The water-soluble extract of *Illicium anisatum* stimulates mouse vibrissae follicles in organ culture

Abstract: It is well known that reduced blood flow in the scalp is a cause of alopecia. We have shown previously that the extract of *Illicium anisatum* increases subcutaneous blood flow in mice. In the present study, we used an organ culture system to examine whether this extract promoted hair follicle elongation. B6C3HF1 mouse vibrissae follicles were cultured in serum-free medium for 7 days at 31°C. Follicles treated with water-soluble (WS) extracts of the leaves, fruits and roots of *Illicium anisatum* or shikimic acid grew significantly longer than controls. In contrast, ethyl acetate-soluble (AS) extracts and n-hexane-soluble (HS) extracts of the leaves, fruits and roots of the plant inhibited hair follicles and shaft growth. Fractionation of the WS fruit extract showed that the number 1 and number 2 fractions possessed hair follicle elongation activity. GC/MS analysis revealed that the number 1 fraction contained shikimic acid, and that the number 2 fraction was a mixture of many components including glycosides and polysaccharides. Reverse transcription-polymerase chain reaction analysis demonstrated that shikimic acid also induced mRNA expression of insulin-like growth factor-1, keratinocyte growth factor, and vascular endothelial growth factor in the hair follicles. These results suggest that the WS extract of *Illicium anisatum* promotes hair growth and may be a useful additive in hair growth products.

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Introduction

Alopecia is characterized by a decrease in anagen hair follicles, an increase in pubescences, and small hair follicles. Several factors including androgen effects (1) depressed metabolic function in the hair follicles, and hereditary factors (2) are thought to be responsible for these changes although the exact causes of alopecia remain poorly understood. One of the first clinical signs of reduced peripheral blood flow is the loss of hair, and it has been suggested that reduced blood flow in the scalp may contribute to hair loss (3,4). The blood supply to the scalp originates from the subcutaneous tissue similar to that found in skin elsewhere on the body. However, hair follicles in body skin are situated in the lower part of the dermis, whereas in the scalp they are found in the upper part of the subcutaneous tissue. The lower part of the hair follicle is enveloped by a rich vascular plexus composed of long, more or less parallel vessels connected by cross-shunts. These parallel vessels are assumed to be terminal arterioles and are connected directly to the subcutaneous plexus. Growth and regrowth of the hair therefore depends on sufficient nutritive blood supply to hair follicles. We have shown previously that the extract of *Illicium anisatum* increased subcutaneous blood flow in mice (unpublished data), a finding that suggested this extract may also promote hair growth.

Recently, several *in vitro* assay systems have been reported as a means of investigating the mechanism of hair growth control. These involve using cultured outer root sheath cells (ORSCs) (5), cultured hair papilla cells (6) or organ culture methods for hair follicles from animals and humans (7–9). These *in vitro* assays are especially useful for investigating the effects of drugs as they evaluate the mechanism of hair growth in a shorter time than *in vivo* assays. In this study, we evaluated the hair growth activity of an extract of *Illicium anisatum* using an organ culture system of mouse vibrissae follicles.

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Materials and methods

Preparation of Illicium anisatum extract

The leaves, fruits and roots of *Illicium anisatum* (Koshiro, Osaka, Japan) were extracted with methanol under reflux for 3 h. The extract was then concentrated in a vacuum evaporator. After removal of lipid by n-hexane, the residue was dissolved in purified water, and then extracted three times with ethyl acetate (10). The n-hexane soluble (HS) extract, water-soluble (WS) extract and ethyl acetate-soluble (AS) extract of the leaves, fruits and roots were prepared for analysis by evaporation of the solvent phase. In addition, the WS extract was separated by preparative thin-layer chromatography (TLC) using Silica-gel G (Analtec, Newark, DE) and a solvent system consisting of benzene-methanol-acetic acid (60 : 35 : 10, by vol.), until a single spot was obtained. The four fractions obtained by TLC were eluted with methanol, and each fraction was then prepared for analysis by evaporation of the solvent phase. The number 2 fraction was separated by TLC using Silica-gel G with a solvent system consisting of ethyl acetate-isopropyl alcohol-acetic acid (75 : 33 : 7, by vol.), and analyzed by gas chromatography/mass spectrometry (GC/MS) using a TRACE GC 2000 series (Thermo Quest, Tokyo, Japan) and GCQ plus system (Thermo Quest) with a BPX-5 column (25 m × 0.22 mm, film thickness 0.25 μm) (SGE, Austin, TX, USA). The carrier gas was helium with split injection being used at a ratio of 1 : 50. Injector temperatures were held at 250°C with GCQ, and mass spectrometry being carried out at an ionizing voltage of 70 eV.

Isolation and culture of mouse vibrissae follicles (8)

Mouse vibrissae follicles were harvested from F1 hybrid mice (female C57BL/6 × male C3H/He) at 9 days of age. These animals were the offspring of pregnant mice purchased from Japan SLC (Hamamatsu, Japan). The mice were killed under diethylether, and then with the aid of a stereomicroscope, normal anagen vibrissae hair follicles were removed from the upper lip region using a knife and tweezers. The isolated vibrissae hair follicles were placed for 20 min in Hank's balanced salt solution (Nissui) containing 500 units/ml of penicillin and 500 μg/ml of streptomycin. After washing with RPMI1640 (Nissui), the follicles were placed in the center well of an organ culture dish (Falcon 3037) containing a stainless steel mesh, lens paper, and 0.75 ml of RPMI1640. Following immersion in RPMI1640, the follicles were cultured in 5% CO_2 for 7 days at 31°C. RPMI1640 serum-free medium and RPMI1640 with 20% fetal bovine serum (FBS) were used to prepare a series of culture media either with or without the WS, HS or AS extract of leaves, fruits and roots of *Illicium anisatum* or shikimic acid. Minoxidil (SIGMA) was used as a positive control in the culture systems. Each experiment used 10–20 follicles.

Measurement of vibrissae hair follicle elongation

Photographs of the cultured mouse vibrissae hair follicles were taken using a stereomicroscope with increases in follicle length being measured over the 7 day culture period.

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis

Ten hair follicles were combined as one sample and then homogenized using a POLYTRON (KINEMATICA, Lucerne, Switzerland). mRNA was prepared from the hair follicles using an mRNA purification kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) and then reverse-transcribed using SUPERSCRIPT™ First Strand Synthesis System for RT-PCR (Gibco/BRL). A real-time quantification PCR method based on the procedure of Griscelli et al. (11) was then performed. The sequences of the PCR primers and probe used to quantify mouse

insulin growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), keratinocyte growth factor (KGF), and hepatocyte growth factor/scatter factor (HGF/SF) were designed using Primer Express™ software (Applied Biosystems, Tokyo, Japan). The primer sequences are listed in Table 1. PCR was performed under conditions that incorporated 2 min at 50°C, 10 min at 95°C, followed by 60 cycles of 95°C for 15 s and 60°C for 1 min. The fluorescence of the PCR products was detected using the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems).

Statistics

Data are expressed as mean values ± the standard deviation (SD). Changes in the variables were evaluated using the unpaired Student's *t*-test with significance being set at a *P*-value < 0.05.

Results

Activity of Illicium anisatum extract on mouse vibrissae follicle elongation in organ culture

Our series of experiments showed that the AS and HS extracts of leaves, fruits and roots at concentrations of 100 and 10 μg/ml for 7 days culture inhibited the growth of mouse vibrissae follicles and shafts (Fig. 1). However, follicles treated with WS extract of leaves, fruits and roots grew significantly longer than controls (Fig. 1). We then investigated the effect of the WS extract of leaves, fruits, roots and shikimic acid on mouse vibrissae follicles grown in organ culture using either serum-free medium or medium containing 20% FBS. In the serum-free medium, the WS extract of leaves, fruits or roots of *Illicium anisatum* stimulated the B6C3HF1 mouse vibrissae follicles leading to significant *in vitro* elongation (Fig. 2a). In medium containing 20% FBS, the WS extract of fruits at a concentration of 100 μg/ml was the only extract that caused significant elongation (Fig. 2c). The results of the mouse vibrissae follicles treated with shikimic acid are shown in Fig. 2b,d. Shikimic acid at concentrations of 0.1 and 0.01 mM promoted significant elongation in both serum-free medium and medium containing 20% FBS. This elongation activity associated with shikimic acid was observed over the concentration range of 0.01–1 mM, but inhibited in 10 mM. While the growth of follicles

Table 1. The PCR primer sequences used in the study

| Growth factor		Primer sequence 5′-3′
Insulin growth factor-1	Forward	CAGGCTATGGCTCCAGCATT
	Reverse	AGCTCCGGAAGCAACACTCA
Keratinocyte growth factor	Forward	CGAGTCCAGCTCCTCCATGA
	Reverse	GTTGAGGACAGACGCGTTGTT
Vascular endothelial growth factor	Forward	CATCTTCAAGCCGTCCTGTGT
	Reverse	CACTCCAGGGCTTCATCGTT
Hepatocyte growth factor/scatter factor	Forward	CCTGACACCCCTTGGGAGTA
	Reverse	TTTCCATAGGGACATCAGTCTCATT
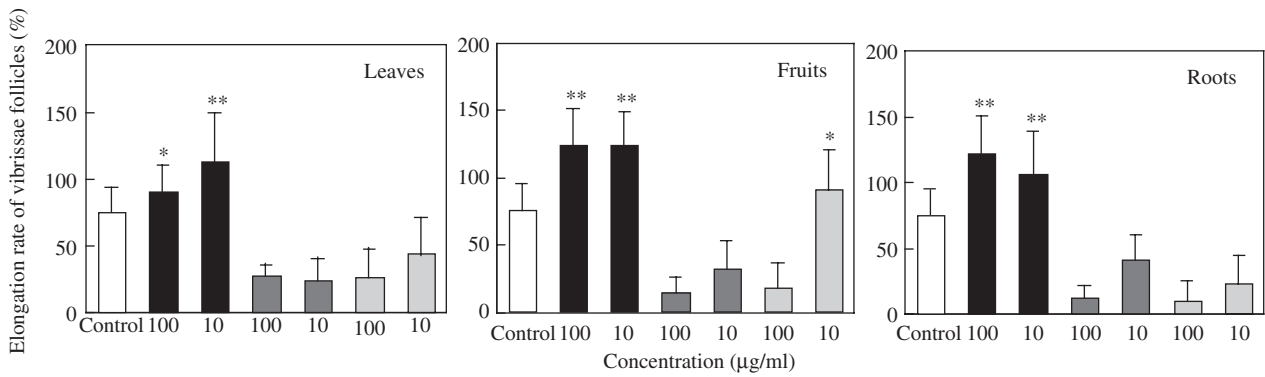
Figure 1. Elongation activity of mouse vibrissae follicles cultured with water-soluble WS (■), ethyl acetate-soluble AS (■), and n-hexane-soluble HS (□) extracts of leaves, fruits, and roots of *Illicium anisatum* for 7 days. (□) control, $n = 10$–20. $*P < 0.05$; $**P < 0.01$ vs. control.

was inhibited by 1 mM minoxidil in medium with and without 20% FBS (Fig. 2b,d), we observed that 0.1 mM of minoxidil in medium containing 20% FBS caused significant elongation (Fig. 2d).

Separation of the fraction of the WS extract of *Illicium anisatum* with elongation activity in mouse vibrissae follicles

The WS extract of the fruits of *Illicium anisatum* was analyzed by TLC and as shown in Fig. 3a, separated into four fractions named number 1, 2, 3, and 4. The elongation activity of each fraction was examined with the number 4 fraction being shown to inhibit the elongation of the follicles in a dose-dependent manner. In contrast, the number 1 and 2 fractions increased follicle elongation, while the number 3 fraction had no effect on follicle growth (Fig. 3b). By TLC analysis, Rf value of number 1 fraction was the same as shikimic acid (Fig. 3a). Moreover, using GC/MS analysis, the number 1 fraction was identified as a shikimic acid (data not shown). The elongation activity of the number 1 fraction was similar to that of shikimic acid. As the number 2 fraction contained many components, we used TLC to separate this fraction into four subfractions (Fig. 4a). As before, the elongation activity of these fractions was examined, with fractions number 2 (1) and 2 (2) having no effect on follicle growth, while fractions number 2 (3) and/or 2 (4) appeared to contain some components with elongation activity (Fig. 4b). Quantitative analysis using the naphtho-resorcinol/phosphate reagent showed that the number 2 (1) fraction contained D-glucose and the number 2 (2) fraction contained D-fructose (Fig. 4a), results that were confirmed by GC/MS analysis (data not shown). In addition, the number 2 (3) and 2 (4) fractions isolated by TLC were found to contain glycosides as they reacted positively with naphthoresorcin/phosphate reagent (Fig. 4a).
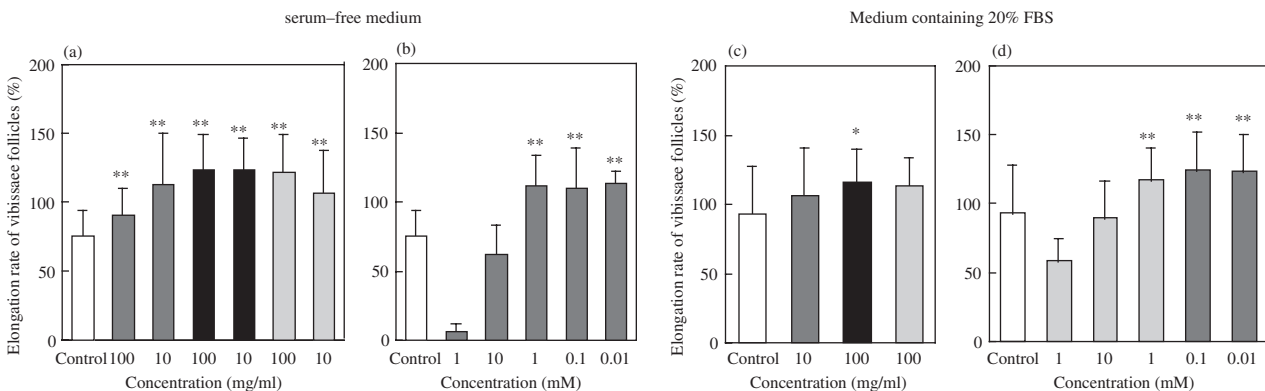

Figure 2. Effect of water-soluble (WS) extract of *Illicium anisatum* in serum-free medium (a) or medium containing 20% fetal bovine serum (FBS) (c) on the elongation activity of mouse vibrissae follicles. (□) control, WS extracts of leaves (■), fruits (■), and roots (□). Effect of minoxidil and shikimic acid in serum-free medium (b) or medium containing 20% FBS (d) on the elongation activity of mouse vibrissae follicles. control (□), minoxidil (□), shikimic acid (■), $n = 10$–20. $*P < 0.05$, $**P < 0.01$ vs. control.
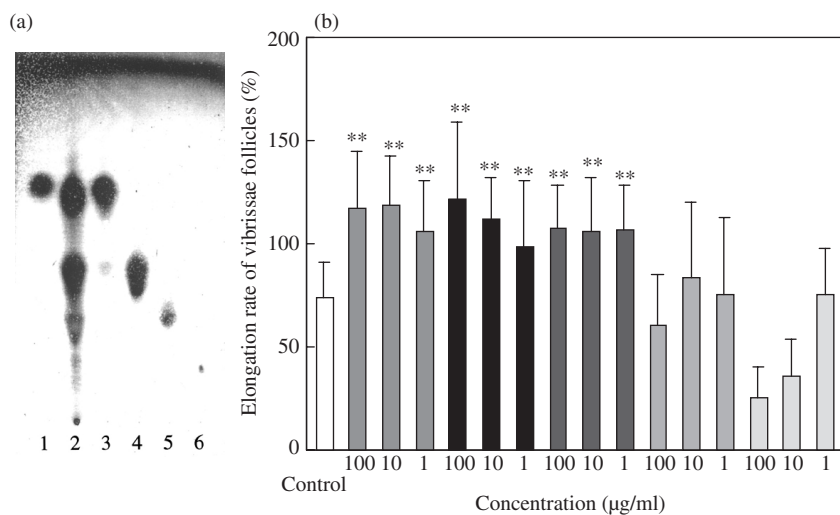
(a)

(b)

Figure 3. (a) Separation by thin-layer chromatography of water-soluble (WS) fruit extract of *Illicium anisatum*. The developing solvent was benzene-methanol-acetic acid (60 : 35 : 10, by volume). After spraying with 50% a sulfuric acid solution, the spots were visualized by charring at 200°C. Lane 1, shikimic acid; lane 2, WS fruit extract; lane 3, number 1 fraction; lane4, number 2 fraction; lane5, number 3 fraction; and lane 6, number 4 fraction. (b) Elongation activity of mouse vibrissae follicles cultured with either shikimic acid or the number 1-4 fractions of WS fruit extract. control, (□); shikimic acid, (■); number 1 fraction, (■); number 2 fraction, (■); number 3 fraction, (■); number 4 fraction, (□); $n = 10$–13. **$P < 0.01$ vs. control.

Growth factor expression of mouse vibrissae follicles by shikimic acid

In order to determine the mechanism of shikimic acid's elongation activity, we studied mRNA expression of IGF-1, VEGF, KGF, and HGF/SF in mouse vibrissae follicles after 1, 2, 3, 4, and 7 days. Comparison of mouse vibrissae follicles treated with 10 μg/ml shikimic acid and controls (Fig. 5) demonstrated an absence of HGF/SF mRNA expression (data not shown), significant increases in the expression of IGF-1 and VEGF mRNA at an early stage, and a more gradual increase in KGF mRNA expression from day 1–7.

Discussion

We have shown previously that the extract of *Illicium anisatum* increases blood flow and promotes hair growth in mice, *in vivo* (unpublished data). Although *Illicium anisatum* is well known as a toxic plant (12,13), we consider that if the toxic substance is removed from extracts of the plant the ability of these extracts to promote hair growth may be enhanced. In the present study, we demonstrated that HS, WS and AS extracts of leaves, fruits and roots of *Illicium anisatum* have a variety of effects on hair elongation activity. We found that the WS extract of *Illicium anisatum* promoted hair elongation, while the AS and HS extracts appeared to have little effect on elongation. The AS extract was found to contain the sesquiterpene compounds anisatin and neoanisatin, both of which have strong toxicity (12) that contributes to the cytotoxic properties of AS. Other studies have shown that the HS extract contains essential oils such as safrole and anethole (14,15), compounds that impart fragrance but do not have elongation activity. While there is evidence that the WS extract contains shikimic acid, the other components have yet to be identified. We therefore separated the extract into four subfractions and
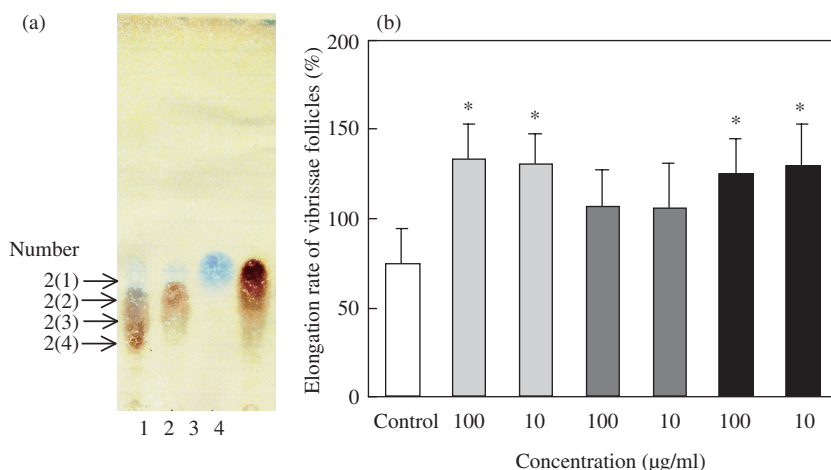
(a)

(b)

Figure 4. (a) Separation by thin-layer chromatography of the number 2 fraction of water-soluble (WS) fruit extract. The developing solvent was ethyl acetate-isopropanol-acetic acid (75 : 33 : 7, by volume). After spraying with naphtho-resorcinol-phosphate solution, the spots were visualized by charring at 105°C. Lane1, number 2 fraction; lane 2, the mixture of number 2 (1) fraction and number 2 (2) fraction; lane 3, D-glucose; lane 4, D-fructose. (b) Elongation activity of mouse vibrissae follicles cultured with number 2 fraction of WS fruit extract. control, (□); number 2 fraction, (■); the mixture of number 2 (1) fraction and number 2(2) fraction, (■); the mixture of number 2 (3) fraction and number 2 (4) fraction, (■); n = 15–20. *P < 0.05 vs. control.
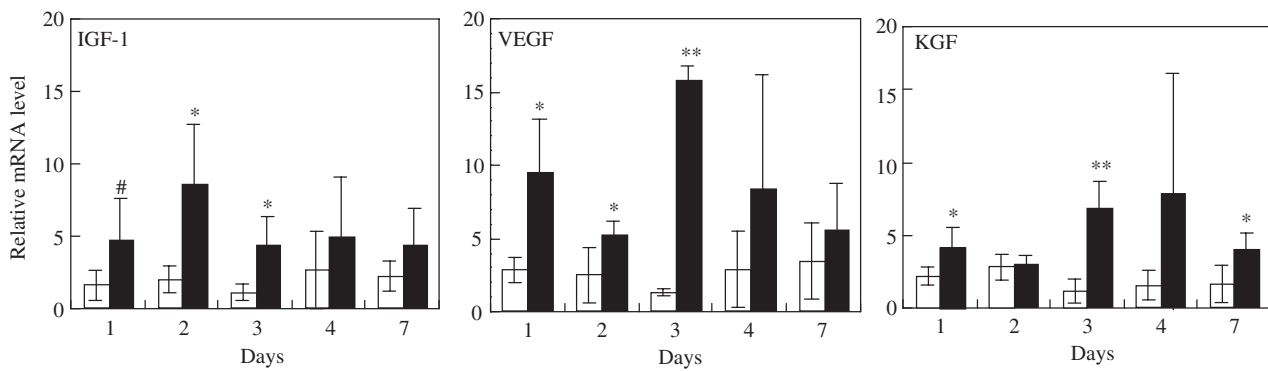
Figure 5. Time course of insulin growth factor-1, vascular endothelial growth factor, keratinocyte growth factor mRNA levels in mouse vibrissae follicles. The amount of reverse transcription-polymerase chain reaction products for each growth factor was corrected according to the quantity of 18s rRNA measured and expressed as a ratio of the day 0 control culture system. control, (□); shikimic acid, (■); $n = 6$. #$P < 0.05$, *$P < 0.01$, **$P < 0.001$ vs. control.

tested these for elongation activity (Fig. 3). This series of experiments showed that the number 1 and 2 fractions had hair elongation activity, whereas the number 3 and 4 fractions had no effect on hair growth. The activity of the number 1 fraction was similar to that of shikimic acid, and using GC/MS analysis, we were able to confirm the identification of the number 1 fraction as shikimic acid. These analyses also demonstrated that the number 2 fraction consisted of a mixture of many components with TLC and subsequent GC/MS showing that the number 2 (1) and 2 (2) subfractions contained D-glucose and D-fructose, respectively. The make-up of the number 2–3 and 2–4 subfractions was not investigated by GC/MS but it appeared these components may have contained polymers such as glycosides or polysaccharides, as they reacted positively with naphthoresorcinol/phosphate reagent following TLC. There is some evidence that glycosaminoglycans may affect hair growth (16), and therefore it is possible that the polysaccharide in the number 2 (3) and 2 (4) subfractions may also induce hair growth.

In our series of experiments, we used minoxidil as a positive control and found that while this compound suppressed hair growth in serum-free medium, it promoted hair growth in medium containing FBS. Similar results have been reported by Uchida et al. (17). While it has been suggested that the hair growth factor be contained in the serum (18), the possibility that the serum also may contain a variety of different factors that suppress hair growth cannot be excluded. FBS is known to contain various factors, and it has been shown that hair growth is suppressed by following the addition of serum (9). We observed that minoxidil inhibited the activity of a factor in the serum that was suppressing hair growth, raising the possibility that this action may lead to the promotion of hair

growth. Minoxidil is well known as a drug for the treatment of alopecia as it opens K^+ channels in dermal papillae cells (DPCs) (19) and up-regulates the expression of prostaglandin synthase-1 (20) and VEGF (21) in these cells. These actions of minoxidil cause relaxation of vascular smooth muscle and increased subcutaneous blood flow. Unlike minoxidil, the WS extract of *Illicium anisatum* and shikimic acid promoted hair follicle elongation in both serum-free medium and medium containing 20% FBS. This difference suggests that the WS extract of *Illicium anisatum* and shikimic acid increases subcutaneous blood flow by an alternative mechanism to that of minoxidil. Both compounds, however, promote hair growth.

In order to examine the mechanism by which shikimic acid promotes hair growth, we examined mRNA expression of IGF-1, VEGF, KGF, and HGF/SF in the hair follicles. There is evidence that IGF-1 expressed in DPCs accelerates the proliferation of epithelial cells in a paracrine manner, and in IGF-1 transgenic animals hair elongation is significantly increased in comparison with littermate controls (22). VEGF is an autocrine growth factor for hair DPCs and is a major mediator of hair follicle growth, with overexpression of VEGF in follicles resulting in accelerated hair regrowth and increased size of hair follicles (23). KGF also promotes hair growth by increasing the proliferation and differentiation of epithelial cells (24). The regulatory role of KGF in hair growth was well demonstrated in a transgenic mouse model, in which KGF was shown to directly affect the development of hair follicles (25). The remaining factor, HGF/SF acts as a modulator of epithelial cell motility (26) and also as an epithelial morphogen (27) with studies in serum-free organ culture systems showing that HGF/SF promotes hair growth

in a dose-dependent manner (28). In the present study, we showed that shikimic acid induced IGF-1, VEGF, and KGF mRNA expression in hair follicles but had no effect on HGF/SF mRNA expression in these cells. Taken together, these results suggest that shikimic acid may have positive effects on hair growth by regulating the levels of IGF-1, VEGF, and KGF in hair follicles.

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