



The hair growth promoting effect of *Asiasari radix* extract and its molecular regulation

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Summary

Background: Hair loss is a distressing condition for an increasing number of men and women. It is of great importance; therefore, to develop new therapies for the treatment of hair loss.

Objective: We examined the effects of 45 plant extracts that have been traditionally used for treating hair loss in oriental medicine in order to identify potential stimulants of hair growth.

Methods: Six-week-old female C57BL/6 and C3H mice were used for evaluating the hair growth-promoting effects of the plant extracts. Topical application onto the backs of the C57BL/6 and C3H mice was performed daily for 30 days and 45 days, respectively. Protein synthesis was measured by the cysteine uptake assay, using cultured murine vibrissae follicles. Proliferation of the immortalized human keratinocyte cell line (HaCaT) and human dermal papilla (DP) cells was evaluated by the MTT and thymidine incorporation assays. The mRNA levels of several growth factors that have been implicated in hair growth control were measured by reverse transcription-polymerase chain reaction (RT-PCR).

Results: Among the tested plant extracts, the extract of *Asiasari radix* showed the most potent hair growth stimulation in C57BL/6 and C3H mice experiments. In addition, this extract markedly increased the protein synthesis in vibrissae follicle cultures and the proliferation of both HaCaT and human DP cells in vitro. Moreover, the *A. radix* extract induced the expression of VEGF in human DP cells that were cultured in vitro.

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Conclusion: These results suggest that the *A. radix* extract has hair growth-promoting potential, and that this effect may be due to its regulatory effects on both cell growth and growth factor gene expression.

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1. Introduction

A unique feature of hair growth is its cyclicity (anagen, catagen, and telogen phases). These cyclic changes involve rapid remodeling of both the epithelial and dermal components of the hair follicles [1–4]. The dermal papilla (DP), which is the main mesenchymal component, is located at the deepest end of the hair follicle, and is thought to play essential roles in the induction of new hair follicles and the maintenance of hair growth [5–7]. For this reason, isolated dermal papilla cells are frequently used for studies of hair growth regulation. Studies have shown that DP size is well correlated with hair growth cycle, and the cell number of DP is increased in anagen phase [8].

Various cytokines and growth factors are involved in the regulation of hair morphogenesis and hair growth, and these include epidermal growth factor (EGF), transforming growth factor- α (TGF- α) and TGF- β , keratinocyte growth factor (KGF), insulin-like growth factor-1 (IGF-1), interleukin-1 (IL-1), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF) [9–14]. It is of interest that dermal papilla cell-derived factors have chemotactic effects on the surrounding cells, which lead, in turn, to hair growth promotion [12].

Aside from growth factors, the most important and well-known factors involved in hair loss are androgens. Androgens are known to cause hair regression and balding in genetically predisposed individuals. Testosterone and dihydrotestosterone (DHT), which is formed from testosterone by the action of 5 α -reductase, are the two major androgens; DHT is considered to be more potent in triggering hair growth and/or hair loss [15,16]. There are two types of 5 α -reductase: type I and type II. Although type I is the predominant form in scalp tissue, type II has a crucial role in the regulation of hair growth. Androgens affect the DP cells by producing paracrine signals, such as the growth factors mentioned above. Interestingly, androgens have diverse effects on human hair growth. According to a recent report, androgen-inducible TGF- β 1, derived from DP cells, is responsible for androgen-induced epithelial cell growth inhibition [17]. In contrast, IGF-1 released from DP cells by androgen

acts as an important positive mediator of the surrounding epithelial cells [18]. Despite the accumulating data on the regulation of hair growth, the precise mechanism underlying hair growth control remains to be elucidated.

The number of men and women who suffer hair loss and/or hair thinning is increasing in accordance with changes in lifestyle and nutritional balance. Therefore, it is of great importance to develop new therapies to prevent hair loss and to enhance hair growth. In this study, we searched for potential therapeutics among those plant extracts that have been used traditionally in oriental medicine for treating hair loss. We examined 45 different plant extracts and discovered that the *Asiasari radix* extract had the most potent hair growth-promoting effect. We report here that an extract of *A. radix* stimulates telogen to anagen transformation in C57BL/6 and C3H mice and increases both cellular proliferation and protein synthesis. Moreover, the *A. radix* extract upregulates the expression of the *VEGF* gene in cultured human DP cells.

2. Materials and methods

2.1. Materials

An ethanol extract of *A. radix* was purchased from OBM Lab (Daejeon, South Korea). *A. radix* is the root and/or rhizome of *Asiasarum heterotropoides* F. Maekawa var. *mandshuricum* F. Maekawa or *Asiasarum sieboldi* F. Maekawa (Aristolochiaceae). The dried root of *A. radix* was crushed and extracted with cold ethanol. The ethanol extract was concentrated in a vacuum evaporator and the resulting residue was weighed and dissolved to a 5% solution in 70% ethanol. C57BL/6 and C3H mice were supplied by Dae-Han Biolink (Eumsung, Korea). Williams medium E, Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were obtained from Gibco-BRL (Gaithersburg, MD), while [methyl- 3 H]thymidine (40–60 Ci/mmol) and L-[35 S]cysteine (1000 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). The reverse transcription system and Taq polymerase were purchased from Promega (Madison, WI).

2.2. Animal test

Six-week-old female C57BL/6 and C3H mice were allowed to adapt to their new environment for one week, with food and water provided ad libitum. The backs of the mice were shaved with animal clipper at seven weeks of age, at which time all of the hair follicles were synchronized in the telogen stage. Starting the following day (day 1), 0.2 ml of a 1% solution of *A. radix* extract in 40% ethanol was applied topically, on a daily basis, for 30 days (C57BL/6 mice) or 45 days (C3H mice). Hair growth promotion was evaluated simply by observing the darkening of the skin color, which indicated telogen-to-anagen conversion [19].

2.3. Isolation and culture of mouse vibrissae tissues

The whisker pads were dissected from the upper lips of the C57BL/6 mice and the vibrissae hairs were trimmed away. After rinsing with copious amounts of 70% ethanol, the snout skins were allowed to air dry for 2 min. Under a dissecting microscope (Nikon SMZ-2T; Nikon Inc., Kanagawa, Japan), the snout skins were cut longitudinally to isolate individual rows of vibrissae. Finally, single follicles were isolated from each row of vibrissae [20]. The follicles were maintained at 37 °C in an atmosphere of 5% CO₂ in Williams medium E that was supplemented with 10 µg/ml insulin and 10 µg/ml hydrocortisone (Sigma–Aldrich, St. Louis, MO).

2.4. Isolation and culture of human dermal papilla cells

Human anagen hair follicles were isolated from the scalp skin of normal men aged from 25 to 30 years who were undergoing plastic surgery. The dermal papilla cells were isolated and cultured as described previously [21], with slight modifications. The DP cells were maintained at 37 °C in an atmosphere of 5% CO₂ in DMEM that was supplemented with 10% FBS.

2.5. Cysteine uptake assay

The isolated murine anagen follicles were allowed to adapt in Williams medium E at 37 °C in an atmosphere of 5% CO₂. After 12 h, the *A. radix* extract and 1 µCi/ml of L-[³⁵S]cysteine were added to the cultures. Following incubation for 72 h, the follicles were washed twice with PBS and then placed individually into scintillation vials. Each follicle was dissolved in 200 µl of Soluene 350 (Packard, Meriden, CT) at 60 °C for 1 h. The level of radioactivity

was measured in a liquid scintillation counter (Beckmann, Fullerton, CA).

2.6. MTT assay

Cell viability was determined by the MTT assay. Cells were seeded at a density of 10⁴ in 0.2 ml of medium into each well of 96-well plates. After 18 h of incubation, the cells were washed with PBS and cultured for 24 h with 200 µl of FBS-free DMEM, together with the indicated concentrations of *A. radix* extract. Then, the medium was removed and 100 µl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] (1 mg/ml) (Sigma–Aldrich, St. Louis, MO) in PBS was added. At the end of 4 h incubation, the plates were centrifuged, and the untransformed MTT was removed. After 100 µl of absolute ethanol was added to each well, the plates were shaken for 5 min. The optical density at 570 nm was determined using an ELISA reader. The cell viability rates were calculated from the OD readings and are represented as percentages of the control value (untreated cells).

2.7. Thymidine incorporation assay

The DP and HaCaT cells were plated in six-well plates and grown for 12–24 h in DMEM supplemented with 10% FBS. After washing twice with PBS, the cells were placed in FBS-free DMEM, together with 1 µCi of [³H]thymidine and the indicated concentrations of *A. radix* extract. Following incubation for 48 h, the cells were washed twice with PBS, and once with 5% cold TCA. The cells were then lysed with 0.1N NaOH, 1% SDS and the levels of radioactivity were measured by liquid scintillation counter (Beckman).

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

The cells were grown in 100-mm tissue culture dishes to about 80% confluency in DMEM supplemented with 10% FBS. After washing twice with PBS, the cells were cultured for 12 or 24 h in FBS-free DMEM, together with the indicated concentrations of *A. radix* extract. Total RNA samples were extracted by acid using the guanidinium thiocyanate, phenol–chloroform method [22]. One microgram of total RNA samples were reverse-transcribed using the Reverse Transcription System (Promega). The RT products were then subjected to PCR cycles as follows: of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min for 33 cycles. The primers used for amplifying the respective fragments are listed in Table 1. After agarose gel electrophoresis, the

Table 1 Nucleotide sequence of the primers and expected size of PCR products

Growth factor	Primer	Expected size (bp)	Size
IGF-1	Forward	CAGCAGTCTTCCAACCCAAT	429
	Reverse	CCTGCACTCCCTCTACTTGC	
HGF	Forward	GCCTGAAAGATATCCCGACA	523
	Reverse	TTCCATGTTCTTGCCACA	
KGF	Forward	GACATGGATCCTGCCAATT	552
	Reverse	GGAAGAAAGTGGGCTGTTTTT	
VEGF	Forward	CTACCTCCACCATGCCAAGT	536
	Reverse	GCGAGTCTGTGTTTTTGCAG	
TGF- β 1	Forward	GTGGAAACCCACAACGAAAT	579
	Reverse	CTACCTCCACCATGCCAAGT	

PCR products were quantified using a densitometer (Imagemaster, Pharmacia Biotech).

2.9. Real-time PCR

For quantitative analysis of VEGF mRNA alternative splice form, real-time PCR was performed on Rotor-Gene 2000 real-time amplification operator (Corbett Research, Mortlake, Australia). Primers were made based on previous report [23] as follows: VEGF

forward, CCCTGATGAGATCGAGTACATCTT (this primer was used for detecting both 121 and 165 species); VEGF-165 reverse, AGCAAGGCCACAGGGATT; VEGF-121 reverse GCCTCGGCTTGTCACATTTT.

The PCR reactions contained a final concentration of $1 \times$ SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), $10 \mu\text{M}$ specific primers, and 2.5 ng of cDNA. Each gene expression were calculated according to the threshold cycle (C_T) value, normalized using the value of the sample

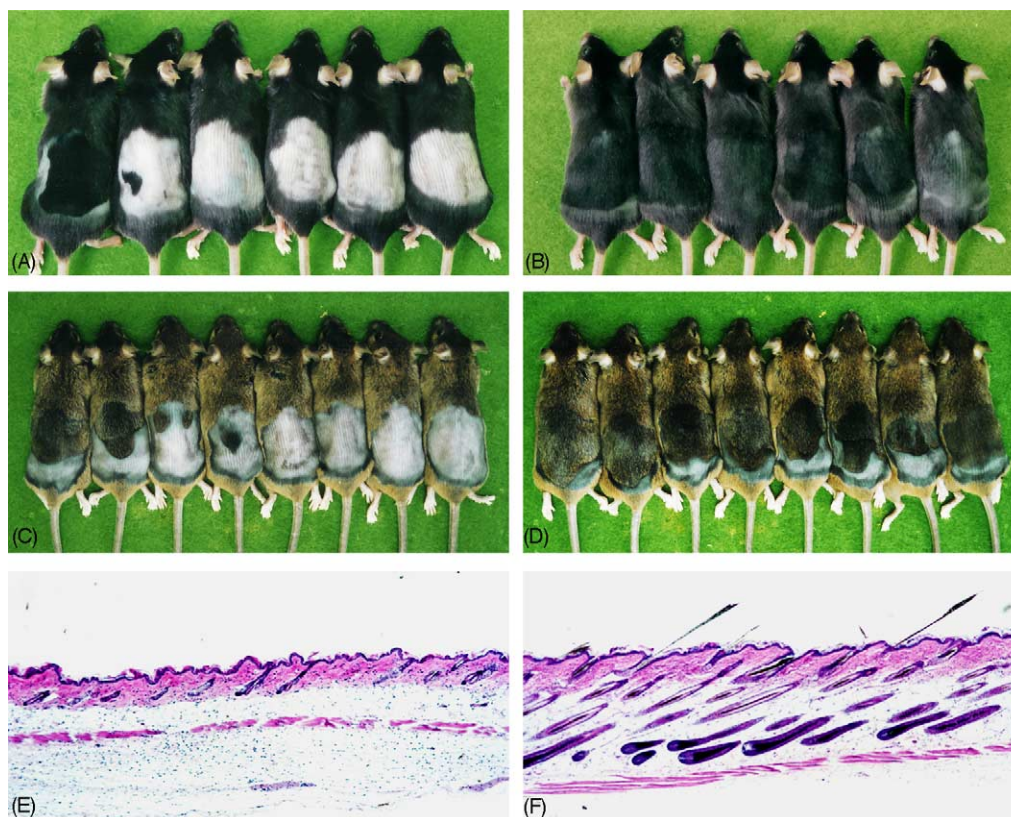


Fig. 1 Hair growth-promoting effects of the *Asiasari radix* extract in C57BL/6 (A and B) and C3H (C and D) mice. The back skins of the mice were shaved and the *A. radix* extract was applied topically. (A and C) Control, 40% ethanol; (B and D) *A. radix* extract, 1% solution in 40% ethanol. The lower panel shows representative histological profiles of skin samples from control (E) and *A. radix* extract-treated (F) C57BL/6 mice.

with the lowest level of each product, and the data were corrected according to the level of GAPDH.

2.10. Statistical analysis

Data were evaluated statistically using Student's *t*-test or one-way analysis of variance followed by Fisher's least significant difference test for a post hoc comparison. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Hair growth-promoting effects of the *A. radix* extract

In order to search for hair growth-promoting agents from alternative medicine, we performed extensive screening of plant extracts using C57BL/6 mice. The *A. radix* extract was superior to all the other plant extracts tested in terms of hair growth promotion. After topical application onto the backs of C57BL/6 mice for up to 30 days, the *A. radix* extract induced earlier telogen-to-anagen conversion than did the vehicle control (Fig. 1A and B). To confirm this result, we subjected C3H mice to the same regimen for 45 days. As shown in Fig. 1C and D, early telogen-to-anagen conversion was also induced by the *A. radix* extract in the C3H mice. Histological studies showed that the *A. radix* extract markedly increased the depth and size of the hair follicles, as compared to the control treatment (Fig. 1E and F). This result supports the notion that the *A. radix* extract induces early onset of anagen and stimulates hair growth. We also tested the effect of the *A. radix* extract on protein synthesis in cultured vibrissae follicles using the cysteine uptake assay. As shown in Fig. 2, the *A. radix* extract significantly increased the uptake of radio-labeled cysteine in the mouse vibrissae hair follicles cultured in vitro, resulting in a 129% uptake, relative to the control at a concentration of 0.0001%. Since it has been found that [³⁵S]cysteine uptake correlates with hair follicle growth in vitro [24], this result implies that the *A. radix* extract stimulates hair growth.

3.2. Effects of the *A. radix* extract on type II 5 α -reductase activity and cell proliferation

It is well known that androgens cause hair regression and balding in genetically predisposed individuals. Several studies implicate the type II 5 α -reductase as the key factor in these processes [15,24]. Previously, we demonstrated that an extract of *Sophora flavescens* had an inhibitory effect on the type II 5 α -

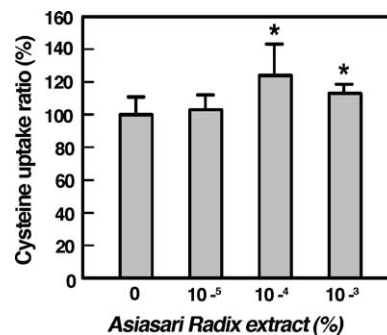


Fig. 2 Effects of the *Asiasari radix* extract on protein synthesis. The isolated murine vibrissae follicles were treated with the *A. radix* extract and 1 μ Ci/ml of L-[³⁵S]cysteine for 72 h. Radioactivity was measured by liquid scintillation counter. The results are shown as the mean values \pm S.E. of quadruplicate measurements ($^*P < 0.05$ vs. control).

reductase [25]. To determine whether the *A. radix* extract had an inhibitory effect on the type II 5 α -reductase, we performed the 5 α -reductase inhibition assay. However, there was no obvious inhibitory effect of the extract on 5 α -reductase (data not shown). Thus, we decided to investigate the effect of the *A. radix* extract on the growth of cells in vitro. To this end, we used two different cell types, immortalized keratinocyte HaCaT cells and primary cultures of human hair DP cells. First, we tested the

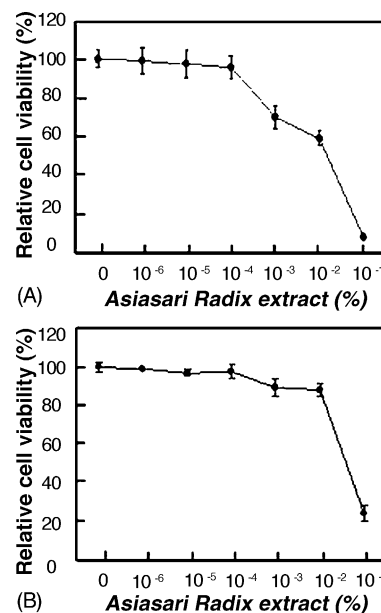


Fig. 3 Concentration-dependent cytotoxicity of the *Asiasari radix* extract in HaCaT (A) and DP (B) cells. The cells were treated for 24 h with the indicated concentrations of the *A. radix* extract. After an additional incubation for 4 h with 1 mg/ml MTT, the formation of formazan was determined at 570 nm in an ELISA reader. The results are shown as the mean values \pm S.E. of triplicate measurements ($^*P < 0.05$ vs. control).

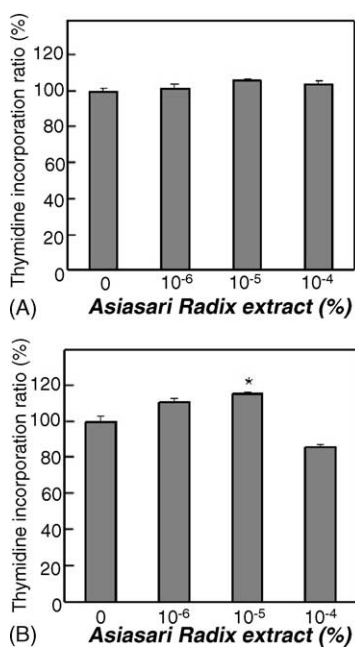


Fig. 4 Effect of *Asiasari radix* extract on [³H]thymidine incorporation. HaCaT (A) and DP (B) cells were treated with the indicated concentrations of the *A. radix* extract and 1 μ Ci of [³H]thymidine, and incubated for 48 h. The radioactivity was measured by liquid scintillation counter. The results are shown as the mean values \pm S.E. of triplicate measurements (* $P < 0.05$ vs. control).

cytotoxicity of the *A. radix* extract using the MTT assay. The *A. radix* extract showed cytotoxic effects for both cells at concentrations higher than 0.0001% (Fig. 3). Based on these results, we evaluated the mitogenic effects of *A. radix* at doses lower than 0.0001%, using the [³H]thymidine incorporation assay. As shown in Fig. 4, the *A. radix* extract at a dose of 0.00001% increased the proliferation of HaCaT and DP cells to 106.5% and 115.6%, respectively, as compared to the control treatment.

3.3. Effect of the *A. radix* extract on growth factor gene expression

Recently, several growth factors related to the hair growth cycle have been reported [9–14]. In this experiment, we used semi-quantitative RT-PCR analysis to search for growth factors that were affected by the *A. radix* extract. We measured the expression levels of IGF-1, KGF, HGF, VEGF (stimulating effect on hair growth), and TGF- β 1 (inhibitory effect on hair growth). There were no significant changes in the growth factor expression of HaCaT cells (Fig. 5A). However, when the DP cells were treated with the *A. radix* extract at dosages of 0.000001% and 0.00001%, the level of VEGF mRNA expression increased significantly at the indicated time-points.

(Fig. 5B). To further verify the induction of VEGF mRNA alternative spliced form, we performed real-time PCR analysis. *A. radix* extract (0.00001%) increased the VEGF 121 and VEGF 165 mRNA level by 129% and 126%, respectively, 24 h after treatment, as compared to control group (Fig. 5C).

4. Discussion

Many people suffer from hair loss or hair thinning, despite the development of several medical treatments. Therefore, it is important to develop novel therapies that prevent hair loss and enhance hair growth. In this respect, alternative medicine has attracted interest. Although it has not yet been incorporated into mainstream of medical care, due to limited scientific evidence and incomplete knowledge of the mechanisms involved, alternative medicine has become an increasingly attractive approach worldwide [26]. *A. radix* is a traditional herb medicine that is used in Korea and China to treat various diseases, such as aphthous stomatitis, local pain, toothache, and gingivitis [27]. It is known to have a number of pharmacological actions, such as analgesic and anti-inflammatory effects and protective effects against brain cell damage [28].

In the present report, we have demonstrated that the *A. radix* extract has outstanding hair growth-promoting effects *in rodent models*. In addition, the *A. radix* extract regulates the expression of growth factors in DP cells that are cultured *in vitro*.

As mentioned previously, various cytokines and growth factors play important roles in hair growth control. IGF-1, VEGF, KGF, and HGF have stimulatory effects on hair follicle growth, whereas EGF and TGF- β have inhibitory effects on hair follicle growth. Our experimental data, therefore, suggest that the *A. radix* extract promotes hair growth by upregulating VEGF expression.

VEGF plays important roles in angiogenesis, as well as in a number of inflammatory and neoplastic diseases that are associated with neovascularization [29]. The vascular network is associated with the hair growth cycle. [30–32]. Lachgar et al. have demonstrated that VEGF mRNA expression in normal human hair follicles varies during the hair cycle [33]. For example, in the anagen phase, VEGF mRNA is expressed strongly in the dermal papillae, whereas VEGF expression is decreased in the catagen and telogen phases. These authors also suggest that VEGF is an autocrine growth factor for DP cells [14]. The importance of VEGF in hair growth stimulation has been further supported by the study on the activity of minoxidil. Minoxidil has been used extensively over the last 30 years to treat androgenic alopecia, but

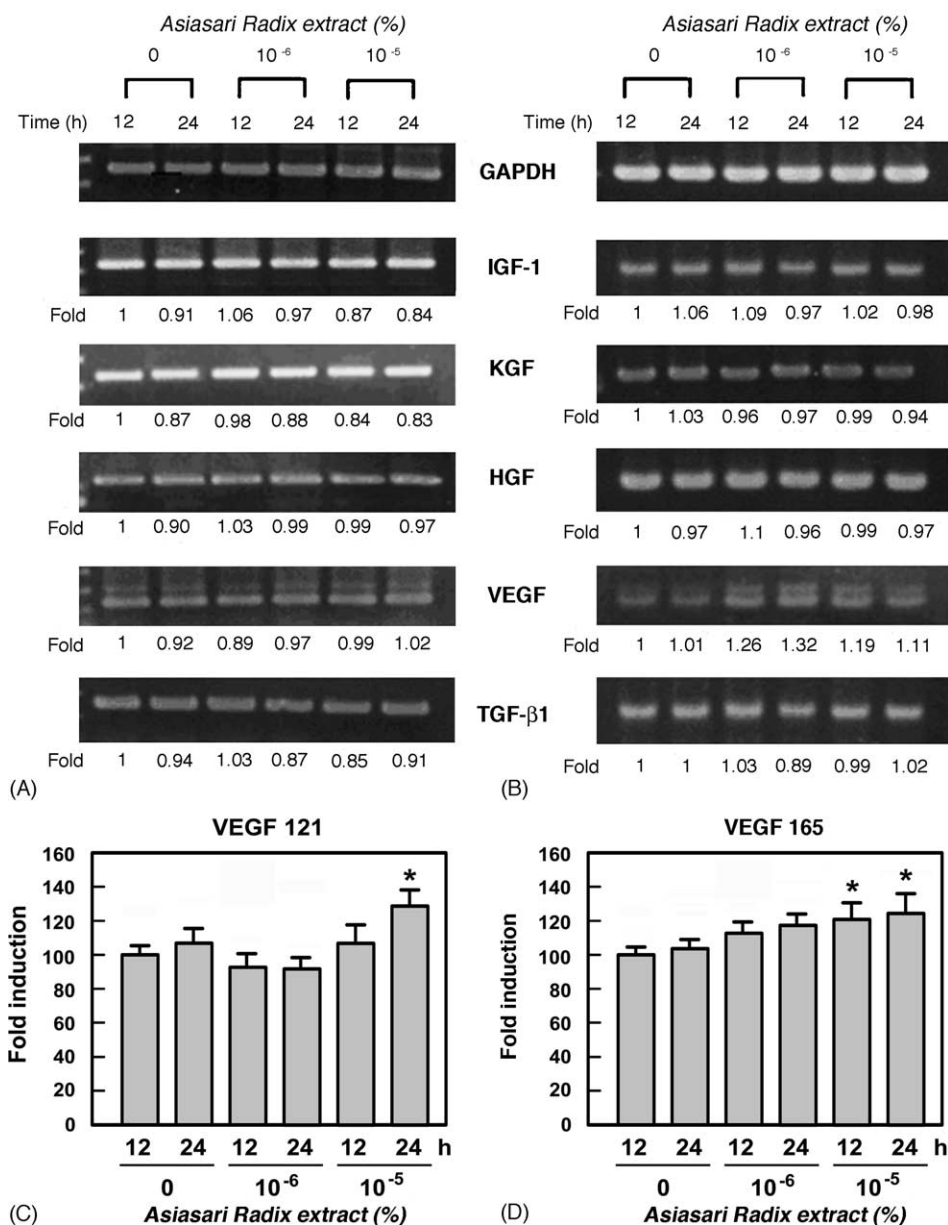


Fig. 5 Changes of mRNA expression levels of growth factors by the *Asiasari radix* extract. After treatment with the *A. radix* extract for 12 h and 24 h, the growth factor mRNA levels in HaCaT (A) and DP (B) cells were evaluated by semi-quantitative RT-PCR. The results were quantified using a densitometer. The level of RT-PCR product for each growth factor is corrected according to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the results are expressed as the fold-induction values. (C) Real-time PCR analysis of VEGF mRNA alternative spliced form. Real-time PCR for VEGF 121 and VEGF 165 was performed as in Section 2. The data are corrected according to the level of GAPDH and shown as the mean values \pm S.E. of triplicate measurements ($P < 0.05$ vs. control).

little is known about its mechanism of action [34]. There are several hypotheses regarding the pharmacological activities of minoxidil, and one hypothesis is that minoxidil regulates the expression of VEGF. The importance of VEGF is strongly supported by Lachgar et al. [35]. In 1998, these authors have demonstrated that minoxidil upregulated the expression of VEGF in normal human hair follicles, thereby promoting the conservation of vascularization of hair dermal

papilla, which may contribute to the extension of the anagen phase in androgenetic alopecia [35]. In this regard, it is noteworthy that the *A. radix* extract has the potential to increase the expression of VEGF in DP cells.

Recently, the role of apoptosis in alopecia has been investigated by several groups [1,36,37]. Finasteride, which is an inhibitor of type II 5 α -reductase, also influences caspase and XIAP

expression in hair follicle cells, thereby signaling the induction of anagen, active growth phase of the hair cycle [37]. To elucidate the precise biological mechanism underlying the effects of the *A. radix* extract on hair growth, additional research on the anti-apoptotic effect of this extract is needed. But, unfortunately, the *A. radix* extract did not show an inhibitory effect on the expression of 5 α -reductase in the present study. However, based on its abilities to stimulate cellular proliferation and VEGF expression, the *A. radix* extract appears to be a good candidate for the promotion of hair growth.

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