Sanguisorba Officinalis Root Extract Has FGF-5 Inhibitory Activity and Reduces Hair Loss by Causing Prolongation of the Anagen Period

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FGF-5 inhibitors with FGF-5-antagonizing activity may be effective against hair loss, as these inhibitors suppress the transition of hair follicles from the anagen to catagen phase caused by FGF-5. We screened 24 substances for their FGF-5-antagonizing activity using both an FGF receptor-1c-transfected Ba/F3 cell line and the C3H mouse, and finally detected *Sanguisorba Officinalis* Root Extract (SO extract) as a reliable FGF-5 inhibitor. Topical application of SO extract elongated the anagen period in C3H mice. In addition, in a clinical study using 39 volunteers with hair loss, the SO extract significantly decreased the telogen /anagen hair ratio and the number of shed hairs. This extract showed a high degree of usefulness clinically. These findings suggest that FGF-5-antagonizing activity of SO extract observed *in vitro* and *in vivo* is closely related to its clinical effects.

INTRODUCTION

The mammalian hair growth cycle is modulated by various agents, including hepatocyte growth factor/scatter factor (HGF/SF), keratinocyte growth factor (KGF), insulin-like growth factor-I (IGF-I), and apoptosis-related factors [1-4]. Fibroblast growth factor-5 (FGF-5), among others, is believed to regulate the anagen-to-catagen transition [5]. It is therefore anticipated that FGF-5 antagonists suppress the transition to catagen, thereby preventing hair loss or maintaining hair growth. In this study we tested 24 natural plant and other extracts, and identified a potent FGF-5 inhibitor candidate. Its clinical effect on human hair loss was also investigated in this study and is reported here. All experimental and clinical studies included in this research were reviewed and approved by the institutional experimental animal ethics committee and the human subject protection committee, respectively.

MATERIALS, METHODS, AND RESULTS

1. Screening for FGF-5 Inhibitors

A) Materials

Twenty-four commercially available extracts (Table 1) were used for testing the FGF-5 inhibitory action.

B) Evaluation of cell culture FGF-5 inhibition

FGF-5 was prepared as described by Clements et al. [6]. The FGF-5 cDNA fragment was inserted into the Ndel site of the pET-3c vector, and the resulting plasmid was transformed into BL21 (DE3)-pLysS strain *E. coli*. The transformed E. coli cells were cultured to produce FGF-5 protein, then disintegrated by supersonic vibration. The FGF-5 fraction was purified by heparin-sepharose (Amersham). The FGF-5 activity of the obtained fraction was determined using BALB/3T3 cells according to the method of Komi et al. [7], and the sample was stored frozen at -80°C.

For the test system, FGF receptor-1c (FGFR-1c)-transfected Ba/F3 cell line (FR-Ba/F3 cells) was used—these cells were reported by Ito et al. [8] to express *c* variants of FGFR-1 and FGFR-1c (FGF-5 receptors) on the cell surface. The FR-Ba/F3 cells exhibited FGF-5- and IL-3-dependent proliferation, because the FGF-5 receptors were transfected into the cells originally expressing the IL-3 receptor. Therefore, in order to confirm whether the test material suppressed specifically FGF-5-dependent proliferation alone, we first evaluated the suppressive effect of the test material on cell proliferation in the presence of both FGF-5 and IL-3. FR-Ba/F3 cells were seeded at a concentration of 50,000 cells per well into 96-well micro-culture plates (Falcon; basal medium: RPMI-1640 medium containing 10% fetal bovine serum [FBS] and 5 μg/mL heparin). FGF-5 (1.0 μg/mL) was added to one group of plates, while IL-3 (0.2 ng/mL, Sigma) was added to the other group. Test materials diluted with the basal medium were added simultaneously (total volume of medium per well: 100 μL), and the plates were incubated at 5% CO_2 and 37°C for 3 days. Then, after 10 μL (1:2 dilution) of the Cell Counting Kit-8 reagent (Wako) was added, the plates were left standing at 37°C for 3 hours, and absorbance values were determined at 450 nm. To calculate the proliferation suppression rates of the test materials, the following equations were used (in which OD refers to the optical density of the plate reader):

Table 1　Test materials

Rosa Multiflora Fruit Extract-R[a)] (hereinafter referred to as 'Rosa multiflora extract')	Fucus vesiculosus extract[a)] (hereinafter referred to as 'brown algae extract')
Garden Burnet Root Extract-R[a)] (hereinafter referred to as 'Sanguisorba officinalis extract')	Eriobotrya Japonica Leaf Extract-J[a)] (hereinafter referred to as 'loquat leaf extract')
Ginko Biloba Leaf Extract BG[a)]	Foeniculum Vulgare (Fennel) Fruit Extract-J[a)]
Malva sylvestris extract[a)]	Ononis Extract BG[a)]
Artemisia princeps leaf extract[a)]	Prunus Armeniaca (Apricot) Kernel Extract LA[a)]
Pearl Extract LA-J[a)]	Cnidium Rhizome Extract A[a)]
Rosa normalis extract[a)]	Citrus Unshiu Peel Extract-J[e)]
Butcher broom extract [a)]	Rose Extract[b)]
Iris Florentina Root Extract ET-50[b)]	Echinacea Leaf Extract ET-50[b)]
Nuphar Japonicum Root Extract ET-50[b)]	Pharcolex Syzygium Aromaticum[c)]
Pharcolex Hawthorn E[c)]	Hop extract[e)]
Murraya koenigii spreng (curry leaf) extract[d)]	
Arctostaphylos uva-ursi leaf extract[f)]	

a) Maruzen Pharmaceuticals, b) Koei Kogyo, c) Ichimaru Pharcos, d) Yamakawa & Co.,
e) Nagaoka Perfumery, f) Koshiro Co.

Table 2　Extracts showing FGF-5–specific inhibitory activity and the results of inhibition evaluation

Extract	IC50 (%)		FGF-5 specificity
	FGF-5	IL-3	
Rosa multiflora extract	0.28	> 3	> 11
Brown algae extract	0.77	3	3.9
Sanguisorba officinalis extract	0.2	0.49	2.5
Loquat leaf extract	1.4	> 3	> 2.1

An IL-3 IC50 value larger than 3% is designated as '> 3.' In this case the FGF-5 specificity value is shown as '> (calculated value)' based on the calculation of 3/(FGF-5 IC50).

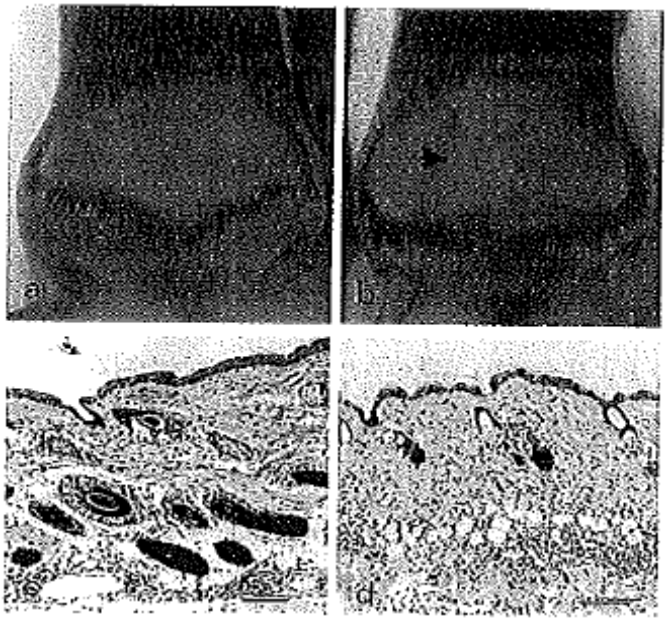

Fig. 1. Representative photographic views of the subcutaneous injection site of mice and HE-stained sections from the same region (scale bar = 100 μm)
a: Control mouse subcutaneously injected with phosphate-buffered saline (PBS) only.
b: Test mouse subcutaneously injected with PBS containing FGF-5.
c: Hematoxylin-eosin (HE)-stained section of a moderately dark area surrounding the injection site of test mouse *b*. d: HE-stained section of the pale-white skin area of test mouse *b* (indicated by the arrow).

Suppression rate of FGF-5-dependent proliferation = [(OD with FGF-5 and test material) – (OD with test material and without FGF-5)/[(OD with FGF-5 alone) – (OD without FGF-5 or test material)] × 100;

Suppression rate of IL-3-dependent proliferation = [(OD with IL-3 and test material) – (OD with test material and without IL-3)/[(OD with IL-3 alone) – (OD without IL-3 or test material)]× 100.

We postulated that test materials having a high specificity for FGF-5 would inhibit the FGF-5-dependent proliferation strongly (i.e., effects observable at low concentrations), while exhibiting a weak suppressive effect against the IL-3-dependent proliferation (i.e., effects not observable at high concentrations) [5]. We therefore prepared a series of test samples containing 0% to 3% of each of the test specimens, and used them to determine the 50% inhibitory concentration (IC50) against FGF-5- and IL-3-dependent proliferation. When evaluated by the (IL-3 IC50)/(FGF-5 IC50) ratio as the specificity index for the suppression of FGF-5-dependent proliferation, the following products were found to present high values (Table 2): Rosa multiflora fruit extract (Rosa Multiflora Fruit Extract-R), brown algae extract (Fucus vesiculosus extract), Sanguisorba officinalis extract (Garden Burnet Root Extract-R),

and loquat leaf extract (Eriobotrya Japonica Leaf Extract-J). Consequently, these extract preparations were selected as potential inhibitors of FGF-5-dependent proliferation, and subjected to subsequent studies using mice.

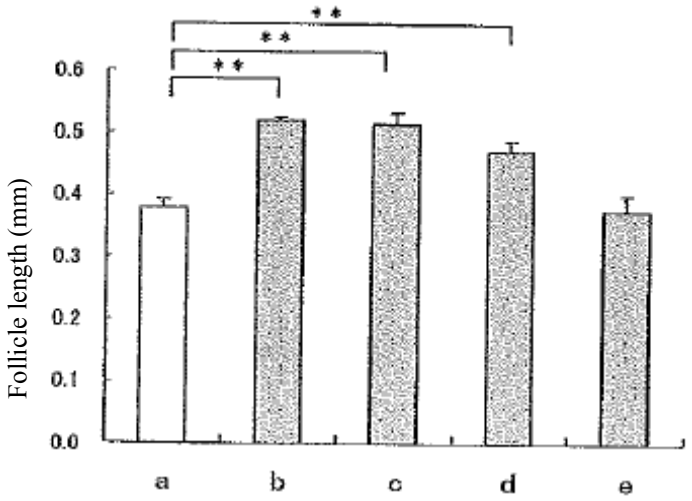

Fig. 2. Comparison of follicle length of mice subcutaneously injected with FGF-5 and selected plant extracts
a: FGF-5 only b: FGF-5 + Rosa multiflora c: FGF-5 + Sanguisorba officinalis
d: FGF-5 + brown algae e: FGF-5 + loquat leaf **: $P < 0.01$

C) Evaluation using mouse *in-vivo* studies

The four extract preparations mentioned above were further examined in *in-vivo* studies. C3H/HeN mice (8-week-old females weighing 22.1 ±1.0 g, purchased from Japan SLC) were dehaired dorsally with a depilatory cream. From the following day onward, the mice were repeatedly injected subcutaneously at the same site once daily for six days with 50-μL solutions of FGF-5 (78 μg/mL PBS) (either FGF-5 alone or FGF-5 plus dry extract). Based on the IC50 values for FGF-5- and IL-3-dependent proliferation obtained in the preceding experiments, the following amounts of dry extracts were dissolved in 50 μL of the FGF-5 solution: Rosa multiflora: 0.97 mg; Sanguisorba officinalis root: 0.18 mg; Eriobotrya japonica leaf: 1.95 mg; and brown algae: 0.72 mg. These concentrations were expected to inhibit FGF-5 activity with negligible cytotoxicities. On day 7 of the experiment, skin strips including the injection site were dissected, fixed with 10% formalin, and embedded in paraffin. The tissue was sectioned into 5-μm-thick slices at 100-μm intervals, and HE-stained for light microscopy. IPLab imaging software (Scanalytics) was used to measure the length of follicles on optical micrographs. The mean follicle length was calculated for each mouse based on 10 follicle measurements, and the group mean was determined (four per group). For statistical analysis, the unpaired t-test was used.

Consequently, mice subcutaneously injected with FGF-5 alone demonstrated pale-colored skin around the injection site, which was apparently different from the neighboring slightly dark-colored skin (Fig. 1). On the other hand, mice administered with a mixture of FGF-5 and one of the extracts developed a variety of skin colors (ranging from pinkish to black) at the injection site, and the skin luminosities were not comparable in a quantitative and straightforward manner. However, the follicle was significantly longer for the groups of mice administered FGF-5 in combination with Rosa multiflora, Sanguisorba officinalis, or brown algae, when compared with FGF-5 alone (Fig. 2). Of these, Sanguisorba officinalis root extract, which showed no major issues involving coloration, precipitation, and other formulation-related problems, was chosen for further examination as shown below.

2. Effect of Sanguisorba Officinalis Root (SO) Extract on Preventing Hair Loss

A) Effect of SO Extract on the Proliferation of Rat Outer Root Sheath Cells of Whisker Follicles and Whisker Dermal Papilla Cells

Rat outer root sheath cells of whisker follicles or rat whisker dermal papilla cells were seeded at 600 cells/well into 96-well culture plates (RPMI-1640 medium containing 10% FBS). The following day, the medium was replaced with a new medium containing 10^{-1}% to 10^{-5}% of the SO extract, and the cells were incubated for three more days. On the final day of incubation, the medium was changed with 100 μL of freshly prepared medium, and 10 μL of the Cell Counting Kit-8 reagent was added to each well. After three-hour culture, the absorbance of the wells was measured at 450 nm. For statistical analysis, the unpaired t-test was used.

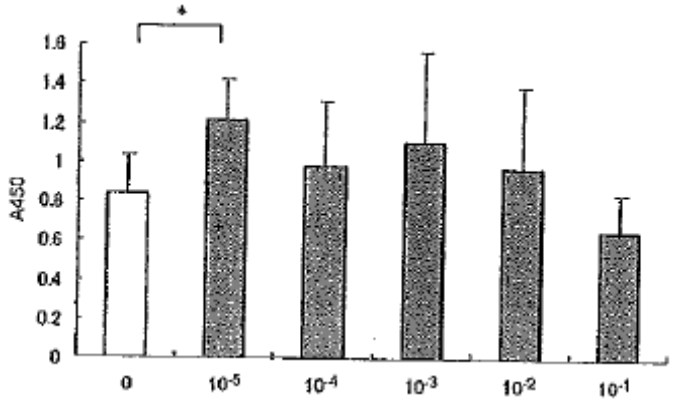

Fig. 3 The effect of Sanguisorba officinalis extract on the proliferation of rat outer root sheath cells of whisker follicles. *$P < 0.05$

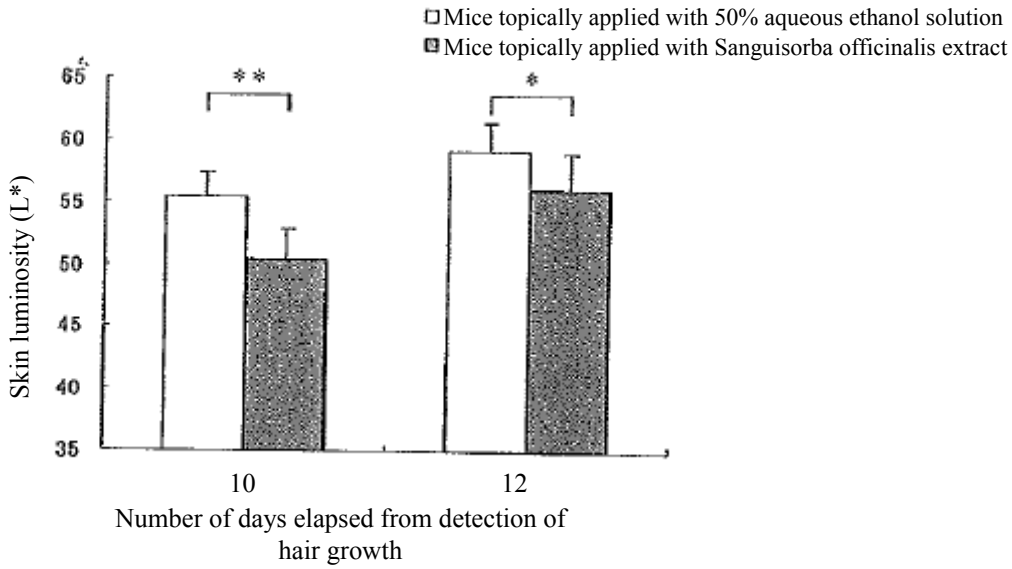

Fig. 4 Evaluation of the anagen-prolonging effect of Sanguisorba officinalis extract—skin luminosity measured 10 and 12 days after detection of hair growth.
*$P < 0.05$, **$P < 0.01$.

The results showed that the SO extract significantly enhanced the multiplication of rat outer root sheath cells (Fig. 3), but not rat whisker dermal papilla cells when the SO extract concentration was 10^{-5}%.

B) Anagen-prolonging effects

C3H/HeN mice (8-week-old males weighing 25.7 ±1.3 g, purchased from Japan SLC) were dehaired dorsally with a hair-cutting device and shaver. The test group (seven mice) was repeatedly given topical skin application of 40 μL of the SO extract once daily five days in a row per week starting from the next day, while the control group received 40 μL of a 50% ethanol-water mixture (extract base) in the same manner.

Skin luminosity (L*) was measured using the Croma Meter CR-200 (Minolta) 10 and 12 days after the date of hair growth detection (anagen V) when new hair shafts were seen protruding from the skin. Another 10 days later, dorsal skin samples were excised, and HE-stained for light microscope examination. The length of follicles was determined using the imaging software mentioned above. The mean follicle length for individual mice was obtained by averaging 20 measurements, and the group mean was derived based on the results of seven rats. For statistical analysis, the unpaired t-test was used.

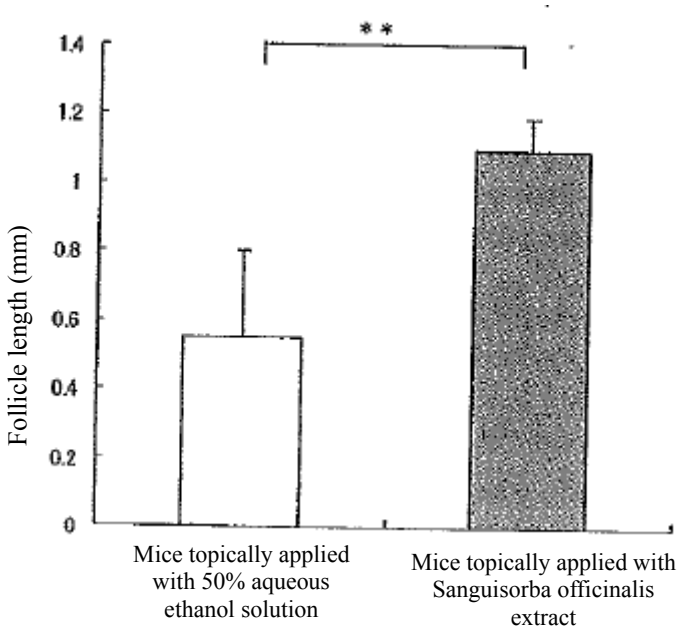

Fig. 5 Evaluation of the anagen-prolonging effect of Sanguisorba officinalis extract—follicle length measured 10 days after detection of hair growth. $^{**}P < 0.01$.

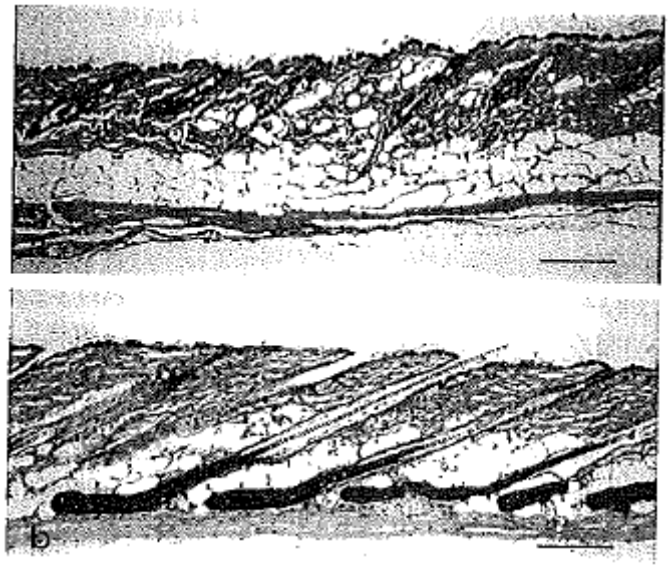

Fig. 6 Evaluation of the anagen-prolonging effect of Sanguisorba officinalis extract—Hematoxylin-eosin stain of skin sections 10 days after detection of hair growth (scale bar: 200 μm). a: mouse topically treated with 50% aqueous ethanol solution, b: mouse topically treated with Sanguisorba officinalis extract.

The results showed that there was no difference in the onset of anagen V between the SO-extract and control groups (21.1 ± 4.1 and 21.5 ± 3.1 days, respectively), indicating no hair growth-promoting effect of the SO extract. On the other hand, skin luminosity showed a significant difference between the groups: 10 days after hair growth detection, nearly all control mice possessed a pale white skin tone that was once dark, whereas most of the SO-

extract–treated mice showed a light-dark tone (Fig. 4). The mean follicle length 10 days after hair growth detection was 1.10 mm for the SO-extract-treated mice, which was significantly longer than 0.53 mm for the control group; telogen-phase follicles were frequently observed in the control mice, whereas the treated mice exhibited a high proportion of anagen-phase follicles (Figs. 5 and 6). No animals in either group presented inflammation or other abnormal histological changes.

3. Human Experience

A) Subjects and test materials

Thirty-nine volunteers with hair loss (males: 30, females: 9, mean age: 46 years old) were recruited for the study. Male volunteers were randomized into two groups after stratification based on the degree of hair loss according to the classification system proposed by Ogata [9]. The female volunteers were randomized into two groups without stratification. The control group was treated with control solution (40 wt% aqueous ethanol solution containing 0.2 wt% l-menthol and 2 wt% 1,3-butylene glycol), while the treatment group was treated with 10 wt% SO-extract solution (prepared by replacing 5 wt% each of pure water and ethanol of the control solution with the same amount of SO extract). These solutions were applied to the scalp using a gentle massaging action in an appropriate amount (2–3 mL/application) twice daily for four months. This study was performed in a double-blind manner. The hair styling products (excluding hair growth stimulants) that the volunteers were using before the start of study were continued during the study. The volunteers were provided with complete study information, and gave their written informed consent to participate in this study.

B) Efficacy evaluation

The hair growth efficacy was evaluated based on the four items shown below.

(1) External examinations by the investigator. The investigator interviewed the subjects and visually examined the condition of the scalp and hair at baseline and the end of study (at zero and four months). Photographic pictures were taken for reference at these two visits.

(2) Subject self-report. Subjects were interviewed by questionnaire for the assessment of the effect at zero (baseline), two, and four months.

(3) Numbers and types of shed hairs. These parameters were measured at baseline, two, and four months. Subjects were instructed to wash their hair as per usual on the night before the measurement day. On the following day, the designated hair specialist washed their hair over a plastic cage covered with a disposable nurse cap (Vilene Medical) to collect

all shed hair shafts; the total numbers of shed hairs as well as the numbers of detached soft hairs of less than 2 cm in length were counted. Attention was paid to the extent possible to maintain the same elapse of time from the hair washing on the previous night to that on the measurement day for each visit.

(4) Phototrichographic measurement of hair growth rate. Hair growth rates were determined at baseline, two, and four months. A circular area of at least 1.3 cm diameter in the parietal or frontal region was specified for each subject, and the hair in the area was cut off to the scalp by a pair of scissors. Scalp images were taken using the Microscope VH-6300 (Keyence, Japan) immediately after the hair was cut and two days later. The same scalp area was subjected to hair cutting at each visit. The corresponding hair shafts on the trichograms taken two days apart were identified, and the rates of hair growth were determined. In addition, the telogen/anagen hair ratio was determined by defining the maximum upper hair growth rate as 0.032 mm/day for telogen-phase hair.

C) Efficacy evaluation

The 4-month hair growth efficacy was evaluated by the investigator with respect to the presence/absence of new hair growth, prevention of hair loss, and global improvement. The presence/absence of new hair growth was assessed on the basis of photographic and visual observation. The preventive effects on hair loss were evaluated based on the questionnaire and clinical interviews of the subjects and shed hairs measurements. Global improvement was evaluated by an overall assessment of the presence/absence of new hair growth, prevention of hair loss, changes in the proportion of lost soft hair shafts, and hair growth rate results. The effectiveness of the test material was judged by taking note of the global improvement and the occurrence of adverse reactions. The subjects that developed adverse reactions were graded as 'deteriorated.'

D) Statistical analysis

The efficacy parameters were analyzed using the Wilcoxon rank-sum test. The measurement parameters on shed hairs and hair growth rate were evaluated by the paired t-test.

E) Results

In this study of 39 subjects, the shed hairs and phototrichogram evaluations demonstrated a significant improvement for the SO-extract-treated group. The numbers of shed hairs were significantly reduced to 86.7% and 68.0% at two and four months, respectively, relative to baseline (Fig. 7). The numbers of soft hairs significantly decreased to 62.6% at four months versus baseline (Fig. 8), and the proportion of soft hair showed a mild trend to decrease. In addition, the hair growth rate was significantly elevated to 111.9% and 117.7% relative to

baseline at two and four months, respectively (Fig. 9). Meanwhile, the telogen/anagen hair ratio decreased to 79.2% and 67.7% relative to baseline at two and four months, respectively (Fig. 10).

According to the global assessment of effectiveness made by the investigator based on the quantitative measurement results, questionnaire and medical interview, and gross observations, treatment with the SO extract was categorized as 'somewhat effective,' 'effective,' or 'very effective' in 73.9% of the treated subjects, though only 25.0% of the control subjects were grouped under any of these categories. Statistical analysis of these data revealed a significant difference ($P < 0.01$) between the treatment and control groups (Fig. 11). No subjects presented with adverse reactions during the study.

When analyzed by sex (details not shown), the results indicated that among the female subjects (N = 9), treatment with the SO extract was 'somewhat effective' in 80% (4/5), while the proportion of this group was 20% for the control group (1/4).

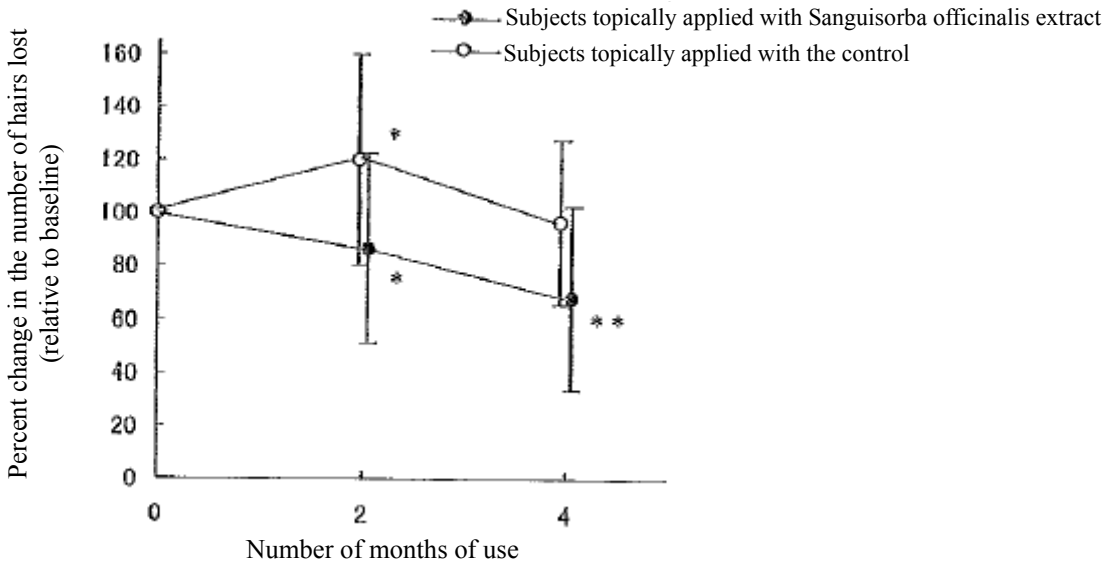

Fig. 7 Human-use test results showing time-course changes in hair loss during hair wash. Changes are shown relative to baseline due to a large inter-subject variability. *$P < 0.05$, **$P < 0.01$ (versus 0 month).

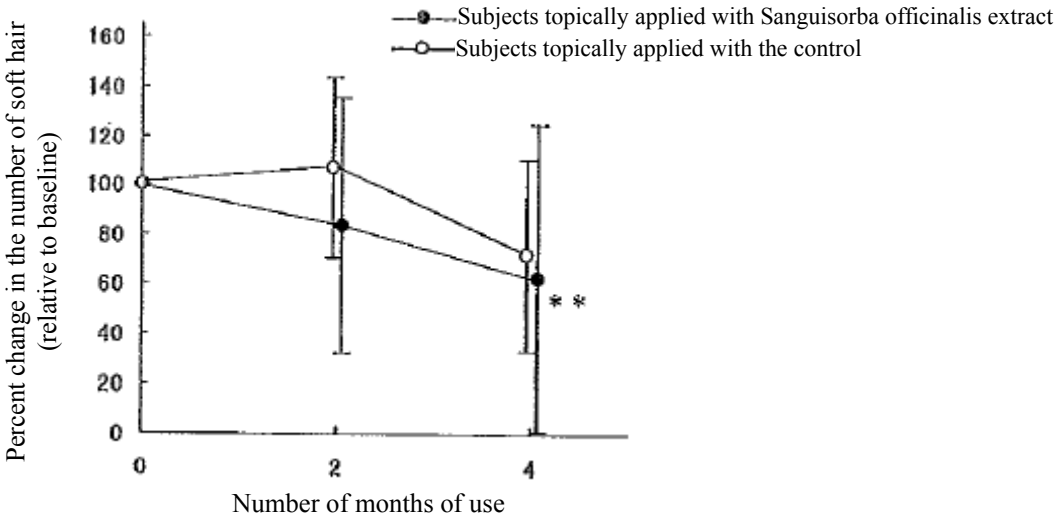

Fig. 8 Human-use test results showing time-course changes in the proportion of soft hair lost during hair wash. $^{**}P < 0.01$ (versus 0 month).

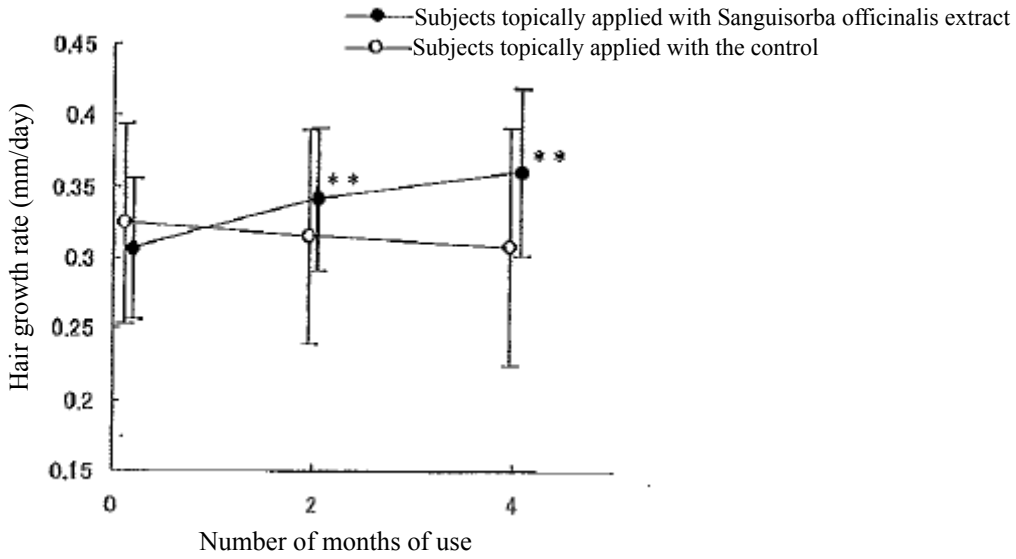

Fig. 9 Human-use test results showing time-course changes in hair growth rate.
$^{**}P < 0.01$ (versus 0 month).

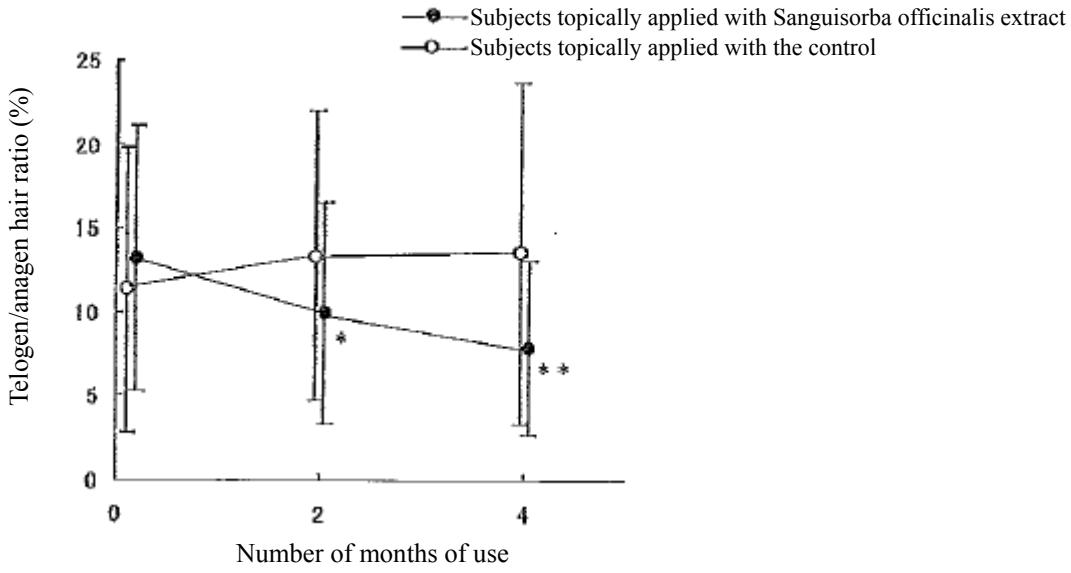

Fig. 10 Human–use test results showing time-course changes in telogen/anagen hair ratio. *$P < 0.05$, **$P < 0.01$ (versus 0 month).

DISCUSSION

Hair grows in cycles consisting of the anagen, catagen, and telogen phases. The hair growth cycle has been shown to be regulated by a variety of agents, including HGF/SF, KGF, IGF-I, apoptosis-related factors, and FGF-5 [1-4]. FGF-5, in particular, is strongly believed to be involved in the anagen-to-catagen transition, because of the fact that *fgf-5*-gene–deleted mice had dramatically longer hair than untreated wild-type mice due to prolongation of the anagen phase [5] as well as other findings. The *fgf-5* gene products include, besides FGF-5, the short form of FGF-5 (FGF-5S protein) as a result of an alternative splicing [10]. In a study that administered FGF-5 and FGF-5S in mice during the latter half of the anagen phase, Suzuki et al. [11] demonstrated that FGF-5 enhanced the transition of hair follicles from the anagen to the catagen phase, whereas FGF-5S inhibited the FGF-5–induced catagen transition. Assuming that FGF-5 binds to the FGF-5 receptor (FGFR-1) of the dermal papilla cell to induce the catagen transition while the action of FGF-5 in the anagen phase is suppressed by FGF-5S, the authors conjectured that the imbalance of the two protein expressions constituted one of the causes of alopecia. On the other hand, Ito et al. [8] synthesized various partial peptide sequences of FGF-5 and FGF-5S, and applied them to cells expressing FGFR-1 and FGFR-1c (major FGF-5 receptors). Consequently, they identified a decapeptide that antagonized FGF-5 activity (the molecule was completely identical to the amino acid sequence between positions 95 and 104 of the FGF-5 and FGF-5S proteins). The authors also found that the decapeptide inhibited FGF-5–induced hair growth suppression *in vivo*, and suggested that FGF-5 inhibitors could prevent hair loss or enhance hair growth.

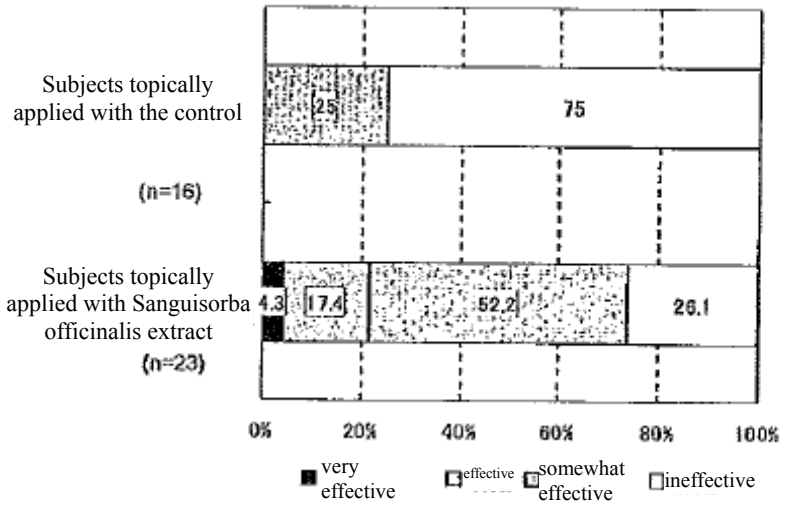

Fig. 11 Human-use test results assessing the effectiveness of Sanguisorba officinalis extract after 4 months of use.

In this study, we screened *in vitro* the FGF-5 inhibitory action of 24 plant and other extract products, and found that Sanguisorba officinalis (SO extract) possesses a potent FGF-5 suppressive action. This finding led to the expectation that the SO extract could prolong the anagen phase (or delay the transition to catagen) by inhibiting FGF-5 activity. Therefore, we investigated the effects of FGF-5 on follicle growth and the effects of SO extract on the FGF-5 action by subcutaneously injecting dehaired mice with FGF-5 alone or in combination with the SO extract. The results showed that subcutaneous injection of FGF-5 alone strongly inhibited the follicle growth, whereas FGF-5 administered in combination with the SO extract significantly increased hair follicle length. In addition, we continually applied the SO extract topically to dehaired second-telogen mice (8 weeks old), and visually examined each animal to determine the date of follicle maturation. We then examined and excised the skin to evaluate the skin conditions 10 and 12 days after this date: we found that the skin of the control mice, which was once dark colored, turned pale white in 10 days, whereas the skin of the SO-extract-treated mice remained dark. We also found that the control group showed a large proportion of telogen-phase follicles, while the treatment group presented a majority of anagen-phase follicles. Moreover, the follicle length was significantly larger for the treatment group than the control. These results suggested that the SO extract prolonged the follicle growth period and/or delayed the transition to the catagen phase.

We conducted a global assessment on the effectiveness of SO extract in preventing human hair loss, based on conventional visual and photographic evaluations, a subject self-report questionnaire, the numbers of shed hairs, and phototrichogram measurements. The results showed significant reductions in the number of shed hairs and the telogen/anagen hair ratio for the SO-extract-treated subjects relative to the control. The global assessment on the effectiveness indicated that the SO extract was effective or very effective in 21.7% of the

treated subjects, and somewhat effective, effective, or very effective in 73.9%, which was significantly higher than the corresponding value of 25.0% for the control. The effects of the SO extract in reducing the number of shed hairs and the telogen/anagen hair ratio demonstrated in the human-use study was probably related to the FGF-5 inhibitory activity observed in the *in-vitro* and animal studies.

Furthermore, we found that the SO extract promoted the proliferation of rat outer root sheath cells of whisker follicles. However, the SO extract did not shorten the time of hair growth in mice. In a similar vein, Inaoka et al. [12] reported that SO extract did not influence the hair growth rate. At present no scientific consensus exists about the effects of SO extract on hair growth, which warrants further investigation.

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