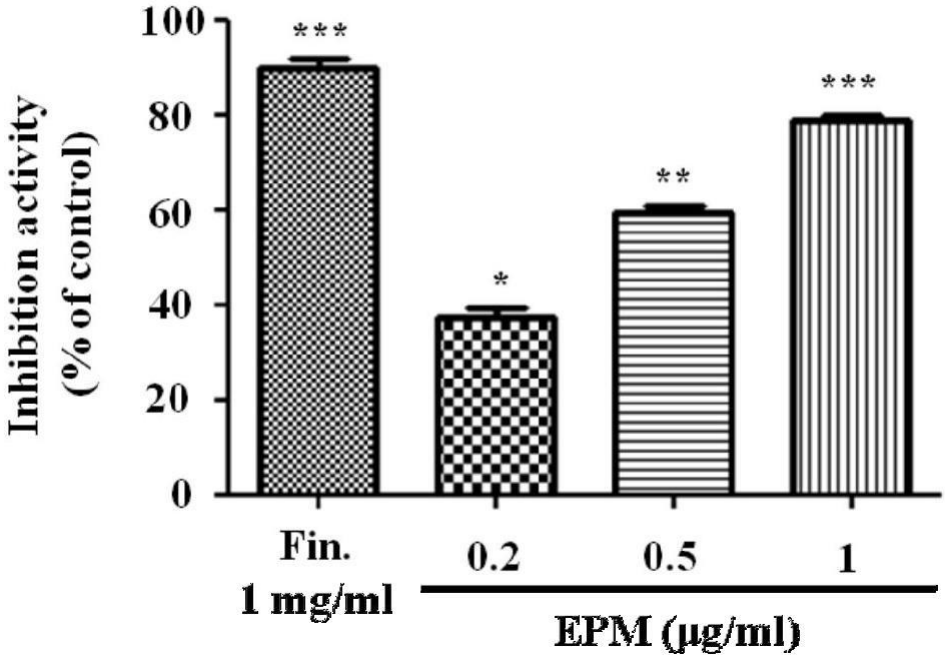

Figure 3. Effect of EPM on the inhibition of 5α-reductase. The assay for 5α-reductase inhibition was performed using rat microsomes as described in materials and methods. The inhibition activity of the control group was regarded as 0% (not shown). Finasteride was used as a positive control. Values are expressed as mean ± S.E.; *p < 0.05, **p < 0.01 and ***p < 0.001 as compared to the vehicle-treated control.

in hair loss. To determine whether EPM could inhibit 5α-reductase activity, the 5α-reductase activities were examined using microsomes from rat liver. As shown in Figure 3, EPM inhibited 5α-reductase activities by 37.1, 59.4 and 78.8% in a dose-dependent manner at concentrations of 0.1, 0.5, and 1 μg/ml. Finasteride, a positive control inhibited 5α-reductase activities by 89% at a concentration of 1 mg/ml.

Effect of EPM on hair regeneration in C57BL/6J mice

The dorsal hair in C57BL/6J mice is known to have a time-synchronized hair growth cycle (Park et al., 2011). Depilated mice skin in telogen phase is pink and darkens along with anagen initiation and then turns gray (Paus et al., 1990). As shown in Figure 4, all mice in EPM groups and minoxidil group exhibited light gray skin from 1 week after depilation, and their hair shafts were visible at 2 week after depilation. Three weeks after depilation, the back dermal tissue was in mature anagen phase in all of the mice. Histologic studies showed that EPM markedly increased the number and morphology of hair follicles as compared to the control group (Figure 5). This result clearly shows that EPM induces early onset of anagen and stimulates hair growth in C57BL/6J mice.

Effect of EPM on growth factors in back dermal tissue C57BL/6J mice

Parallel to *in vitro* assay, gene and protein expressions responsible for growth factors in the back dermal tissue were examined. As shown in panels A and B of Figure 6, gene and protein expressions of IGF-1, VEGF, KGF and HGF increased in EPM-treated mice as compared to those in control mice.

DISCUSSION

Androgens regulate hair growth, and 5α-reductase plays a pivotal role in the action of androgens on target organs. In the preliminary study on screening plants with 5α-reductase inhibition effect, extract of *S. buergeriana* showed the most potent 5α-reductase inhibition activity on high performance liquid chromatography (HPLC) assay (data not shown). On the basis of our previous results, we decided to explore whether EPM of *G. uralensis, A. gigas, A. calamus, C. officinale, P. ginseng, C. sinensis, S. miltiorrhiza, Z. schinifolium, C. tinctorius, P. persica* and *S. buergeriana* (1:1:1:1:1:1:1:1:1:1:2.5) has hair growth effects in HHDPCs and C57BL/6J mice. In the meantime, EPM did not effect toxicity (data not

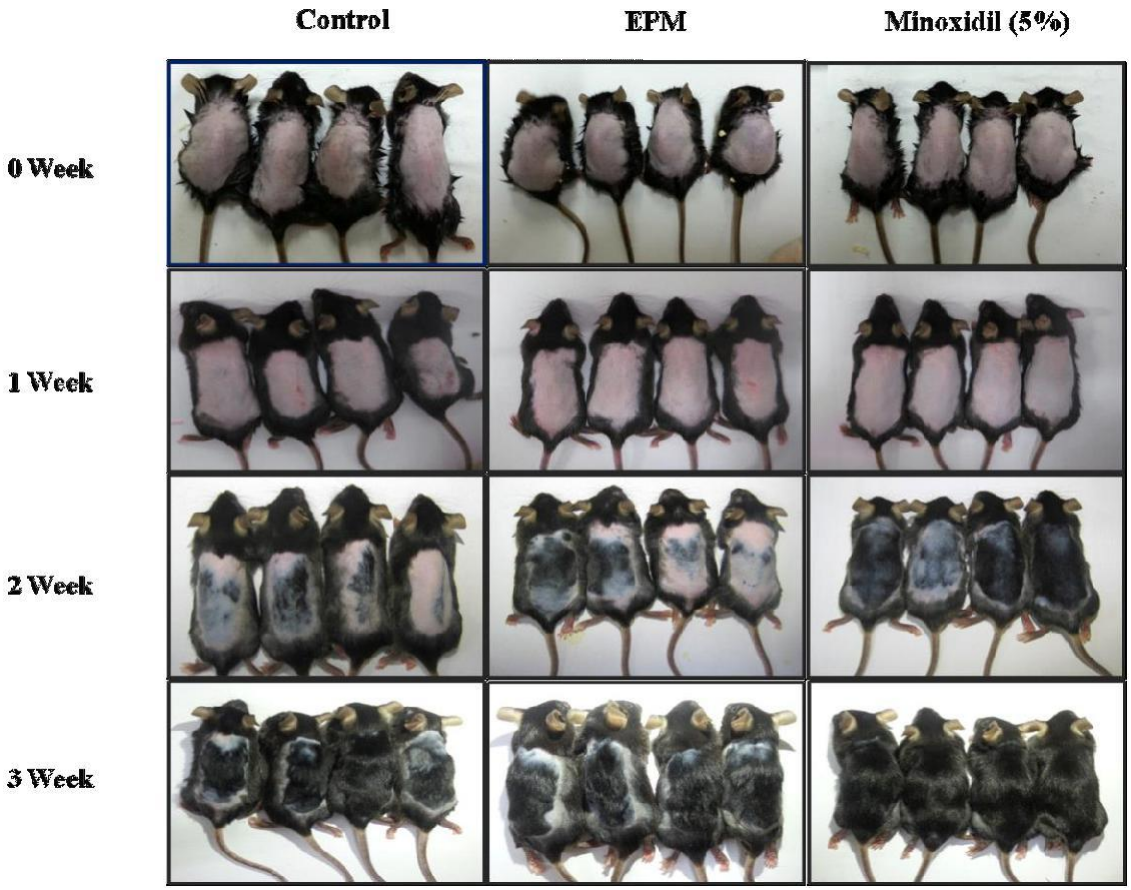

Figure 4. Effects of EPM on hair regeneration in C57BL/6J mice. Hair regeneration is induced by depilating the hairs with a wax/rosin mixture in 5 week-old male mice. The back skin was treated with 1 mg/ml of EPM every day for 30 days. The control group was treated with vehicle (30% ethanol) or 5% minoxidil (positive control).

shown). Our results demonstrated that EPM has an outstanding hair growth promoting effect as well as a regulatory role on the expression of growth factors and inhibitory effect on 5α-reductase.

The mesenchyme-derived dermal papilla (DP) cells play a pivotal role in hair growth regulation. The morphology of DP cells can be altered through the hair growth cycle, being highest in volume in the growing phase and lowest in the resting phase. In the present study, the mesenchyme-derived DP cells are well characterized to have a regulatory role in hair growth, and it is believed that diffusible factors like IGF-1 and KGF from the DP could directly affect follicular epithelium (Itami et al., 1995). Moreover, in IGF-1 transgenic animals, hair elongation was significantly higher than that in their littermates (Roh *e*t al., 2002). KGF, which belongs to the fibroblast growth factor (FGF) family, is an important paracrine mediator of proliferation and differentiation in a wide variety of epithelial cells (Danilenko, 1999). VEGF is a marker of angiogenesis, stimulating hair growth by facilitating the supply of nutrients to the hair follicle, increasing follicular diameter (Gnann et al., 2013). The regulatory role of KGF in hair growth is well implicated in transgenic mice models in which KGF directly affects the development of hair follicles (Werner et al., 1994). Accordingly, the regulation of growth factors like IGF-1 and KGF in DP cells is fundamental to the control of hair follicle growth. In this study, we have shown that EPM increased levels of IGF-1, VEGF, KGF, and HGF mRNA and protein in the HHDPCs and C57BL/6J mice. These results suggest that EPM may have positive effects on the hair growth promotion, partly through the regulation of growth factors in the HHDPCs and C57BL/6J mice.

In all animals, including humans, two different 5α-reductase isozymes have been characterized. Although type I is predominant in scalp, type II has been identified to have a crucial role in the hair growth regulation. The important role of type II 5α-reductase is supported by a well-documented male pseudohermaphroditism where type II 5α-reductase is deficient and shows no androgenetic alopecia (Hoffmann and Happle, 2000). Moreover, the FDA USA approved the use of finasteride, an inhibitor of type II 5α-reductase, in men with androgenetic

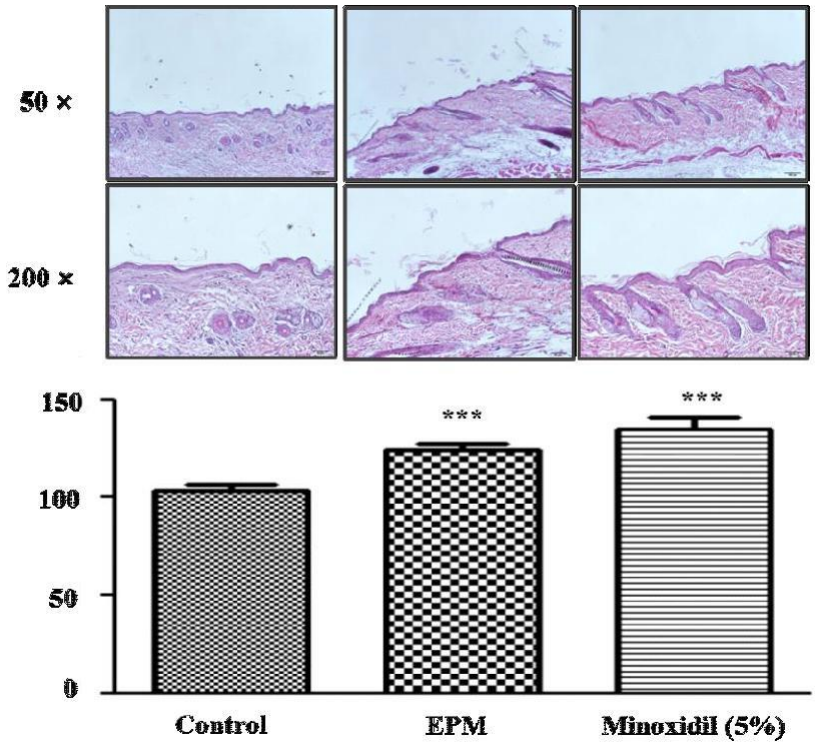

Figure 5. Effects of EPM on anagen prolongation in C57BL/6J mice. The back dermal skin of mice at 14 days after depilation were obtained and shaved then stained with hematoxylin and eosin. Values are expressed as mean ± S.E.; ***p < 0.001 as compared to the vehicle-treated control.

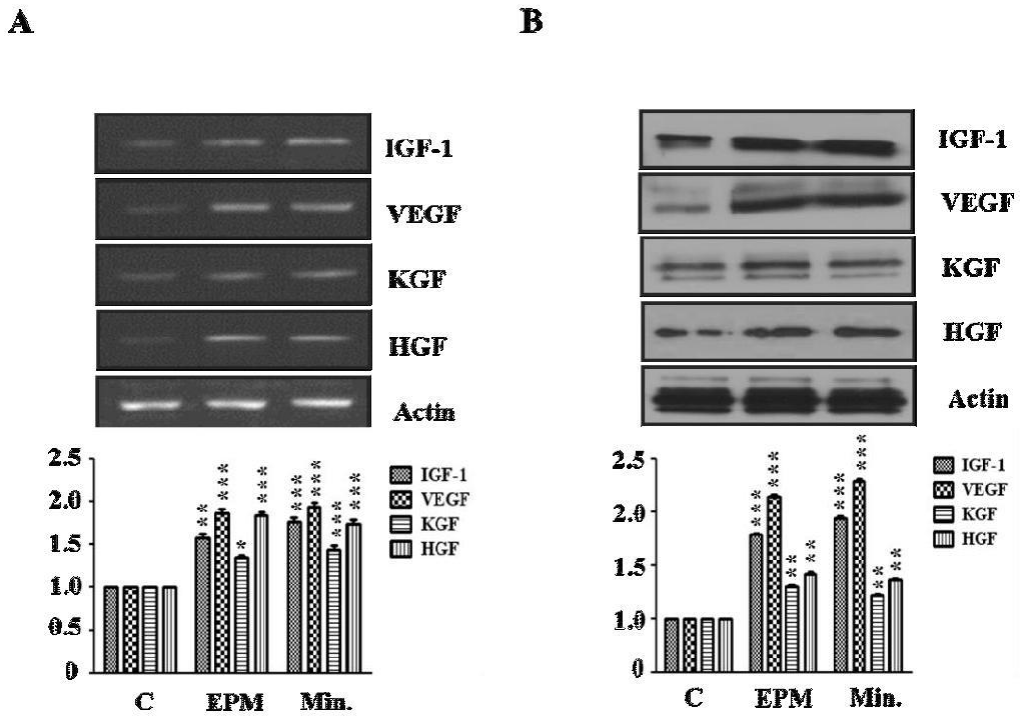

Figure 6. Effects of EPM on the mRNA and protein levels of growth factors in C57BL/6J mice, as determined by RT-PCR analysis (A) and Western blot (B). Values are expressed as mean ± S.E.; *p < 0.05, **p < 0.01 and ***p < 0.001 compared to the vehicle-treated control.

alopecia. Thus, it is noteworthy that EPM has a potent inhibitory effect on type II 5α-reductase activity.

Overall, it has been demonstrated that EPM is capable of preventing hair loss via proliferation of HHDPCs, inhibition of 5α-reductase activity and putative molecular regulatory roles, thus suggesting that EPM may be a good candidate for promoting hair growth.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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Abbreviations

EPM, Extract of plant mixture; **DHT,** dihydrotestosterone; **HHDPCs,** human hair dermal papilla cells; **IGF-1,** insulin-like growth factor-1; **VEGF,** vascular endothelial growth factor; **KGF,** keratinocyte growth factor; **HGF,** hepatocyte growth factor; **HPLC,** high performance liquid chromatography.

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