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Nanotechnology Application in Ethanol Extract of Fenugreek Seeds (*Trigonella foenum-graecum* L.) in Development of Hair Tonic Formulation

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ABSTRACT

The objective of this research was to formulate ethanol extracts of fenugreek seeds with nanotechnology to a look at the effects of nanotechnology on hair growth activity and safety of hair tonic. Fenugreek seeds will be extracted using the soxhletation method. Fenugreek seeds extract will be formulated with nanotechnology, where this study wants to look at the effects of nanotechnology on hair tonic preparations on hair growth activity and safety of hair tonic. Hair growth activity is determined by the measurement of hair length and hair weight, while the safety test is determined by irritation test. The activity test results of formula A (nanoemulsion of fenugreek seed extract 2,5%) showed a significant difference (*P <0.05) compared to formula C (nanoparticles of fenugreek seed extract 2,5%), formula F (fenugreek seed extract 5%), as well as the positive control (minoxidil 2%). The irritation test results for all formulas didn't show any irritating effects (erythema and edema). The results obtained in this research work clearly indicated the modification with nanotechnology provides increased effectiveness in hair growth, where, using a lower concentration of fenugreek seed extract can provide better hair growth results and doesn't irritate the skin.

INTRODUCTION:

Hair grows on almost the entire surface of the skin except for the palms and soles of the feet that have a role in the function of skin protection against adverse environmental conditions and support the appearance of a person. In physiological conditions, hair has a period of growth, rest and release so that at some point a number of hairs (around 100 or more a day) will fall out. If the release of hair from the scalp exceeds the physiological limit, it indicates hair loss. Hair loss is the reduction of hair volume which causes hair thinning and even baldness. Hair treatment using merely shampoo and conditioner is not enough as hair roots are living cells that need to be nourished in order to stay healthy; therefore, the administration of hair tonic is also required[1].

Hair tonic is a cosmetic hair preparation that is used to intensify or stimulate hair growth in baldness or hair loss[2]. Herbal and synthetic hair tonics have been developed to overcome hair loss and baldness. Along with technological developments, Indonesian people tend to use herbal products since the side effects are less as compared to those of synthetic products, such as minoxidil, which often cause hypersensitivity of the scalp[3]. Kalb et is one of the Indonesian plants, also known as fenugreek, which has phytoestrogen compounds. Phytoestrogens in fenugreek seeds are believed to reduce hair loss and increase hair growth rate. Fenugreek seed extracts 10% in hair tonic preparations shows the effect of significantly increasing hair growth rates compared to placebo and minoxidil 2%[4]. The formulation containing 7.5% of each herbal oil (*Emblica Officinalis*, *Bacopa monnieri*, and *Trigonella foenum-graecum*) and 5% of *Murraya koenigii* oil showed excellent hair growth activity with standard (2% minoxidil ethanolic solution) by an enlargement of follicular size and prolongation of the anagen phase[3].

In the last decade of the early 21st century, in the field of developing cosmetology the use of nanotechnology for the manufacture of cosmetics[5]. Nanotechnology-based innovations are aimed at improving the stability of cosmetic ingredients, enhancing the aesthetic appearance of products and targeting active ingredients to the focal structures with controlled release and sustained effects[6]. In its application, nanotechnology has contributed to a health and beauty product on the skin and face. The cosmetics industry uses nano-sized materials because they have different characteristics in color, transparency, solubility, deeper skin penetration, long-lasting effects, improved color and quality of finished products, and increase the stability of

active ingredients which may be broken down by oxidation[7]. Nanotechnology can also help the absorption of substances needed by the skin to accelerate the efficacy of the skin[8].

The purpose of this study was to prove whether fenugreek seeds have an effect on hair growth, to formulate the ethanol extract of fenugreek seeds with nanotechnology, to see the effects of nanotechnology on the activity of hair growth, and to determine the safety of hair tonic.

MATERIALS AND METHODS:

Materials:

Fenugreek seeds (*Trigonella foenum-graecum* L.) was obtained from PT. Phytochemindo Reksa, Bogor, West Java. Regrou Minoxidil 2% Hair Restorer is produced by PT. Surya Dermato Medical Laboratories, Surabaya, Indonesia as a positive control. Ethanol 98%, methanol, ether, ethyl acetate, ethanol 95%, magnesium (Mg), ethanol 70%, distilled water, HCl 2 N, KOH 5%, Liebermann-Bouchard reagent, Mayer reagent, Dragendorff reagent, concentrated HCl, $CHCl_3$, H_2SO_4, anhydrate acetic acid, $FeCl_3$ solution, Lead (II) acetate 0.4 M, isopropanol, anhydrate sodium sulphate, Molish LP, chitosan 1%, propylene glycol, dimethyl sulfoxide, glycerin, Mackaderm® MCT (INCI name : Medium Chain Triglyceride), Carbowax PEG-400 (INCI name : Polyethyleneglycol 400), Peceol™ (INCI name : Glycerol monooleate) , Rheodol TW-O120V (INCI name : Polysorbate-80), Titriplex® III (INCI name : Disodium edetate), sodium metabisulfite, and Microcare PE (INCI name : Phenoxyethanol).

Methods:

Extraction of Fenugreek seeds[4]

This study uses hot soxhlet extract, which is an extraction process with a relatively constant solvent along with a condenser. First, fenugreek seeds were ground using a mixer and using a weighing scale, 100 grams were taken and put into a parchment paper and then extracted with 350 ml 98% ethanol using a Soxhlet. The extraction process was done until the solution color in the circulator became colorless. The sample extract was then concentrated using rotary evaporator and weighed.

Characterization of Fenugreek seeds and Ethanol extract of Fenugreek seeds

Flavonoid

0.5 g of fenugreek seed extract and then added 10 ml of methanol, refluxed for 10 minutes, filtered hot through filter paper. Filtration was diluted with 10 ml of distilled water, cooled, and then added 5 ml of ether, shaken carefully, and then left for a while. The methanol layer was taken and evaporated, the rest was dissolved in 5 ml of ethyl acetate, filtered (solution A). 1 ml of solution A was evaporated to dryness, then dissolved in 2 ml of ethanol 95%, then added 0.1 g of magnesium powder and 10 drops of concentrated HCl. If there is a red-orange color to red-purple color indicates flavonoid.

Terpenoid and Steroid

1 g of fenugreek seed extract was extracted with 20 ml of ether for 2 hours and filtered. 3 drops of filtrate are added with 2 drops of Liebermann-Bouchard reagent. If a purple or red color turns blue or blue or green-blue indicates terpenoids/steroids.

Tanin

1 g of fenugreek seed extract was extracted with 20 ml of ethanol 70 %. 1 ml solution then added 2 drops of $FeCl_3$ 1% solution. A positive reaction is indicated by the formation of the black blue-green color showed the presence of tannins.

Saponin

0.5 g of fenugreek seed extract was diluted with 10 ml of hot water into a reaction tube, cooled, then shake it strong for 10 seconds. If the foam is formed around 1-10 cm, it is stable not less than 10 minutes and doesn't disappear with the addition of 1 drop of HCl 2 N indicating the presence of saponins.

Alkaloid

0.5 g of fenugreek seed extract was dissolved with 5 ml HCl 2 N. The solution obtained was then divided into 3 test tubes. The first tube is used as blank, the second tube is added with 3 drops of Dragendorff reagent, and the third tube is added with 3 drops of Mayer reagent. Formation of orange deposits in the second tube and white to yellowish deposits on the third tube indicate the presence of alkaloids.

Phenolic

1 g of fenugreek seed extract is heated with an amount of water over a water bath, then filtered. The filtrate is added with 2-3 drops of KOH 5% solution. The presence of phenolic compounds is indicated by the formation of yellow to red in solution.

Glycoside

Three grams of fenugreek seed extract was mixed with 30 ml solution of 7 parts ethanol (95%) and 3 parts water inside a water cooler for 10 minutes and then cooled down and filtered. Afterward, 25 ml distilled water and 25 ml Lead (II) acetate 0.4 M was added to 20 ml filtrate, shaken, and left for 5 minutes before filtered. The filtrate was filtered 3 times, each time with 20 ml mixture of chloroform P and isopropanol P of 3:2 ratio. Then, anhydrate sodium sulfate was added to the filtrate, filtered, and evaporated in a temperature of not more than 50^{o}C and the remaining was diluted with 2 ml methanol. This solution was then called solution A. To examine the presence of glycoside, about 0,1 ml solution A was evaporated and the remaining was diluted in 5 ml anhydrate acetic acid and 10 drops of sulphuric acid were added. If the solution turned blue or green, it showed a positive result. To examine the presence of carbohydrate, about 0.1 ml solution A was evaporated and 2 ml of distilled water, 5 drops of Molish LP, and 2 ml of sulphuric acid were added into the remaining. If a purple ring was formed, the test (molish reaction) was said to be positive.

Formulation of hair tonic

Six hair tonic formulations consisted of hair tonic containing nanoparticle fenugreek seed extract (formula A), placebo of formula A (formula B), hair tonic containing nanoemulsion fenugreek seed extract (formula C), placebo of formula C (formula D), hair tonic containing 2,5% fenugreek seed extract (formula E), and hair tonic containing 5% fenugreek seed extract (formula E). The composition of the hair tonic is listed in Table No. 1.

Table No. 1: Hair tonic formulation

Ingredient	Concentration (% b/b)					
	Formula A	Formula B	Formula C	Formula D	Formula E	Formula F
Fenugreek seed extract	2.5	-	2.5	-	2.5	5
Dimethyl sulfoxide	1	1	1	1	1	1
Propylene glycol	30	30	30	30	30	30
Glycerin	20	20	-	-	-	-
Rheodol TW-O120V	2	2	8	8	2	2
Chitosan 1%	10	10	-	-	-	-
Carbowax PEG-400	-	-	16	16	-	-
Peceol	-	-	5	5	-	-
Mackaderm MCT	-	-	5	5	-	-
Sodium Metabisulfite	0.1	0.1	0.1	0.1	0.1	0.1
Titriplex III	0.1	0.1	0.1	0.1	0.1	0.1
Microcare PE	0.8	0.8	0.8	0.8	0.8	0.8
Water	33.5	36.0	32.3	34.8	64.3	61.8

Formula A and B

10 ml of chitosan 1% solution was stirred with a magnetic stirrer at a speed of ± 300 rpm. Add little by little to the fenugreek seed extract which has been dissolved with DMSO, propylene glycol and glycerin, stir until homogeneous. Add the sodium EDTA solution which has diluted with 10 ml of hot water, stir until homogeneous. Add the sodium metabisulfite solution which has diluted with 10 ml of water, stir until homogeneous. Add the Microcare PE, stir until homogeneous. Add little by little to the Rheodol TW-O120V solution which has diluted with 10 ml of water, stir until homogeneous. Add the remaining water little by little until the volume reaches 100 ml, stir until homogeneous. After completion, mixing continued for 60 min with a constant speed of ± 300 rpm so that the resulting particle size was stable.

Formula C and D

Combine Rheodol TW-O120V added with Carbowax PEG-400, stir using a magnetic stirrer with a speed of ± 300 rpm to homogeneous. Add Peceol and Mackaderm MCT little by little, stir until homogeneous. Add little by little to the fenugreek seed extract which has been dissolved with DMSO and Propylene glycol, stir until homogeneous. Add the sodium EDTA solution which has diluted with 10 ml of hot water, stir until homogeneous. Add the sodium metabisulfite solution which has diluted with 10 ml of water, stir until homogeneous. Add the

Microcare PE, stir until homogeneous. Add the remaining water little by little until the volume reaches 100 ml, stir until homogeneous. After completion, mixing continued for 30 min with a constant speed of ± 300 rpm so that the resulting particle size was stable.

Formula E and F

Dissolve the fenugreek seed extract with DMSO and propylene glycol until it dissolves completely, stirs until homogeneous. Add the sodium EDTA solution which has diluted with 10 ml of hot water, stir until homogeneous. Add the sodium metabisulfite solution which has diluted with 10 ml of water, stir until homogeneous. Add the Microcare PE, stir until homogeneous. Add little by little to the Rheodol TW-O120V solution which has diluted with 10 ml of water, stir until homogeneous. Add the remaining water little by little until the volume reaches 100 ml, stir until homogeneous.

Evaluation of hair tonic[9]

Identification using the senses and covered the smell and color of hair tonic.

pH test

A pH meter was calibrated using a buffer solution with pH 4, pH 7 and pH 9. The electrode was immersed into the hair tonic and left for a few minutes until the pH stabilized.

Viscosity

Viscosity measurement using viscometer Brookfield type LV (spindle 1, RPM 3.0)

Specific gravity

Select a scrupulously clean, dry pycnometer that previously has been calibrated by determining its weight and the weight of recent water contained in it at 25°C. Adjust the temperature of the liquid to about 25°C, and fill the pycnometer with it, remove any excess liquid, and weigh. Subtract the tare weight of the pycnometer from the filled weight. The specific gravity of the liquid is the quotient obtained by dividing the weight of the liquid contained in the pycnometer by the weight of water contained in it, both determined at 25°C.

Characterization of nanoparticles[10]

Particle size, polydispersity index, and zeta potential

The measurements of particle size, polydispersity index, and zeta potential of nanoparticles were performed on a Delsa™ Nano C on the basis of photon correlation spectroscopy and electrophoretic light scattering.

Morphology and surface charge

The measurements of morphology and surface charge, using transmission electron microscopy (TEM) type JEOL 1010.

Hair growth activity test[4]

Testing of hair growth of hair tonic on rabbits using the method of Tanaka et al. Shaved back of rabbits was divided into 8 regions, each of a rectangular shape with 2.5 cm x 2.5 cm size and 1 cm spacing between boxes. Before applying the hair tonic, the rabbit's back was smeared with 70% ethanol as an antiseptic. The application area is as follows:

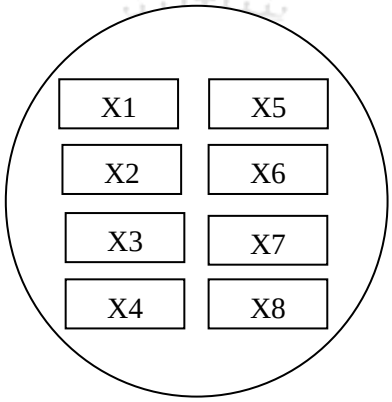


Figure No. 1: The application area to the rabbit's back is done by scrambling the position of X1-X8

X1: formula A was applied

X2: formula B was applied

X3: formula C was applied

X4: formula D was applied

X5: formula E was applied

X6: formula F was applied

X7: positive control (Regrou® Minoxidil 2%)

X8: negative control (no applied)

1.0 ml of each formula was given twice a day for 35 days. The first day was considered as day 0. Observations were made by taking 6 pieces hair on each box on day 7, 14, 21, 28 and 35. The hair is taken by cutting, then straightened and placed on a dark colored base and taped. Measured was done with vernier caliper Mitutoyo. On the 35th day, all hair in each box was cut and weighed.

Skin irritation test

Tests carried out to determine the presence of disturbances of side effects of preparations on rabbit skin which are characterized by the absence of redness or swelling so that the preparation is safe. The test animals used were male or female albino rabbits weighing around 2 kg. Test animal hair shaved with a 10 x 15 cm on the back area of at least 24 hours prior to the tests. Shaving starts from the shoulder blades area (shoulder) to the groin bone (waist bone) and half down the body on each side. Animals used for experiments are animals that have a healthy skin. Skin irritation testing procedures as follows:

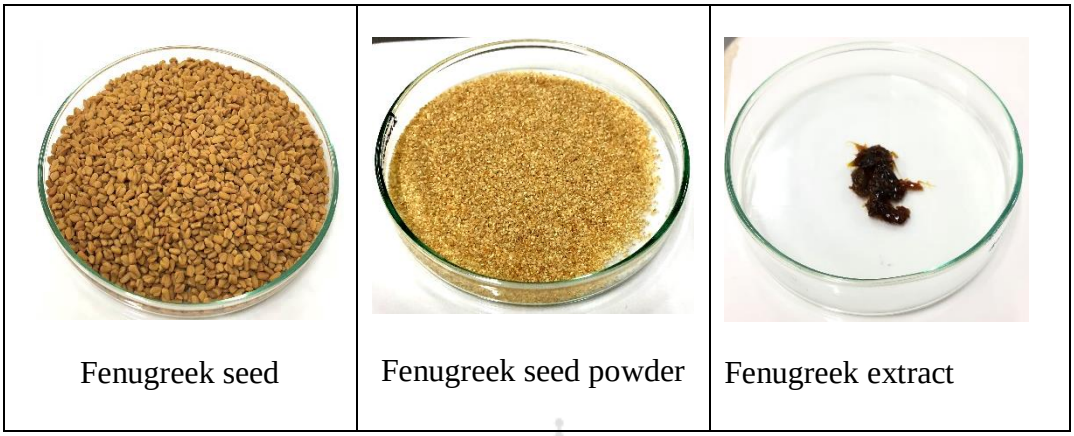
Apply a hair tonic preparation (0.1 ml) and cover with plaster for 24 hours. After 24 hours lift the patch and clean the area then evaluated for redness and swelling. Then the skin is evaluated again at the 48th and 72nd hours after the patch is opened.

RESULTS AND DISCUSSION:

Ethanol extraction of fenugreek

The material used in this study was fenugreek seed obtained from PT. Phytochemindo Reksa, Bogor, West Java. Fenugreek seed extraction process performed using soxhletation, which is a process of continuous extraction with a relatively constant amount of solvent in the presence of cooling behind (condenser). A total of 2.8 kg of crude fenugreek seeds mashed with a blender, then sieved with a mesh of 40 and generated as much as 2.65 kg of fenugreek seed powder.

Fenugreek seed powder as much as 2.65 kg dried in an oven at 40°C. Dry powder fenugreek seed crushed with machine disk mill and sieved with a 120 mesh so as to obtain as much as 2.495 kg of finely powdered fenugreek seeds. A total of 2.495 kg of finely powdered fenugreek seeds were extracted with 17.5 L of ethanol 96% for 48 hours at 78-79°C, then evaporated for 36 hours at 50°C, resulting in a viscous ethanol extract as much as 870.2 g fenugreek seeds. The extract is a dark brown viscous liquid with a distinctive smell and a rather bitter taste.



Fenugreek seed | Fenugreek seed powder | Fenugreek extract

Figure No. 2: Seeds, powder and ethanol extract of fenugreek

Fenugreek extract phytochemical test

Phytochemical examination by Balittro Bogor on January 12th, 2018. Phytochemical test results fenugreek extract can be seen in Table No. 2 and it showed that the extract contained alkaloids, saponin, tannin, phenolic, flavonoid, triterpenoids, steroids, and glycosides.

Table No. 2: Phytochemistry screening of the fenugreek seed extract

Compounds in the Fenugreek seeds extract	Presence
Alkaloids	+
Saponin	+
Tanin	+
Phenolic	+
Flavanoid	+
Triterpenoids	+
Steroids	+
Glycosides	+

Evaluation of hair tonic

The characteristics of the hair tonic preparations produced are in Table No. 3 and Figure No. 3.

Table No. 3: Evaluation of hair tonic

Parameter	Formula A	Formula C
Description	The colloidal liquid is yellow with a distinctive aromatic odor.	Slightly viscous liquid translucent yellow with a distinctive aromatic odor.
pH	4.80	5.50
Viscosity (spindle 1; RPM 3,0)	20 cps	100 cps
Specific gravity	1.0832 g/ml	1.0576 g/ml
Particle size	215.5 nm	25.3 nm
Polydispersity index	0.485	0.452
Zeta potential	-40.74 mV	-1.39 mV

Figure No. 3: Nanoemulsion and nanoparticle hair tonic

Determination of particle analyzer preparations carried out by using Scanning Transmission Electron (TEM), is a characterization tool for directly imaging obtain quantitative particle and/or grain size, size distribution, and morphology. TEM images the transmission through a sample, forming an image in a light microscope. The morphology of the nanomaterial can be seen in Figure No. 4.

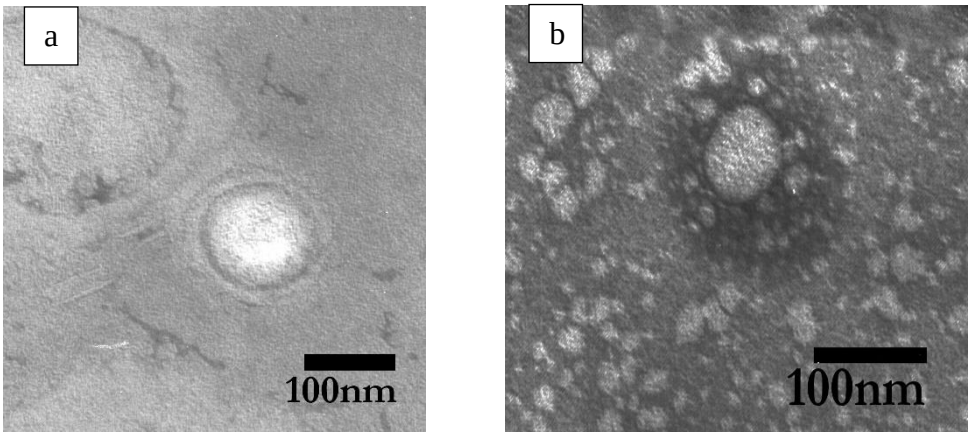


Figure No. 4: TEM results of formula A[a)] and formula C[b)]

Hair growth activity test

Hair growth activity test using rabbits as experimental animals has received ethical approval from Health Research Ethics Committee of the Faculty of Medicine, University of Indonesia, Cipto Mangunkusumo Hospital with No. 1004/UN.2/F1/ETIK/2017. Hair growth activity test against hair tonic carried out by observing two test parameters with an average hair length and weight of rabbit hair.

Hair length

In Table No. 4 and Figure No. 5 shows that since the first week until the fifth week, formula A provides the best hair growth results compared to other formulas and positive control, this can be seen from the average length of hair produced each week. To see the difference in hair growth activity in each week on each of the treatment groups can be determined by statistical calculation. Statistical calculation in every week showed the data wasn't normally distributed, and thus Kruskal Wallis test was run. It was shown there was a significant difference between the treatment group (P-Value <0.05) at weeks 1, 2, 3 and 5, this means that all treatments provide different hair growth activity. However in the fourth week, the Kruskal Wallis test showed no significant difference between the treatment groups (P-Value> 0.05), this means that all treatments didn't provide different hair growth activities.

Table No. 4: Average of hair length on 35 days

Treatment	Average length (mm) ± SD				
	7th Day	14th Day	21st Day	28th Day	35th Day
Formula A	0.98 ± 0.14	2.15 ± 0.43	5.20 ± 0.60	8.88 ± 1.44	15.11 ± 1.22
Formula B	0.71 ± 0.21	1.90 ± 0.33	4.18 ± 0.56	6.92 ± 1.89	10.91 ± 0.78
Formula C	0.86 ± 0.10	2.25 ± 0.44	4.09 ± 0.78	8.79 ± 2.05	11.54 ± 1.34
Formula D	0.64 ± 0.11	1.86 ± 0.53	3.56 ± 0.89	5.88 ± 0.46	9.11 ± 1.20
Formula E	0.55 ± 0.09	1.85 ± 0.41	4.07 ± 0.67	6.70 ± 1.26	9.32 ± 1.98
Formula F	0.67 ± 0.15	1.87 ± 0.53	3.80 ± 0.62	7.33 ± 1.34	11.13 ± 2.26
Positive control	0.82 ± 0.09	2.03 ± 0.50	4.67 ± 0.93	6.24 ± 1.23	9.52 ± 1.12
Negative control	0.49 ± 0.24	1.57 ± 0.43	3.04 ± 0.52	5.14 ± 1.46	6.85 ± 0.91

Statistical calculations continued with the Mann Whitney test to see differences between groups. In the results of Mann Whitney test between formula A vs formula C, formula C vs positive control, formula C vs formula F, formula C with formula D, formula C with formula F, and formula C vs negative control on day 35 shows that only formula C vs formula F which is not significantly different (P-Value> 0.05).

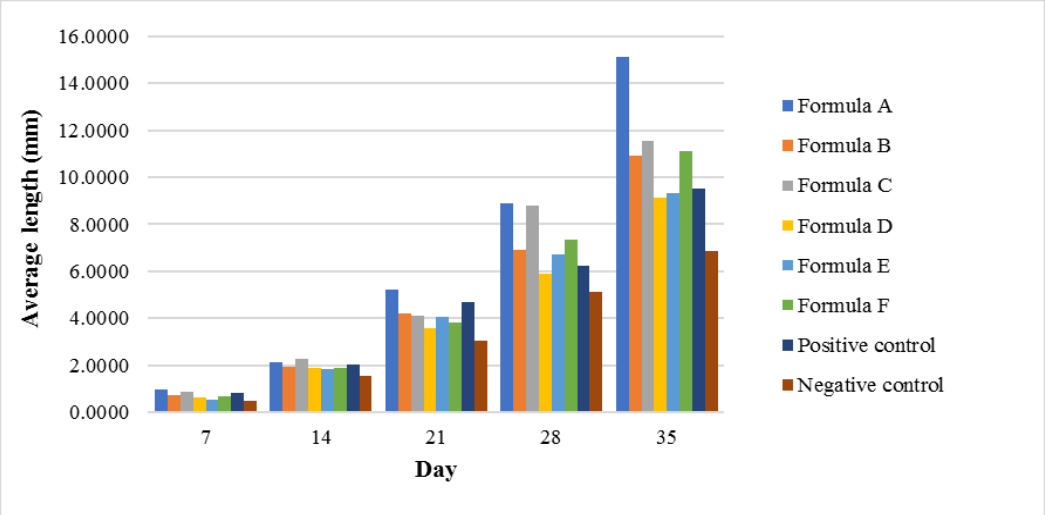


Figure No. 5: Hair tonic activity test against the average hair length on 35 days

Weight of hair

Hair weight was also observed in the fifth week by shaving each test area and then weighted. The weight of the hair is used to see the effect of each treatment on the density of rabbit hair. The test results of hair growth activities based on the weight of the rabbit hair can be seen in Table No. 5 and Figure No. 6.

Table No. 5: Average weight of the hair on 35 days

Treatment	Hair weights (mg)					SD
	Rabbit I	Rabbit II	Rabbit III	Rabbit IV	Average	
Formula A	159.70	210.90	185.30	225.80	195.43	29.10
Formula B	98.50	104.70	109.90	104.50	104.40	4.66
Formula C	141.30	179.40	150.10	144.20	153.75	17.49
Formula D	80.40	90.10	99.00	89.40	89.73	7.60
Formula E	93.80	100.40	102.80	108.20	101.30	5.97
Formula F	103.90	118.60	112.90	131.10	116.63	1139
Positive control	118.80	123.50	152.90	147.00	135.55	16.91
Negative control	63.60	73.40	62.40	67.60	66.75	4.96

Statistical calculations were performed to see the difference in average hair weights in each treatment group. Statistical results show data is normally distributed. Statistical calculations with the one-way ANOVA test showed that the results of the average hair weight of each treatment differed significantly (P-Value <0.05).

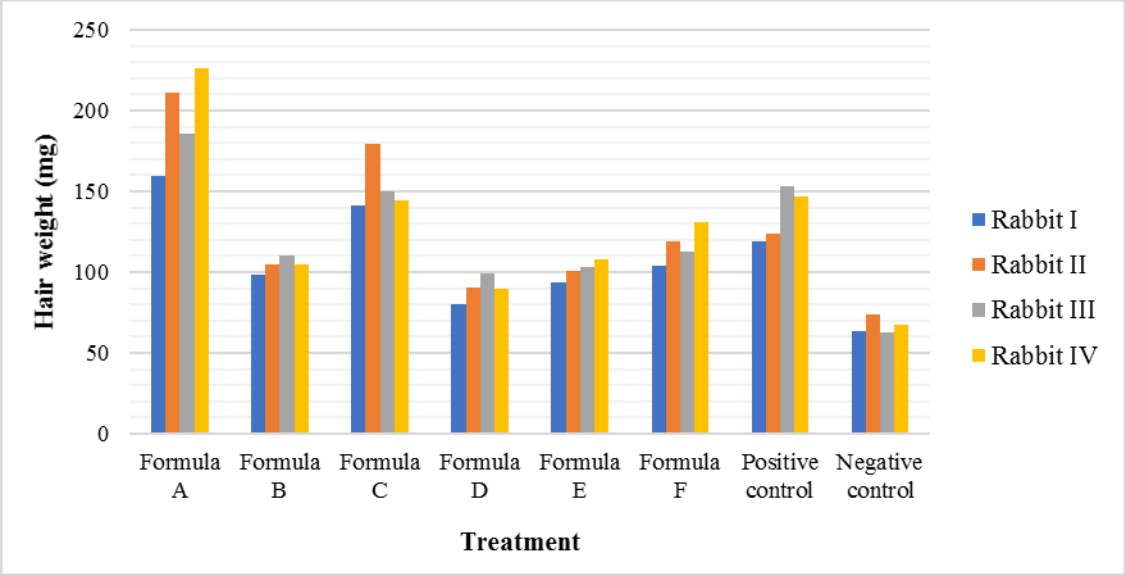


Figure No. 6: Hair tonic activity test against the average hair weight

Skin irritation test

From the results of observation and calculation of the irritation index on rabbit skin for 72 hours, it can be concluded that all formulas and positive controls didn't give the effect of erythema and edema.

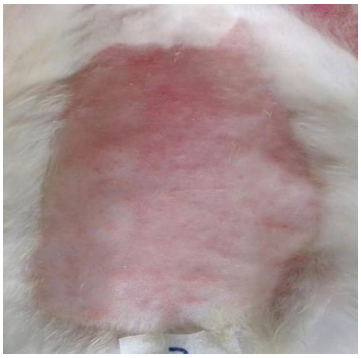
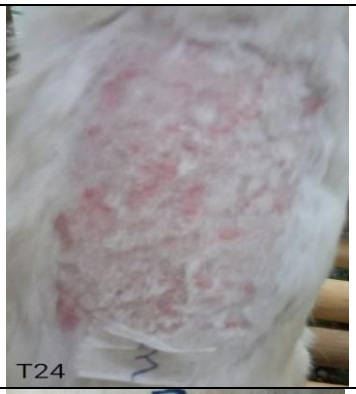
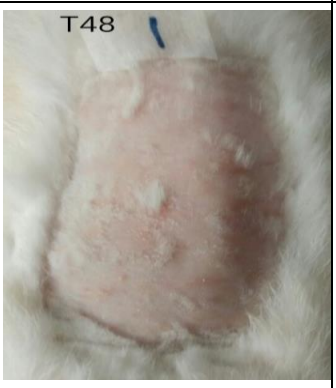
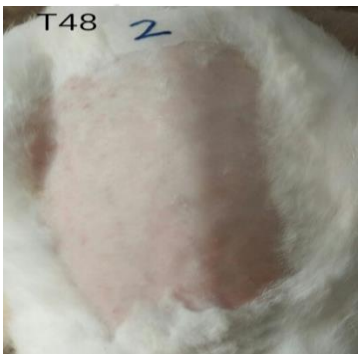
	Rabbit No.1	Rabbit No.2	Rabbit No.3
0 hours			
24 hours			
48 hours			
72 hours			

Figure No. 7: Skin irritation test of hair tonic

CONCLUSION:

Formula A has better effectiveness compared to Formula F, Formula C and positive control Minoxidil 2% (Regrou®). Skin irritation test of all formulas and positive control had no effect on erythema and edema. This study shows that the modification with nanotechnology provides increased effectiveness in hair growth, were using a lower concentration of fenugreek seed extract can provide better hair growth results and doesn't irritate the skin.

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Review

Hair-Growth Potential of Ginseng and Its Major Metabolites: A Review on Its Molecular Mechanisms

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Abstract: The functional aspect of scalp hair is not only to protect from solar radiation and heat/cold exposure but also to contribute to one's appearance and personality. Progressive hair loss has a cosmetic and social impact. Hair undergoes three stages of hair cycle: the anagen, catagen, and telogen phases. Through cyclical loss and new-hair growth, the number of hairs remains relatively constant. A variety of factors, such as hormones, nutritional status, and exposure to radiations, environmental toxicants, and medications, may affect hair growth. Androgens are the most important of these factors that cause androgenic alopecia. Other forms of hair loss include immunogenic hair loss, that is, alopecia areata. Although a number of therapies, such as finasteride and minoxidil, are approved medications, and a few others (e.g., tofacitinib) are in progress, a wide variety of structurally diverse classes of phytochemicals, including those present in ginseng, have demonstrated hair growth-promoting effects in a large number of preclinical studies. The purpose of this review is to focus on the potential of ginseng and its metabolites on the prevention of hair loss and its underlying mechanisms.

Keywords: ginseng; human-hair-follicle dermal papilla cells; WNT/β-catenin; Shh/Gli; TGF-β; BMP/Smad; mouse-hair growth

1. Introduction

The hair-growth cycle comprises three distinct phases, the anagen, catagen, and telogen phases of independent hair follicles. Hair continues to grow during the anagen phase, followed by a transitional period of the catagen phase, which enters into the telogen phase, when hair is released from the follicle and falls. The anagen phase can be classified into a propagating anagen phase that involves the activation of new hair follicles, and an autonomous anagen phase, when hair growth and differentiation of hair follicles actively occur [1]. The normal hair-growth cycle is repeated about 20 times; however, it can be modified or shortened by internal or external factors such as hormones, stress, concurrent disease, exposure to environmental pollution, and smoking. Changes in the growth cycle leading to hair loss may be represented with the shortening of the anagen phase, premature ingression of the catagen phase, and the prolongation of the telogen phase. Early hair loss is medically termed as alopecia [2,3]. The number of people suffering from alopecia is increasing and approaching approximately 10 million throughout the world. Considering the pathological background of alopecia and its impact on an individual's health and social value, there is now a growing interest in the development of novel therapeutics for its medical management. To date, the United States Food and Drug Administration (US-FDA) has approved two medications, minoxidil and finasteride, for the treatment of alopecia. Finasteride has been shown to prevent male pattern hair loss through the inhibition of type II 5α-reductase, which affects androgen metabolism. Although the exact mechanism of minoxidil has still not been elucidated, available research findings suggest that the hair-growth promotional effects of minoxidil are mediated through enhanced nutrient supply to hair follicles

through vasodilation, opening of the K^+ channel, and the activation of extracellular signal-regulated kinase (ERK) and protein kinase B (AKT/PKB) signaling, resulting in increased cell proliferation and inhibition of apoptosis in dermal papilla cells [4,5]. However, these drugs exhibit certain adverse effects, such as allergic contact dermatitis, erythema, and itching. While discontinuation of minoxidil leads to recurrence of alopecia, prolonged use of finasteride causes male sexual dysfunction and appears as a major cause of infertility and teratogenicity in females [6,7]. Thus, nontoxic chemicals with persistent hair-growth promoting effects have long been sought from the vast resources of natural products [8–10].

Ginseng is an ancient herbal remedy that was recorded in The Herbal Classic of the Divine Plowman, the oldest comprehensive Materia Medica, which was scripted approximately 2000 years ago. Contemporary science has revealed that ginseng contains a wide variety of bioactive constituents, especially a group of saponin compounds collectively known as ginsenosides, which are accredited with diverse biological activities, including the hair-growth potential of ginseng. Depending on the number of hydroxyl groups available for glycosylation via dehydration reactions, ginsenosides can be classified as protopanaxadiol (PPD) and protopanaxatriol (PPT). Common PPD-type ginsenosides include ginsenosides Rb1, Rb2, Rc, Rd, Rg3, F2, Rh2, compound K (cK), and PPD, whereas PPT-type ginsenosides include Re, Rf, Rg1, Rg2, F1, Rh1, and PPT [1]. Ginseng extract or its specific ginsenosides have been tested for their potential to promote hair growth. This review sheds light on the potential of ginseng and ginsenosides in promoting hair growth and delineating the mechanisms by which they function.

2. Biochemical Basis of Hair-Growth Promotion by Ginseng

There has been mounting evidence suggesting that ginseng and its major bioactive constituents, ginsenosides, promote hair growth by enhancing proliferation of dermal papilla and preventing hair loss via modulation of various cell-signaling pathways [11–13]. While the role of 5α-reductase enzyme in the hair-loss process has been well-documented [14,15], the emerging biochemical mechanisms of hair-follicle proliferation and the hair-loss process unravel new targets for designing novel therapeutics for the management of hair loss and alopecia (Figure 1). These targets include, but are not limited to, WNT/Dickkopf homologue 1 (DKK1), sonic hedgehog (Shh), vascular endothelial growth factor (VEGF), transforming growth factor-beta (TGF-β), matrix metalloproteinases (MMPs), extracellular signal-regulated protein kinase (ERK), and Janus-activated kinase (JAK). The following section summarizes the role of ginseng and its metabolites on hair growth.

2.1. Prevention of Radiation-Induced Skin Damage

Photoaging is one of the long-term effects of chronic sun exposure characterized by different inflammatory responses to ultraviolet radiation (UVR). Although exposure to solar UVR induces the synthesis of vitamin D, melanocortins, adrenocorticotropic hormone, and corticotropin- releasing hormone in human skin, and shows a beneficial effect, excessive UV irradiation is known to cause skin photodamage by inducing reactive oxygen species (ROS), precipitating skin inflammation, and promoting keratinocyte cell death. The impact of UVR exposure further leads to skin photoaging and carcinogenesis. However, the influence of UVR on skin appendages such as hair follicles is still in progress in many aspects. Accumulating evidence suggests that UVR exposure not only causes the damage of the hair shaft as an extracellular tissue, but also alters the hair-growth cycle by affecting keratinocyte and dermal papilla growth [16]. UV irradiation causes accumulation of ROS and activates MMPs, a class of tissue-degrading enzymes, thereby compromising dermal and epidermal structural integrity. Irradiation of normal human dermal papilla cells (nHDPC) with ultraviolet B (UVB) ($\geq$50 mJ/cm^2) exhibited ROS-mediated induction of apoptotic cell death [17]. Ginsenosides Rb2 [4] and 20 (S) PPD, but not 20 (R) PPD [4], have been reported to reduce the formation of ROS and MMP-2 secretion in cultured human keratinocytes (HaCaT) cells after exposure to UVB radiation. Likewise, ginsenoside Rg3 20 (S), but not 20 (R), reduced ROS generation in HaCaT

cells and human dermal fibroblasts without affecting cell viability. The 20 (S) Rg3 also attenuated UVB-induced MMP-2 levels in HaCaT cells [6]. In another study, ginsenoside Rh2 epimers reduced UVB radiation-induced expression and activity of MMP-2 in HaCaT cells, but UVB-induced ROS formation was only suppressed by 20 (S)-Rh2 [7]. Because the extracellular matrix plays a critical role in hair-follicle function, degradation and matrix remodeling by MMPs affect the hair cycle [18,19]. The inhibitory effect of ginsenosides on UVB-induced activation of MMP2 suggests the potential of these ginseng saponins in hair-growth regulation.

Ginsenosides have also been shown to improve hair growth by attenuating radiation-induced cell death in the skin. Total-root saponins and ginsenoside Rb1 diminished apoptotic cells, as revealed by the accumulation of Ki-67-positive cells and elevated expression of Bcl-2, an antiapoptotic protein, in UVB-exposed human keratinocytes [20]. Ginsenoside F1, an enzymatically modified derivative of ginsenoside Rg1, also protected keratinocytes from radiation-induced apoptosis by maintaining a constant level of Bcl-2 and Brn-3a expression in UVB-irradiated HaCaT cells [21].

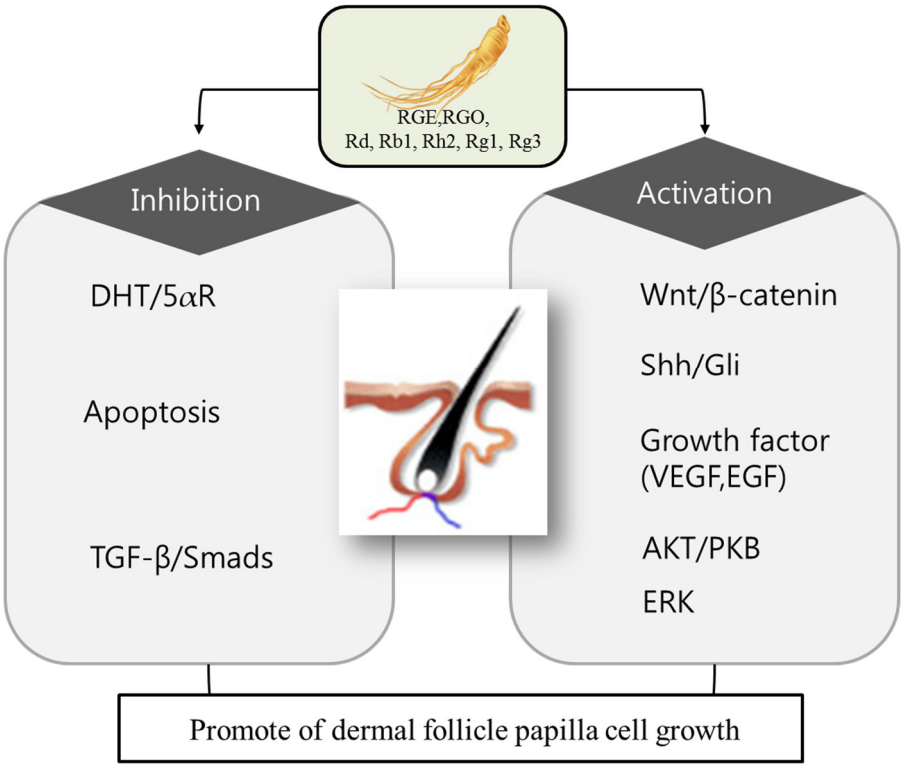

Figure 1. Potential molecular targets of ginseng in hair growth and loss. Ginseng exhibits therapeutic potential for hair growth and preventing hair loss by preventing the apoptosis of dermal follicle papilla cells. Ginseng components: RGE (red ginseng extract), RGO (red ginseng oil), ginsenoside Rd, Rb1, Rh2, Rg1, Rg3. antiandrogenic: DHT (dihydrotestosterone), 5-aR (5α-reductase). Apoptosis inhibition: TGF-β (transforming growth factor beta), Smads (homologues of the Drosophila protein, mothers against decapentaplegic (Mad) and the *Caenorhabditis elegan* sprotein Sma). Proliferation activation: WNT (wingless-type MMTV integration site family member), Shh (Sonic hedgehog), Gli (glioma-associated oncogene homolog), VEGF (vascular endothelial growth factor), EGF (epidermal growth factor), AKT/PKB (protein kinase B), ERK (extracellular-signal-regulated kinases).

2.2. Antiaging Effects of Ginsenosides

Several studies have reported on the antiaging effects of various ginsenosides [22,23]. As a general outcome of antiaging effects, ginseng extract and ginsenosides maintain skin structural integrity and regulate hair-growth promotion. For instance, incubation of cultured human dermal fibroblasts with *Panax ginseng* for three days significantly increased cell proliferation and collagen synthesis [24].

The antiaging effects of *P. ginseng* root extract were attributed to the induction of type-1 pro-collagen via phosphorylation of Smad2 and activation of human collagen-A2 promoter in human dermal fibroblast. According to this study, *P. ginseng* root extract did not exhibit any sensitivity reaction to human skin [25]. Another marker of the aging process is wrinkle formation, which is often associated with a reduced level of hyaluronan in the dermis. Topical application of a major ginseng metabolite (compound K) on mouse skin elevated the expression of hyaluronan synthase-2, an enzyme that catalyzes the synthesis of hyaluronan, through Src kinase-dependent activation of ERK and AKT/PKB kinases in the dermis and papillary dermis of mice [26,27]. These antiaging effects result in improved skin health, thereby ensuring hair-follicle health and a regular hair cycle.

2.3. Modulation of TGF-β Signaling

The role of TGF-β in hair loss has been documented through the study revealing that treatment with a TGF-β antagonist can promote hair growth via preventing catagen progression [28]. Since TGF-β1 induces catagen in hair follicles and acts as a pathogenic mediator of androgenic alopecia [28], red ginseng extract can delay the catagen phase and holds the potential to promote hair growth. Administration of red ginseng extract at a dose of 20 or 60 mg/kg twice daily by gavage decreased TGF-β1 levels in UVB-irradiated mouse skin [29]. Likewise, topical administration of ginsenoside Re on to the back skin of nude mice for up to 45 days significantly increased hair-shaft length and hair existent time, and stimulated hair-shaft elongation in the ex vivo cultures of hair follicles isolated from C57BL/6 mouse. The hair-growth-promoting effects of ginsenoside Re were associated with the downregulation of TGF-β-pathway-related genes, which are involved in the control of hair-growth phase-transition-related signaling pathways [30]. It has been reported that brain-derived neurotrophic factor (BDNF) enhances transition from the anagen to the catagen phase through the activation of TGF-β [31]. Protopanaxatriol-type gisenoside Re promotes hair growth through the inhibition of TGF-β signaling pathways [30]. TGF-β-induced hair loss is associated with the hyperactivation of the c-Jun-N-terminal kinase (JNK) pathway [32]. The inhibition of JNK by Korean red ginseng has been attributed to the protective effects of ginseng on radiation-induced apoptosis of HaCaT cells [33].

Moreover, hair-follicle regression is partly regulated by the p75 neurotrophin receptor (p75NTR), which is a classical BDNF [34]. Since neurotrophins elicit their effects by interacting with high-affinity neurotrophin receptors, it would be a rational approach to develop neurotrophin-receptor antagonists as potential therapy for the treatment of hair loss, particularly androgenetic alopecia. A recent study has demonstrated that *P. ginseng* hexane extracts, which largely contain polyacetylenes, strongly inhibited β-nerve growth factor (β-NGF) interaction with p75NTR. Thus *P. ginseng*-derived polyacetylenes would be a potential therapeutic choice for the treatment of hair-growth disorders [35].

2.4. Inhibition of 5α-Reductase Enzyme

Progressive hair loss, also known as alopecia, occurs due to alternations in cell-signaling pathways in hair follicular cells resulting in the induction of apoptosis, changes in usual pattern of hair cycling and thinning, or fracture of the hair shaft. One of the major triggers for hair loss is the exposure to androgens, which in most cases are genetically predetermined among the individuals who have androgenic alopecia. The androgen that mainly plays a role in altering hair cycling is 5α-dihydrotestosterone (DHT), which is a metabolite of testosterone. The conversion of testosterone to DHT is mediated by the 5α-reductase (5αR) enzyme in each follicle [27]. Treatment with 5α-reductase inhibitors, e.g., finasteride, prevents the development of alopecia and increases scalp-hair growth. In several in vivo experiments, topical application of ginseng extract or ginsenosides was reported to enhance hair growth. Rhizomes of *P. ginseng* (red ginseng) containing a considerable amount of ginsenoside Ro inhibited the activity of 5α-reductase. Ginsenoside Rg3 and Rd also exhibited similar inhibitory effects on this enzyme [36]. The inhibition of 5αR enzyme activity was more pronounced with extracts of red-ginseng rhizomes as compared to that of ginseng main-root extract. Ginsenosides Ro

derived from rhizome extract and ginsenoside Rg3 obtained from main-root extract attenuated the 5αR enzyme activity with IC_{50} values of 259.4 and 86.1 μm, respectively. Another variety of ginseng, the *Parribacus japonicas* rhizome extract that contains a larger quantity of ginsenoside Ro also inhibited 5αR enzyme activity. Topical administration of red-ginseng rhizome extracts (2 mg/mouse) and ginsenoside Ro (0.2 mg/mouse) onto shaved skin of C57BL/6 mice abrogated testosterone-mediated suppression of hair regrowth [36].

2.5. Modulation of Wnt/Dickkopf Homologue 1 (DKK1) Signaling

Wingless-type integration-site (WNT) signaling plays a key role in hair-follicle development. The blockade of Wnt signaling by overexpression of the WNT inhibitor, DKK1, prevents hair-follicle formation in mice [37]. β-catenin signaling is essential for epithelial stem-cell fate since keratinocytes adopt an epidermal fate in the absence of β-catenin [38]. Treatment with ginsenoside F2 resulted in a 30% increase in the proliferation of HHDPC and HaCaT cells as compared to that of finasteride. Ginsenoside F2 increased the expression of β-catenin and its transcriptional coactivator Lef-1, while it decreased the expression of DKK-1 in HHDPC as well as in the skin of C57BL/6. Administration of ginsenoside F2 promoted hair growth as compared to finasteride, as revealed by an increase in the number of hair follicles, thickness of the epidermis, and follicles of the anagen phase, suggesting that F2 induces the anagen phase and stimulates hair growth through the modulation of the Wnt signal pathway [39]. In another study by Matsuda et al., the hair growth-stimulating activity of the methanol extract of red ginseng in an organ culture of mouse vibrissal follicles was attributable to ginsenosides Rg3 and Rb1 [40]. Treatment of cultured outer root sheath (ORS) keratinocytes with *P. ginseng* extract in the presence or absence of DKK-1 has revealed that *P. ginseng*-extract treatment increased the Bcl-2 to Bax ratio, and the anagen to catagen ratio, and reversed DKK-1-mediated suppression of the Bcl-2/Bax ratio. *P. ginseng* extract antagonizes DKK-1-induced catagen-like changes, in part, through the regulation of apoptosis-related gene expression in hair follicles [41].

2.6. Modulation of Sonic Hedgehog (Shh) Signaling

Shh/Gli (glioma-associated oncogene homolog) regulates hair-follicle development during embryonic life and influences the cycling and growth of hair follicles in adults by promoting telogen-to-anagen transition of follicular cells and epidermal growth [42–44]. Mice harboring the mutant form of Shh have small dermal papillae characterized by the presence of abnormal hair follicular cells that are incapable of maintaining normal hair morphogenesis [44]. Attenuation of Shh activity by a monoclonal antibody targeting Shh diminished hair growth in mice, indicating the importance of Shh signaling in hair-growth promotion [45]. Treatment with red-ginseng oil reversed testosterone-induced suppression of hair regeneration in C57BL/6 mice by increasing the expression of Shh/Gli pathway-related proteins, including Shh, Smoothened (Smo), and Gli1. Additionally, two major compounds in red-ginseng oil, linoleic acid and β-sitosterol, were also found to activate the Shh/Gli signaling pathway in testosterone-treated mice. Topical application of bicycle (10.1.0) tridec-1-ene was unlikely to significantly accelerate protein levels of Shh and Gli1, but likely to increase Smo expression [46].

2.7. Modulation of JAK2-STAT3 Signaling

Cytokines, such as interleukins (ILs) and interferons (IFN), are inflammatory-signaling molecules that, upon overexpression and/or secretion, cause skin inflammation. Hair follicles are usually immune-tolerated areas, where natural killer (NK) cells remain suppressed [47]. Such immune activation is supported by the presence of CD8+ T cells and NKG2D+ cells around the peribulbar area of the affected hair follicles [48] and upregulation of several ILs, such as IL-2, IL-7, IL-15, and IL-21, and IFN-γ [49]. Loss of immune tolerance or immune activation, due to the upregulation of major histocompatibility complex (MHC class I) or UL16-binding protein 3 (ULBP3) molecules, leads to the activation of a cytotoxic cluster of differentiation 8-positive (CD8+) and NK group 2D-positive

(NKG2D+) T cells to the hair follicles [50,51], thereby leading to hair-follicle dystrophy and acceleration of the catagen phase [52]. Since JAK/Signal transducer and activator of transcription-3 (STAT3) pathway plays a critical role in mediating the activation of CD8+ NKG2D+ T cells, the inhibition of JAK appears as a plausible target for developing a therapy for hair loss [49]. In fact, a number of JAK inhibitors, such as tofacitinib and roxulitinib, are in the progress of developing a therapy for alopecia [53]. Ginsenoside Rk1 inhibited the lipopolysaccharide- stimulated phosphorylation of JAK2 and STAT3 in murine macrophage cells [54]. It would be interesting to investigate whether ginsenoside Rk1 or other ginsenosides can target JAK2 signaling in dermal papilla and diminish activation of NKG2D+ T cells. Moreover, the pathogenesis of alopecia areata is believed to involve inflammatory cytokines IL-17A and monoclonal antibodies against IL-17A secukinumab-caused hair regrowth in human volunteers [55]. Treatment of Th17 cells with *Panax notoginseng* saponins diminished the proliferation and differentiation of Th17 cells and decreased IL-17 expression [56]. Topical application of ginsenoside F2 also ameliorated phorbol ester-induced dermal inflammation by inhibiting the production of IL-17 and ROS in γδT cells and neutrophils, respectively, in mouse-ear skin [57]. These findings suggest that ginsenosides may enhance hair growth in alopecia areata by regulating IL-17 secretion.

2.8. Activation of Dermal Papillary Cell Proliferation

Various intracellular signaling molecules, including kinases and growth factors, play a critical role in stimulating hair growth by promoting dermal papillary-cell proliferation. VEGF, which is released from the epithelium, is a signaling protein that increases the vascular network surrounding the hair follicle [58]. Ginsenoside Rg3 promotes hair growth by upregulating VEGF expression [36]. Shin et al. also demonstrated that Rg3 increased the proliferation of human dermal papillary cells, which was associated with elevating the mRNA level of VEGF. In mouse-hair follicles in vivo, Rg3 not only increased the expression of VEGF but also stimulated stem cells by upregulating factor-activating CD34, and promoted hair growth even more than minoxidil [39].

Signaling pathway ERK, usually activated by mitogens, plays an important role in the proliferation of human hair-follicle dermal papillary cells (HHDPCs) [59]. Both red-ginseng extract (RGE) and ginsenoside-Rb1 activated the ERK signaling pathway. Thus, the proliferation of HHDPCs by red ginseng may be mediated by the ERK signaling pathways [12]. Another intercellular kinase, AKT/PKB, transmits critical signals for cell survival, and also regulates the survival of dermal papillary cells (DPCs) as an antiapoptotic molecule [60]. Therefore, the activation of AKT/PKB by red-ginseng extract and ginsenoside-Rb1 may prolong the survival of HHDPCs [12].

The Bcl-2 family proteins consists of more than a dozen members, which are either antiapoptotic or proapoptotic in nature [61]. During the hair cycle, the DPC is the only region where Bcl-2 is expressed consistently and is considered to resist apoptosis [62]. *Fructus panax ginseng* extract increases the expression of Bcl-2 but decreases Bax expression, a proapoptotic species, in cultured DPCs [63].

3. Evidence from In Vivo Animal Studies

Ginsenosides Rb1 and Rd from *P. ginseng* also exert a stimulating effect on hair follicles, and thus, appear as potential therapeutic agents. One suggested mechanism for this effect of ginsenosides Rb1 and Rd is the induction of p63 [64]. Topical application of *P. ginseng* extract (2.5%) failed to stimulate hair growth as compared to minoxidil in athymic nude mice [8]. The lack of the hair growth-promoting effect of ginseng in this study compared to other herbal products exhibiting hair growth may not be appropriately judged, as the nude mice are basically hairless or have limited fine hairs with poorly defined hair cycles. However, application of *P. ginseng* extract by intraperitoneal or per oral prior to gamma irradiation to adult N:GP mice diminished apoptosis and promoted hair medullary-cell repair [65]. In another study, C57BL/6 mice were subjected to treatment with ginsenoside F2 or finasteride. As compared to the finasteride-treated group, the ginsenoside F2-treated group showed 20% higher hair-growth rates as evidenced by increased number of hair follicles, epidermal thickness,

and proportion of follicles in the anagen phase. This hair-growth promoting effect of ginsenoside F2 was mediated, at least in part, through the activation of the Wnt-β-catenin pathway via blockade of Dkk [39]. Truong and colleagues [46] also reported that hair-regenerative capacity was significantly restored by treatment of red-ginseng oil and its major compounds in testosterone-treated mice.

4. Human Clinical Studies

Although individual ginsenosides are yet to be investigated for hair-growth promotion in human clinical trials, there have been few interesting human studies documenting the potential of Korean red ginseng in hair-growth promotion. Oh et al. studied hair-growth efficacy and safety of Korean red ginseng (KRG) in alopecia areata (AA), a model of androgenic alopecia, in human subjects. According to this study, human volunteers were treated with corticosteroid intralesional injection (ILI) with or without treatment with KRG. Hair growth in both the ILI-alone and ILI-plus-KRG patient group was monitored using Folliscope 2.5 for 12 weeks. Average hair density and hair thickness were significantly increased upon addition of KRG with ILI, suggesting that KRG may be considered as a useful complimentary food for gaining efficacy in the treatment for AA [66]. Kim et al. reported the effectiveness of Korean red ginseng in increasing the thickness and density of hair in human volunteers [11]. Moreover, combination treatment with topical minoxidil and oral KRG is more effective than topical minoxidil treatment alone for promoting hair growth. Therefore, KRG is expected to be a helpful supplement in the treatment of hair loss [67]. Keum et al. examined the potential of KRG in preventing premature hair-follicle dystrophy using a human hair-follicle organ-culture model. According to this study, human occipital scalp-skin specimens were obtained from patients undergoing hair-transplantation surgery, and follicular keratinocytes cells (FKC) were cultured in vitro. Treatment of FKCs with 4-hydroxycyclophosphamide (4-HC), a metabolite of chemotherapeutic agent cyclophosphamide, attenuated human hair growth, induced premature catagen development, diminished proliferation, and stimulated apoptosis of hair matrix keratinocytes. Pretreatment with KRG protected against 4-HC-induced hair-growth inhibition and premature catagen development partly by blocking 4-HC-induced p53 and Bax/Bcl2 expression [13].

5. Conclusions

The use of plant products in therapy has long been practiced and has appeared to be generally safe. Ginseng is a multipurpose natural medicine with a long history of medical application throughout the world, particularly in Eastern countries. The medical use of ginseng is not only restricted to the improvement of general wellness, but also extended to the treatment of organ-specific pathological conditions. In the field of dermatology, ginseng and ginsenosides have been shown to regulate the expression and activity of major proteins involved in hair-cycling phases. The promotion of hair growth and prevention of hair loss by ginseng and its metabolites are associated with the induction of anagen and delaying of catagen phases. Although the underlying mechanisms by which ginseng and its metabolites regulate hair cycling have been explored to a limited extent, further studies, especially focusing on extended human trials, are required to establish this natural remedy for hair loss. Alopecia, originating from a variety of causes, including hyperactivation of androgenic signals, exposure to chemotherapeutics, aging, or skin photodamage, is considered as a skin pathology and has great psychosocial impact. Thus, it would be a plausible approach to develop hair growth-stimulating formulations, either as FDA-approved therapeutics or as cosmeceuticals, by using the index component of red ginseng (Table 1).

Table 1. Molecular mechanisms underlying hair-proliferative and antiapoptosis-inducing activity of ginseng.

Type	Study Model	Dosage	Action Mechanism	Target	Reference
Fructus panax ginseng extract (FPG) (95% EtOH)	Human hair dermal papilla cells Male six-week-old C57BL/6 mice	0.8, 4, 20, 100, 500 μg/mL 1 mg/mL	FPG elicited the proliferation of DPC by the upregulation of antiapoptotic Bcl-2 accompanied by the inhibition of apoptotic Bax expression	Apoptosis	[4]
Ginsenoside Re	Male six-week-old C57BL/6 mice Cultured C57BL/6 mouse HFs HeLa cells	1 or 5 mg/d 10 or 50 mg/L 10 mg/L	Ginsenoside Re is the effective constituent in Panax ginseng that promotes hair growth through inhibition of transition related TGF-β signaling pathways.	TGF	[5]
Polyacetylenes isolated from *P. ginseng*	Neurotrophin receptor-binding inhibition assay	sample solution (10, 30, and 100 μM)	Inhibits BDNF-TrkB binding.	Growth	[6]
Ginsenoside F2	Human hair dermal papilla cells Human keratinocyte (HaCaT) cells Male six-week-old C57BL/6 mice	0.01, 0.1, 1, and 10 μM 0. 5 and 2.5 mg/kg	(1) Stimulates proliferation of HHDPC and HaCaT; (2) increases β-catenin and Lef-1 expression and decreases DKK-1 expression in HHDPC; (3) hair anagen induction and acceleration of hair growth in mouse model; (4) increases β-catenin expression and decreases DKK-1 expression in mouse tissue.	WNT	[7]
Root of PG extract (70% EtOH)	Human ORS keratinocytes Anagen HFs from human scalp-skin specimens	20 ppm	PG extract may enhance ORS and hDPC stimulation of hair follicle growth despite the presence of DKK-1, a strong catagen inducer	WNT	[29]
Korean Red Ginseng (KRG)	Human (patients diagnosed with AA)	Treated with corticosteroid ILI while taking KRG	KRG can result in improved hair regrowth in AA patients.		[20]
KRG extract	Follicular keratinocytes (FKCs) Human anagen hair follicles	0~1000 μg/mL 500 μg/mL	KRG may protect against 4-HC-induced premature dystrophy as it occurs in CIA in vivo. Possible mechanisms include the stimulation of hair matrix keratinocyte proliferation and inhibition of hair matrix keratinocyte apoptosis, which are possibly mediated through modulation of p53 and Bax/Bcl-2 expression.	Apotosis	[21]

Table 1. *Cont.*

Type	Study Model	Dosage	Action Mechanism	Target	Reference
Red ginseng extract (RGE)	Six-week-old female C57BL/6 mice	3%	RGE and its ginsenosides may enhance hDPC proliferation, activate the ERK and AKT/PKB signaling pathways in hDPCs, upregulate hair matrix keratinocyte proliferation, and inhibit DHT-induced androgen receptor transcription.	Growth 5aR	[16]
RGE, insenoside-Rb1	Human hair follicles	100 μg/mL			
Red ginseng oil (RGO)	C57BL/6 mice	RGO 10%	Upregulates Wnt/-catenin and Shh/Gli pathways-mediated expression of genes such as β-catenin, Lef-1, Sonic hedgehog, Smoothened, Gli-1, Cyclin D1, and Cyclin E in TES-treated mice. RGO and its major components reduce the protein level of TGF-β but enhance the expression of antiapoptotic protein Bcl-2.	WNT Shh Growth TGF Apoptosis	[25]
KRG	Human (patients with female pattern hair loss)	Oral	Patients about the size of the vertex spot, hair loss on the top of scalp, bitemporal recession, hair shedding, hair quality, and overall satisfaction; group 2 was more satisfied at 24 weeks.		[68]
Ginsenoside Rg3	Human DP cells	1, 5, 10 μM	Dose-dependent increases in VEGF, CD8, CD34 Rg3 might increase hair growth through stimulation of hair-follicle stem cells	Growth	[69]
	Female C57BL/6 mice	1000 μM			
Red ginseng (RGE) White ginseng (WGE) Ginsenoside-Rb1 (G-Rb1), Rg1 (G-Rg1), -Ro (G-Ro)	B6C3F1 mice	20, 50 μg/mL	Hair growth-promoting assay using mouse vibrissal follicles in organ culture	Growth	[26]
	Mouse vibrissal hair follicles	10 μg/mL			
Ginseng rhizome Ginsenoside Ro	C57BL/6 mice	extracts of red ginseng rhizomes (2 mg/mouse) and ginsenoside Ro (0.2 mg/mouse)	Inhibitory activity against 5αR in the androgenetic alopecia model.	5aR	[27]
Ginsenosides Rb1, Re, and Rg1	Cultured hHFs	2, 5, and 10 mg/mL PG extracts and 1 mM of the ginsenosides Rb1, Re and Rg1	PG extract using hHF organ culture, and promoting hair growth through similar mechanisms to those of minoxidil.	5aR	[36]

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Hair Growth Promoting Effect of Urticadioica L

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Objectives: We investigated the effect of *Urticadioica L* extracts on hair growth by using in-vitro and ex vivo study methods. **Methods:** Human single hair follicle and dermal papilla cells obtained from scalp skin samples of healthy volunteers. We evaluated the effect of *Urticadioica L* on hDPCs and on ex vivo hair follicle organ culture. Hair follicle matrix cell's proliferation marker Ki-67 identified by immunoflurescence staining. **Results:** *Urticadioica L* ethanol extracts promoted elongation of the hair shaft and reduced catagen transition of human hair follicles in organ culture model. E.extract of *Urticadioica L* increased Ki-67 positive matrix keratinocytes. **Conclusions:** *Urticadioica L* ethanol extract enhanced human hair growth in ex vivo organ culture model. Future study is needed related to its mechanism of hair growth.

Keywords: Dermal Papilla Cells (hDPCs), Hair Follicle, Urdicadioica L, Ki-67

Introduction

Stinging nettle (*Urticadioica L*) belongs to the family *Urticaceae* [1]. About 50 species of Stinging nettle (*Urticadioica*) are grown in the world. Three species of *Urticaceae* (*Urtica cannabina, Urtica angustifolia, Urtica dioica L*) grow in Mongolia [2]. *Urticadioica L* is a herbaceous perennial flowering plant that native to Europe, Asia, northern Africa and North America. The maximum typical height of this plant species ranges from 2 to 4 meters [3]. It produces pointed leaves and white to yellowish flowers [4]. The plant has many hollow stinging hairs called trichomes on its leaves and stems, which act like hypodermic needles, injecting histamine and other chemicals that produce a stinging sensation when contacted by humans and other animals [3]. Flavonoids, tanins, scopoletin, sterols, fatty acids, polysaccharides, isolectins andsterols are phytochemicals which are reported from this plant [5].

The whole plant is used in folk medicine to treat allergies, kidney stones, burns, anemia, rashes, internal bleeding, diabetes, etc. However only a few of these pharmacological activities have been experimentally proved [6]. Stinging nettle extracts has been different studies reported to have various pharmacological antioxidant, anti-microbial, anti-inflammatory, anti-ulcer, anticolitis and analgesic [7, 8]. As well as this plant

has been also used in human nutrition such as food or tea and also gathered commercially due to high content of chlorophyll, which is used as green coloring agent (E140) in food and medicines [5]. Despite the fact that science has not been proven its effectiveness, Mongolians have been using extracts from this plant to prevent hair loss and as a hair loss treatment.

Hair loss problem have approximately 2% of the world population [9]. According French scientists, one out of every five people have hair loss [10]. Depending on the clinical form, approximately 78% of men and 57% of women over the age of 80 are affected by androgenetic alopecia and 58% of men over the age of 50 are affected [11]. In Mongolia, the prevalence of hair loss has not yet been studied, but between in 2011-2015 a total of 1325 hair loss cases diagnosed in out-patient clinic of National Dermatology Center of Mongolia.

The hair follicle (HF) is a very small-organ [12]. In normal hair growth, the hair follicles cycle through successive phases: anagen phase of growth, catagen phase of involution, and telogen phase of rest [13]. The hair grown from the follicle will pass through the 4 stages of hair cycle to repeat hair growth and loss [12]. When generalized loss is present it is useful to assess the ratio of anagen (growing) hair to that of the telogen hair. In hair loss, there appears to be a dysfunction and disproportion in hair cycling, resulting in a reduced length of anagen phase, increased proportion of hair in catagen/telogen phase [14]. Many various factors including stress as social, psychological and mental, eating habits, and living habits, drugs etc can affect hair loss [15]. The most common form of human hair loss is androgenic alopecia.

Alopecia has few physically harmful effects, but may lead to psychological consequences, including high levels of anxiety and depression [16]. However, only two drugs so far have been approved for hair loss treatment by the Food and Drug Administration (FDA), finasteride and minoxidil [11]. Researchers are still studying low-cost therapeutic methods that are effectives against hair loss.

This study aimed to determine the effect of *Urticadioica L* on human hair growth. To accomplish this, we investigated the effect of *Urticadioica L* extracts on hair growth by using in-vitro and ex- vivo study methods.

Material and Method

Ethical statements

Study protocols were approved by the institutional research board of Mongolian National University of Medical Sciences [IRB approval number], and written informed consent was obtained from all subjects. All experimental procedures using human tissues were performed according to the principles described in the declaration of Helsinki.

Human hair follicle samples

A total of 479 human scalp HFs were obtained from 7 different healthy male volunteers 20 to 40 years of age without current or prior scalp diseases. The samples were obtained from 1.5x1.0 cm of scalp from their occipital region yielding greater than 60 HFs per subject. The samples were carefully dissected into single HFs under a stereo microscope (Motic K Series). In this study, only anagen HFs were used.

Preparation of Plant extract

The aerial part of the *Urticadioica L* was harvested according to the standards of "A" Pharmaceutical Factory (Ulaanbaatar, Mongolia). *Urticadioica L* extract was obtained by adding water, ethanol, hexane as solvents to macerated method *Urticadioica L* at the room temperature for 72 hours [17]. The solvent and extraction ratio were same (1:10). After filtration through filter paper, the filtrates were concentrated and dried by vacuum evaporation and freeze drying method.

Cell culture

As previously described [18, 19], the hair dermal papilla cells (hDPCs) were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Welgene, Daegu, Korea), supplemented with 10% fetal bovine serum (Welgene), 10 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN, USA), and antibiotic/antimycotic 1x solution (100 mg/ml streptomycin and 100 U/ml penicillin).

Thiazolylblue (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) (MTT) assay

Cell viability was measured by the MTT assay. Cells at $1x10^4$ cellsper well were seeded into 96-well plates, serum-starved for 24 hours, and then treated for 24 hours with the vehicle

(ethanol at a diluted to 1:1,000 in serum-free DMEM) or with *Uricadioica L* extracts (0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml [final concentration], diluted to 1:1,000 in serum-free DMEM). Subsequently, 15 µl of MTT solution was added to each well, and the plate was incubated for 4 hours at 37°C. The supernatant containing the MTT was removed, and the formazan crystals in the wells were dissolved by adding 100 µl of dimethyl sulfoxide. The samples were finally incubated for 30 minutes at room temperature before a plate reader enabled quantitative analysis for viable cells by optical density measurement at 570 nm.

Human hair follicles organ culture and immunofluorescence staining

Isolated human scalp hair follicles were cultured as described previously [20, 21]. Each HF was cut at the level of the sebaceous duct, and then cultured for 12 days at 37°C (5% CO_2) in Williams' E medium (Gibco-BRL laboratories, Grand Island, NY, USA), supplemented with 10 ng/ml hydrocortisone, 10 µg/ml insulin, 2 mM L-glutamine, and antibiotic/antimycotic 1x solution (penicillin and streptomycin, Gibco-BRL laboratories). *Urticadioica L* water, hexane and ethanol extracts was added to culture medium at final concentrations of 0.125 mg/ml. At every third day, elongation of the hair shaft was measured, and HFs in anagen phase were determined according to their growth.

Immunofluorescence staining was performed to evaluate proliferation of HF matrix keratinocytes. As previously described, immunoreactivity for Ki-67 (DAKO, Carpinteria, CA, USA) was used as an indicator of cell proliferation [22]. A DAPI mounting media kit (Vector Laboratories, Burlingame, CA, USA) was used to counterstain the nuclei.

Statistical analysis

Statistical significance was determined using Student's t-test. Paired t-test was used to compare the HF culture results. All tests

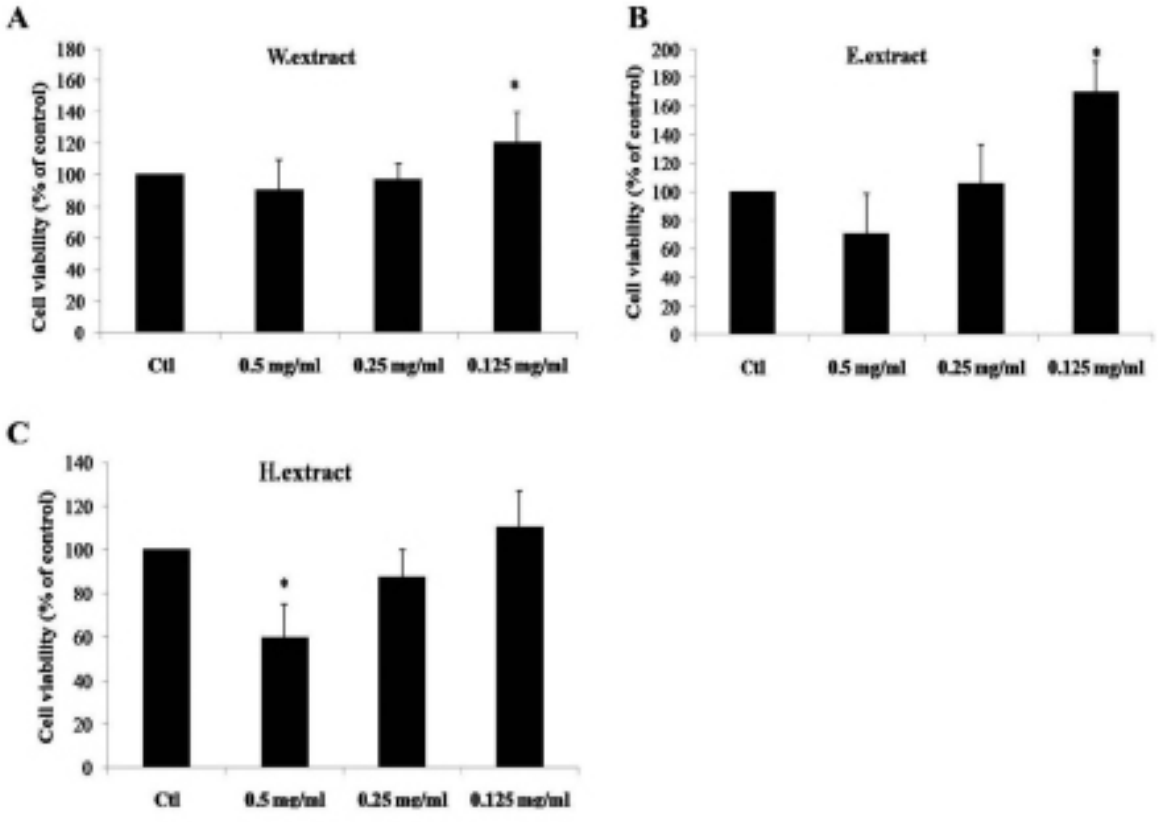

Figure 1. The viability of cultured human dermal papilla cells (hDPC's) on treatment of *Urdicadioica L* extracts. Cells were treated with *Urticadioica L* extracts, or vehicle for 24 hours. (A) water extract (W.extract) of *Urticadioica L*, (B) ethanol extract (E.extract), and (C) hexane extract (H.extract). The results are expressed as mean ± standard error.

were two-tailed, and differences with a p-value of <0.05 were considered statistically significant. The statistical analyses were done by using the STATA 13 software package.

Results

Effect of Urticadioica L extracts on DPC proliferation

We performed the MTT assay to evaluate the effect *Urticadioica L* extracts on hDPCs' survival. In this experiment, *Urticadioica L* water, ethanol, hexane extracts (0.125 mg/ml) significantly enhanced viability of hDPCs compared to the vehicle-treated controls. However, the hDPCs' viability decreased at all extracts concentrations that were greater than 0.250 mg/ml (Figure 1 A,B,C).

Effect of *Urticadioica L* extracts on matrix cell proliferation and hair shaft elongation in cultured human hair follicles

We performed in vitro culture of the healthy human scalp HFs to examine the effect of *Urticadioica L* extracts at the organ level. Hair shaft elongation by treating culturing human HFs with 0.125 mg/ml concentration of *Urticadioica L* water, ethanol and hexane extracts for 12 days was analyzed. HFs treated with *Urticadioica L* ethanol extract significantly enhanced hair shaft elongation after 6, 9, and 12 days, compared to the vehicle-treated controls. As well, *Urticadioica L* hexane extract grew longer than HFs treated with vehicle (Figure 2A). *Urticadioica L* ethanol extract reduced catagen transition compared to the vehicle control and other extracts (Figure 2B).

To analyze the proliferation of human HFs matrix cells, immunofluorescence staining was performed for Ki-67 (a proliferation marker) after HFs were cultured with 0.125 mg/ml concentration of *Urticadioica L* water, ethanol and hexane extracts for 3 days. The number of Ki-67+ matrix keratinocytes were counted and normalized by using the number of DAPI positive cells. We found that treatment with *Urticadioica L* ethanol extract (0.125 mg/ml) significantly increased Ki-67+ matrix keratinocytes than vehicle control (Figure 3A and B).

Discussion

The hair follicle (HF) is composed of epidermal (epithelial) and dermal (mesenchymal) compartments and their interaction has an important role in the morphogenesis of hair, growth of the hair follicle, and the hair cycle [23]. The dermal papilla, composed of specialized fibroblasts located in the bulb of the hair follicle, contributes to the control of hair growth and the hair cycle [21].

Single follicle organ culture method, which is an ex vivo model for the study of freshly isolated human scalp HFs under serum-free conditions developed by Philpott et al, [22]. This

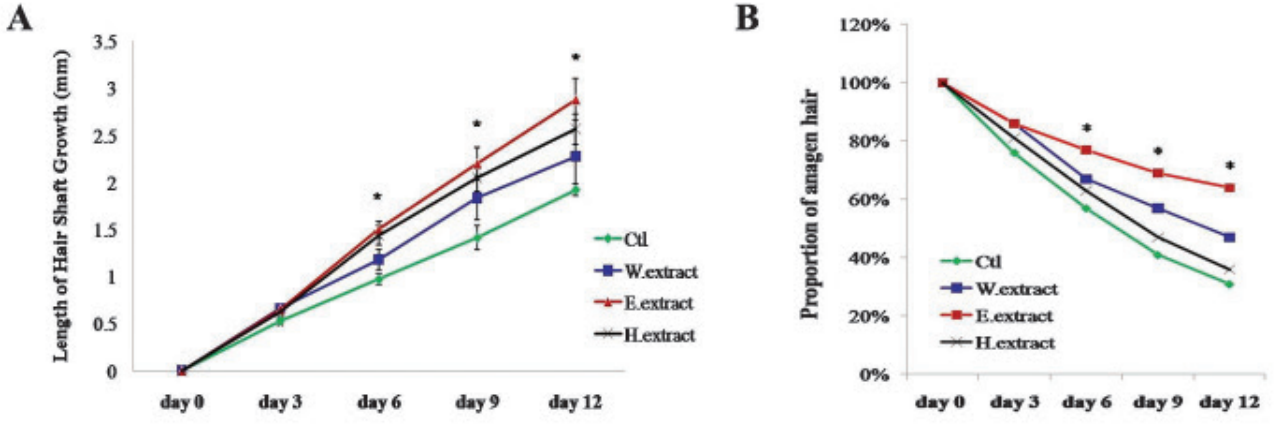

Figure 2. The effect of *Urticadioica L* extracts onhair shaft elongation and catagen transition in ex vivo hair follicle organ culture. HF's cultured with 0.125 mg/ml concentration of *Urticadioica L* water, ethanol and hexane extracts for 12 days (n = 7). (A) The effect of E.extract of *Urticadioica L* on hair shaft elongation and (B) The effect of E.extract of *Urticadioica L* oncatagen transition as compared to the vehicle control. The results were expressed as mean ± standard error. * p ≤ 0.05, vs. the control group. Ctl: Control, W.extract: Water extract of *Urticadioica L*, E.extract: Ethanol extract of *Urticadioica L*, H.extract: Hexane extract of *Urticadioica L*.

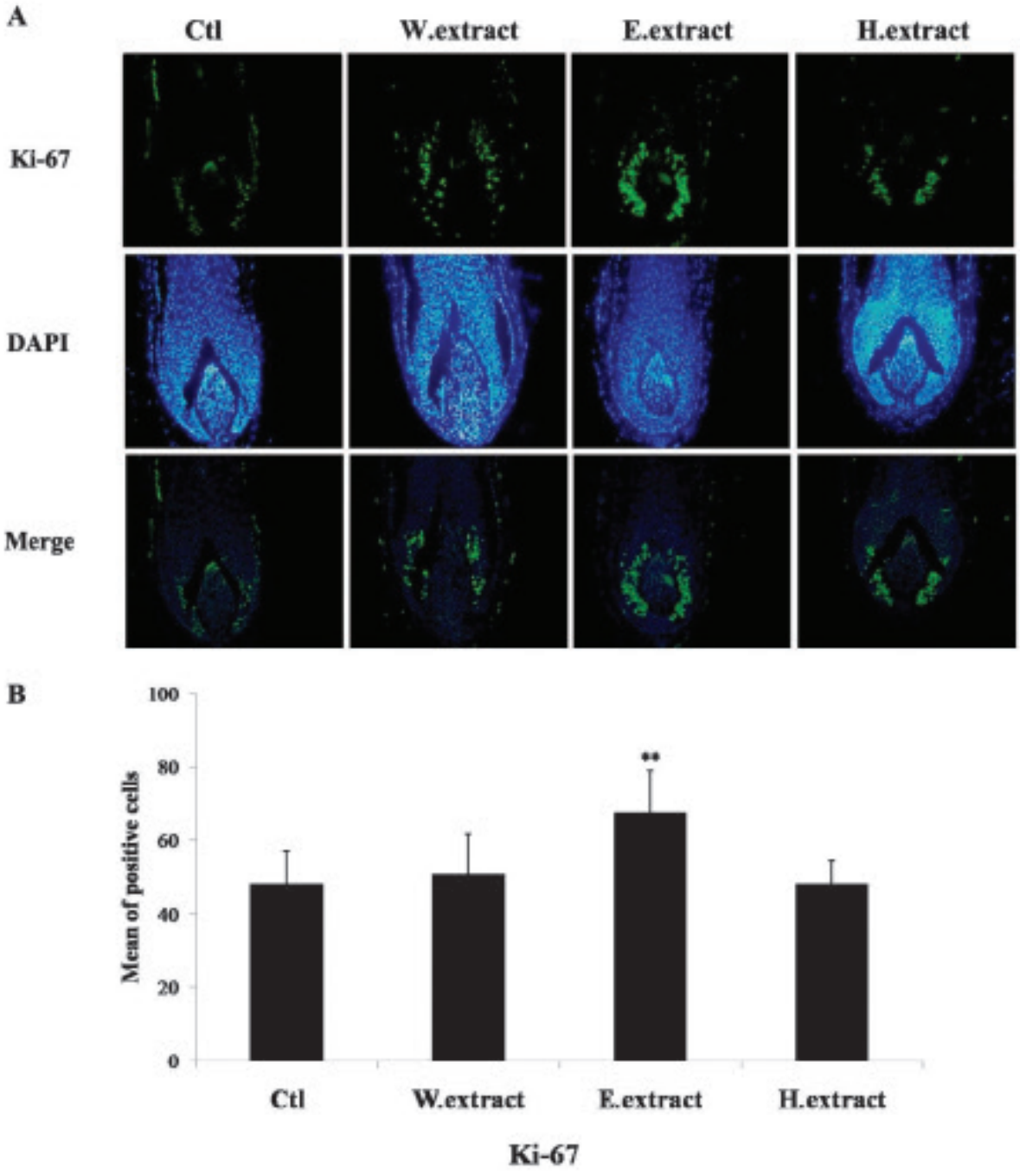

Figure 3. The proliferation of matrix keratinocytes in *Urticadioica L* ethanol extract treated HFs. (A) Human HFs were cultured with the vehicle or water, ethanol and hexane extracts (0.125 mg/ml) for 3 days, and then subjected to immunofluorescence staining, to examine proliferation in the hair matrix keratinocytes, with Ki-67 (proliferation, green fluorescence), and 4',6-diamidino-2-phenylindole (DAPI, blue fluorescence) to counterstain the nuclei (X200). (B) For quantitative analysis, Ki67+ cells were counted and normalized to DAPI-stained cells. E.extract of *Urticadioica L* increased proliferation in the hair matrix keratinocytes. The results were expressed as mean ± standard error. * $p \leq 0.05$, **$p \leq 0.01$, vs. the control group. Ctl: Control, W.extract: Water extract of *Urticadioica L*, E.extract: Ethanol extract of *Urticadioica L*, H.extract: Hexane extract of *Urticadioica L*.

method, not only compares the single hair shaft elongation and hair cycle change within the study groups and also has the advantage using immunohistochemical and immunofluorescence staining evaluate the desired markers related proliferation and differentiation [24].

Ki-67 immunoreactivity (IR) is a useful tool to obtain information on cell proliferation in situ, with the significant advantage that it can be used on both cryo and paraffin embedded sections [25]. The changes in Ki-67 IR during the hair cycle transition are well demonstrated. During anagen VI, Ki-67 IR is most prominent in hair matrix keratinocytes and the onset of catagen is marked by the reduction in the percentage of Ki-67+ matrix keratinocytes, and no Ki-67+ keratinocytes present in late catagen [26-28]. Therefore, in our study we also hair shaft elongation and examined Ki-67 IR by immunofluorescence staining.

Urticadioica L elaborates different classes of organic compounds of medicinal importance including phytosterols, saponins, flavanoids, tannins, sterols, fatty acids, carotenoids, chlorophylls, proteins, amino acids, macro, micro-elements and vitamins [5].

In our study, we confirmed that *Urticadioica L* ethanol extracts enhanced hair shaft elongation, reduced catagen transition and significantly increased the Ki-67+ matrix keratinocytes compared with the vehicle control and water and hexane extracts.

Previous research has shown the *Urticadioica L* chemical components which are mainly soluble in ethanol are flavonoid, polyphenol, lectins, sterol, lignans, beta carotene, keton, palmitic and steric acid, oleic acid, etc [29]. Polyphenols and some flavonoids has been reported to hair growth promoting effect by the proliferation of hDPCs with ROS scavenging and increasing of growth factors IGF-1 and VEGF, also confirmed hair re-growth promotion in mice [30-32].

Our study has some limitation. First, we used only whole plants and single proliferation marker Ki67. Using additional proliferation marker could make our study more effective. Second, our sample size was small.

Further researches are required for finding the effective molecules or compounds and related mechanism.

We conclude above mentioned components such as polyphenols and flavonoids which are soluble in ethanol of *Urticadioica L* may promoteon hair growth.

Conflict of Interest

The authors state no conflict of interest.

Acknowledgement

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Research Article

USING HAIR GROWTH ACTIVITY, PHYSICAL STABILITY, AND SAFETY TESTS TO STUDY HAIR TONICS CONTAINING ETHANOL EXTRACT OF LICORICE (*GLYCYRRHIZA GLABRA* LINN.)

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ABSTRACT

Objective: The purpose of this study was to determine the safety, physical stability, and hair growth activity of ethanol extract of licorice.

Methods: In this study, 2.5%, 5%, and 10% licorice extract was formulated into a hair tonic as a tonic is easier to use and is not sticky like a semisolid dosage. The hair growth activity test was conducted by rubbing the hair tonic preparations on rabbit's backs; subsequently, the hair length, hair thickness, hair weight, and hair density were measured. Quantitative analysis of glycyrrhizic acid from the licorice ethanol extract with a ultraviolet spectrophotometer showed a level of about 156.65 mg/g or 15.665%. The physical stability test was performed on samples of the tonic stored at low (4±2°C), room (25±2°C), and high (40±2°C) temperature, and a cycling test was also performed. The safety test was performed using an eye irritation test that employed the Hen's egg test–chorioallantoic membrane (HET-CAM) method and a skin irritation test that employed the patch test method.

Results: The hair tonics containing 5% and 10% licorice extract had an equivalent activity of hair growth and even better than the positive control containing 2% minoxidil. The physical stability test showed that the licorice extract hair tonic has good physical stability. The results of the safety test showed no skin irritation, whereas the HET-CAM test showed that the hair tonic containing licorice extract showed mild eye irritation.

Conclusions: Licorice ethanol extract hair tonic solutions in concentrations of 2.5%, 5%, and 10% had hair growth activity similar to that of the positive control (minoxidil). They have a good physical and chemical stability, also safe for topical use, except the 2.5% licorice ethanol extract hair tonic solution which caused mild eye irritation.

Keywords: Licorice, Hair growth activity, Glycyrrhizic acid, Mild irritation.

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INTRODUCTION

Hair protects against environmental factors, such as harsh temperatures and ultraviolet (UV) light. Similar to how eyelashes protect the eye from dust and nose hair protects the nose by filtering inhaled hair, hair also protects the scalp against irritants. Hair also has a role in perspiration and temperature regulation and has sensitive tactile senses [1]. Hair has a growth and loss cycle that differs between each strand [2]. Although hair loss is part of the natural hair cycle, an increase in the quantity and frequency of hair loss results in balding. This increased quantity and frequency of hair loss usually stem from stress, food consumption, hormonal disturbances, and the side effects of medications.

The use of shampoo and conditioner is usually not enough as a hair treatment to counter hair loss because hair is a vital cell that needs high care and maintenance to stay healthy. Therefore, hair tonics can be used for hair treatment [3]. Hair tonics are used to thicken hair or to stimulate hair growth in people experiencing balding or hair loss [4]. In general, hair tonics contain irritants, such as alcohol, camphor, capsicum, cantharidin, cinnamon, garlic, vitamin, ginseng, or nicotinic acid [3]. Currently, most Indonesians choose to use herbal products due to their minimal side effects compared with synthetic products, such as minoxidil, which has side effects such as scalp sensitivity [5].

Licorice has been used in Ayurveda and Chinese medicine for its anti-hair loss effect and its ability to restore dry hair. Its main components, glyceric acid and its derivatives, are suspected to produce an anti-hair loss effect by inhibiting the production of dihydrotestosterone (DHT) through the suppression of 5-alpha-reductase enzyme activity, which subsequently increases hair growth [6-8]. A study by Khalaf *et al.* (2010) on methanol extract from licorice showed that there were eight components with phytoestrogen activity (seven isoflavone components and one coumestan component): Daidzein, daidzin, genistein, glycitein, formononetin, ononin, and coumestrol. The phytoestrogen components contained in licorice (isoflavone, daidzin, genistein, and glycitein) act as active components in hair loss prevention by blocking the production of DHT (a DHT blocker) and a β-sitosterol compound, which is a hair regrowth promoter [9]. Glabridin and glabrene are flavonoids contained in licorice that is suspected to have estrogen-like activity. One study proved that petroleum ether extracted from licorice roots had the potential to act as an agent to promote hair growth in female white rats (*Rattus norvegicus*) [10].

The glyceric acid compound and its derivatives and phytoestrogen in licorice have been suspected to promote hair growth. However, a study on the activity of the compounds derived from licorice on hair growth has not been conducted. This study was expected to produce raw materials for a hair growth product with minimum side effects. The study involved experimentation and observation of the hair growth of New Zealand rabbits. This study also uses a stability and safety test using the patch test and Hen's egg test–chorioallantoic membrane (HET-CAM).

MATERIALS AND METHODS

Materials

This study used the following materials: Licorice extract (PT Tri Rahardja Javaplant, Karanganyar, Indonesia), 2% minoxidil (Regrou®, obtained from PT Surya Dermato Medica Laboratories, Surabaya, Indonesia), 96% ethanol (PT Brataco, Jakarta, Indonesia), distilled water, propylene glycol (PEG) (PT Brataco, Jakarta, Indonesia), Microcare®, PEG-40 hydrogenated castor oil and water, BHT (PT Brataco, Jakarta, Indonesia), 2N hydrochloride acid (HCl), Veet® cream (PT Reckitt Benckiser, Bogor, Indonesia), Lieberman-Burchard reagent, Mayer reagent, Dragendorf reagent, 50% methanol, magnesium (Mg), dense HCl, Chloroform ($CHCl_3$), sulfuric acid (H_2SO_4)p.a., sodium hydroxide (NaOH), acetate anhydride acid, Iron(III) chloride ($FeCl_3$) solution, 0.4 M lead (II) acetate, isopropanol, anhydrous sodium sulfate, molish LP, and standard glyceric acid (Sigma Aldrich, Nucleos, Singapore).

Production of licorice ethanol extract
First, 5 L of ethanol was added to 500 g of dry licorice extract powder and macerated at room temperature for approximately 48 hrs. Then, after the filtrate and residue were separated, the filtrate was condensed using a rotary evaporator at 60°C or lower and was macerated again for 12 hrs by adding 3 L of ethanol and filtering it. This procedure was repeated thrice to obtain a combination filtrate. Next, 1 L of 70% ethanol was added and was maintained at a cool temperature for at least 48 hrs. The residue and filtrate were separated through filtration. The obtained filtrate was condensed to produce licorice ethanol extract. This procedure was expected to attract 3-4% or more glyceric acid as its main active component [11].

Determination of the glyceric acid concentration
A standard solution was produced by dissolving 10 mg of standard glyceric acid in 100 mL of distilled water to obtain a master solution of 100 ppm. The master solution was diluted into solutions of differing concentrations, and the maximum wavelength and absorption scores of the solutions were calculated using a UV spectrophotometer. A calibration curve was made from the absorption scores.

A licorice extract sample solution was produced by dissolving 1 g of the licorice extract sample in 100 mL of distilled water to obtain a master solution of 1000 ppm. The solution was then diluted, and the absorption scores were calculated using a UV spectrophotometer with the maximum wavelength that had been previously determined based on the calculations of the glyceric acid standard solution. The obtained absorption scores were added to a formula on the calibration curve to obtain the concentration of glyceric acid contained in the licorice extract sample.

Production of the hair tonic
The formula was divided into three groups of differing licorice extract concentrations: 2.5%, 5%, and 10%. The materials were weighed according to the formula. In the oil phase, 1.2% PEG-40 hydrogenated castor oil was mixed with a little ethanol. In another container, 0.5% BHT, 0.1% menthol, and 0.5% microcare were dissolved with ethanol, PEG was added little by little, and the solution stirred until it was thoroughly mixed. The oil phase, the licorice extract solution, and the water phase were mixed thoroughly, and the volume of the mixed solution was increased by adding distilled water.

Evaluation of the hair tonic

Organoleptic examination

A visual examination of the odor, shape, and color of the produced hair tonic was conducted.

Viscosity test
To test the hair tonic's viscosity, 250 ml of the tonic was poured into a Becker glass, and a spindle 1 was inserted to a previously determined limit. Measurements were conducted with a Brookfield viscometer at speeds of 5, 10, 20, 50, and 100 rpm. The obtained data were plotted to sliding pressure (dyne/cm^2) and sliding speed.

pH measurement
The measurement of the pH of the solutions was conducted using a pH meter, and the pH was measured once every 1 weeks for 12 weeks.

Testing the physical stability of the hair tonic
The physical stability of the hair tonic tested in this study included its odor, color, homogeneity, pH, and viscosity. The hair tonic's physical stability was evaluated at room (25±2°C), warm (40±2°C), and cold (4±2°C) temperatures every 2 weeks for 12 weeks. A cycling test was also conducted (six cycles) [12].

Hair growth activity test
This study used male New Zealand rabbits from Balai Penelitian Hewan Ternak, Ciawi, that weighed 2-2.5 kg. Tanaka *et al.* modification was used to measure hair growth [13]. The hairs on the rabbits' backs were divided into 5 ×10 cm test areas. After the rabbits' backs were shaved, six squares were made: Three squares on the left side and three squares on the right side of the rabbits' backs with measurements of 2.2 cm × 2.5 each. Every test area was marked with a marker. Each test involved the application of a different treatment. The rabbits were left for 24 hrs before the experiment. 1 mL of each sample was applied twice a day for 6 weeks. The first day of the application was considered day 0. Hair growth was measured based on hair length, density, weight, and thickness.

Safety test
The safety test was conducted using the HET-CAM as an alternative to the mucous membrane safety test method (for eye irritation) to avoid the use of animals. The safety test was conducted by 20 volunteers who were 17-30 years old [14]. The volunteers were chosen by inclusion and exclusion criteria for participation in the study.

RESULTS

Determination of the concentration of glyceric acid in licorice ethanol extract
The determination of glyceric acid in licorice ethanol extract was done using a Hitachi U-2910 UV spectrophotometer with glyceric acid from Sigma-Aldrich as a standard. Distilled water was used as a solvent because of its dissolving properties and its ready availability [15].

The standard glyceric acid solution was diluted into concentrations of 10 ppm, 20 ppm, 30 ppm, 40 ppm, and 50 ppm. The maximum wavelength measurement, which was 256.5 nm, was then obtained with the UV spectrophotometer, and the absorption scores of each concentration were determined to be 0.140, 0.289, 0.433, 0.606, and 0.729, respectively. The obtained absorption scores were plotted with the concentration to obtain a calibration curve formula.

The licorice extracts weighed 1 g, and the sample concentration of the standard was 313.3 µg/ml. Finally, the glyceric acid concentration in the licorice extract, 156.65 mg/g or 15.665%, was obtained.

Hair growth activity test

Hair length parameter

Hair length was measured every week from the 1st week of application to the 6th week. The measurement was done by randomly taking 10 strands of hair from each test area and measuring their length using a digital caliper. The mean hair length of each week's samples is shown in Table 1.

The results of the normality distribution test (Shapiro–Wilk test) and the homogeneity test (Levene test) on the mean length of the hair in each test group each week showed that the distribution of data was normal and homogeneous every week (6 weeks in total). Therefore, analysis of variance (ANOVA) testing was performed. The results of the ANOVA test showed that there was no significant difference between the test groups in weeks 4, 5, and 6. To find whether there was a significant difference between the test groups, a least significant difference (LSD) test was conducted. In week 2, the normality distribution test showed that the data were normally distributed. However, the homogeneity test showed that the data were not homogenous, so a non-parametric data analysis was conducted using the Kruskal–Wallis test.

Hair weight parameter
Hair weight was measured on the 42nd day (the end of week 6) by pulling all the hair from a 1×1-cm area in each test area and weighing the hair. The weights of the samples are shown in Table 2.

The mean weights of the normal control, negative control, positive control, formula 1, formula 2, and formula 3 groups were 93.7±8.88 mg, 97.26±6.15 mg, 115.3±15.00 mg, 114.56±7.34 mg, 130.66±11.38 mg, and 118.52±8.50 mg, respectively. Statistical analysis was needed to assess whether there was a difference between the weights of

Table 1: The mean length of rabbit hair each week

Test group	Treatment	Mean hair length (mm) ± SD					
		M1	M2	M3	M4	M5	M6
Group 1	Normal control	5.13±1.42	5.79±2.26	10.18±3.26	13.70±1.86	16.26±1.92	16.37±5.69
Group 2	Negative control	7.82±4.07	9.25±6.56	12.98±5.71	17.23±5.61	19.45±3.49	19.87±7.92
Group 3	Positive control (2% minoxidil)	9.23±3.84	10.18±7.27	13.57±6.06	19.22±5.20*.**	21.45±4.81*.**	22.80±6.46
Group 4	Formula 1 (2.5% licorice)	5.81±4.51	7.45±3.73	11.64±4.17	15.27±0.93	20.40±5.01	16.24±4.50
Group 5	Formula 2 (5% licorice)	6.78±4.28	8.86±4.73	12.80±5.35	18.57±2.98	21.70±5.31	22.78±5.66
Group 6	Formula 3 (10% licorice)	8.85±5.27	11.30±8.63	16.67±6.90	21.66±5.02*	24.45±4.86*	29.18±8.97*.**

SD: Standard deviation. *Significantly different (p<0.05) from the normal control, **significantly different (p<0.05) from the negative control

hair. Based on a normality distribution test (Shapiro–Wilk test) and a homogeneity test (Levene test) of the mean weights of the hair, the data distribution was normal and homogeneous. The results of the LSD test showed that the normal and negative controls did not show a significant difference, meaning that the activity of the negative control on the weight of the hair was similar to that of the normal control. The three formulas showed a significant difference when they were compared with the negative control. However, formulas 1 and 3 were not significantly different from the positive control, whereas formula 2 differed significantly from the positive control.

Hair density parameter

Hair density was measured by counting the amount of hair per cm^2 area on the 42nd day (the end of week 6). The results are shown in Table 3.

The mean amounts of hair of the normal control, negative control, positive control, formulas 1-3 groups were 1202±49, 1300±102, 1502±82, 1457±263, 1621±286 and 1322±146, respectively. Statistical analysis was needed to assess whether there was a difference between the hair densities. The normality distribution test (Shapiro–Wilk Test) and the homogeneity test (Levene Test) on the mean amounts of hair showed that the data distribution was normal and homogeneous. The LSD test showed that the normal control and negative control did not have a significant difference, meaning that the normal control and negative control resulted in similar hair densities. When the positive control was compared with the results of formulas 1-3, the three formulas did not differ significantly from the positive control. This result shows that formulas 1-3 showed hair densities similar to that of the positive control. However, formula 2 differed significantly from formula 3.

Parameter of hair thickness

Hair thickness was assessed by measuring the thickness of hair strands (diameter) at week 1 and week 6 using a scanning electron microscope. The results are shown in Table 4. The diameters of the hair strands of the normal control, negative control, positive control, formulas 1-3 groups in week 1 were 11.00±0.96 µm, 16.75±0.73 µm, 64.63±1.57 µm, 67.32±4.64 µm, 31.80±3.14 µm, and 71.72±3.39 µm, respectively. The diameters of the hair strands in week 6 were 47.83±9.22 µm, 109.72±3.87 µm, 119.12±5.88 µm, 90.35±5.06 µm, 60.19±7.61 µm, and 112.65±.24 µm, respectively.

Evaluation of the hair tonic

Measurement of viscosity and rheology

The viscosity of the hair tonic was measured at weeks 0 and 12, and the solutions were stored at temperatures of 4±2°C, 25±2°C, and 40±2°C. The obtained data were then plotted with sliding pressure (dyne/cm^2) and sliding speed (rpm) until a flow property (rheology) was obtained. The measurement of the viscosity at week 12 showed that there was no change in the viscosity of the hair tonic. The rheogram showed that the flow type from the hair tonic was Newton.

Physical stability test of the hair tonic

The three hair tonic formulas were stable at the low (4±2°C), room (25±2°C), and high (40±2°C) temperatures. The cycling test of the hair tonic formulas resulted in neither a physical change nor a separation of phases.

Table 2: The mean weight of rabbit hair in week 6

Test group	Treatment	Mean hair weight (mg)±SD
Group 1	Normal control	93.7±8.88
Group 2	Negative control	97.26±6.15
Group 3	Positive control	115.3±15.00*.**
Group 4	Formula 1 (2.5% licorice extract)	114.56±7.34*.**
Group 5	Formula 2 (5% licorice extract)	130.66±11.38*.**
Group 6	Formula 3 (10% licorice extract)	118.52±8.50*.**

SD: Standard deviation. *Significantly different (p<0.05) from the normal control, **significantly different (p<0.05) from the negative control

Table 3: The mean amount of hair at week 6

Test group	Treatment	Mean amount of hair±SD
Group 1	Normal control	1202±49
Group 2	Negative control	1300±102
Group 3	Positive control	1502±82*.**
Group 4	Formula 1 (2.5% licorice extract)	1457±263*.**
Group 5	Formula 2 (5% licorice extract)	1621±286*.**
Group 6	Formula 3 (10% licorice extract)	1322±146

SD: Standard deviation. *Significantly different (p<0.05) from the normal control, **significantly different (p<0.05) from the negative control

Table 4: The mean hair diameter at week 6

Test group	Treatment	Mean hair diameter (µm)±SD	
		Week 1	Week 6
Group 1	Normal control	11.00±0.96	47.83±9.22
Group 2	Negative control	16.75±0.73	109.72±3.87
Group 3	Positive control	64.63±1.57	119.12±5.88
Group 4	Formula 1 (2.5% licorice extract)	67.32±4.64	90.35±5.06
Group 5	Formula 2 (5% licorice extract)	31.80±3.14	60.19±7.61
Group 6	Formula 3 (10% licorice extract)	71.72±3.39	112.65±6.24

Measurement of pH

The pH of the three hair tonic formulas was stable at around 5.6-5.9.

Safety test

HET chloriallantoic

The HET-CAM can observe hemorrhaging, lysis, and coagulation. Hemorrhaging is defined as bleeding or blood leaving the circulatory system. Lysis is the absence or the disappearance of small, fine arteries after a hemorrhage. Coagulation, which can happen intra- or extra-cellular, is the clotting of blood, which can appear a dark stain and which may happen as a reaction of opacifying or turbidity of the CAM.

The results of the HET-CAM showed that, during the 5 minutes of observation time, a lysis and coagulation reaction did not occur for the three test groups. According to the irritation score obtained, the negative control was non-irritative, whereas the positive control and the 2.5% licorice ethanol extract were irritative. The irritative properties contained in the hair tonic solution stemmed from the properties of PEG and menthol, which might be slightly irritative to the eye.

Patch test

The safety test for skin irritation was conducted with a single closed patch test for 48 hrs and was attended by 20 volunteers who filled the inclusion criteria. The solutions used for the irritation safety test were a basic hair tonic and a 2.5% licorice ethanol extract hair tonic solution. Before treatment, the volunteers signed an informed consent form. A dose of 0.1 mL of the two solutions of hair tonic was applied to the upper back of each volunteer, which was observed after 30 minutes, 24 hrs, and 48 hrs to assess whether there were any erythema or edema reactions.

The results of the primary irritation index (PII) scoring for the basic hair tonic and 10% licorice ethanol extract showed that the PII was 0.125, so the irritation was classified as insignificant (0-0.4). Based on the results, the basic hair tonic and the 10% licorice ethanol extract were safe for topical use.

DISCUSSION

Based on the hair growth activity test, formula 1 (2.5% licorice ethanol extract), formula 2 (5% licorice ethanol extract), and formula 3 (10% licorice ethanol extract) had hair growth activity as compared to the activity of positive control, minoxidil. However, formulas 2 and 3 showed significantly better hair growth activity than that of the positive control. For hair weight, formulas 1 and 3 had results similar to that of the positive control. However, formula 2 (5% licorice ethanol extract) had better results than the positive control.

Based on the mean amount of hair, formula 2 had the best results of the three formulas. The bigger and rougher texture of the hair strands seen in subjects to which formulas 1 and 3 were applied might cause the amount of hair per cm^2 to be less than that seen in formula 2. The hair density parameter has shown that the positive control and the three formulas stimulate hair follicles through the growth of new hair. Based on the graphic seen in attachment 49 and Table 4, an increase in the diameter (thickness) of each hair strand between week 1 and week 6 was evident. The difference in hair diameter between the test groups was also evident. The positive control group had the largest hair strands according to diameter, followed by the formula 3, formula 1, negative control, and normal control groups. Based on the four hair growth parameters, the three licorice ethanol extract hair tonic formulas had activities similar to the positive control. However, formulas 2 and 3 had significantly better hair growth activity than the positive control.

The mechanism of action or chemical compound responsible for the hair growth activity of licorice ethanol extract might be glyceric acid and its derivatives and glycyrrhizic acid and its derivatives, which are suspected to have anti-hair loss effects resulting from the inhibition of the production of DHT through suppression of 5-alpha-reductase activity [8,16,17]. According to phytochemistry identification, licorice ethanol extract contains flavonoid and terpenoid, which can increase hair growth by strengthening the capillary walls of the small blood vessels that supply the hair follicles and by increasing blood flow to hair follicles [18]. Alkaloids can enlarge hair strands, increasing the food supply and nutrition to hair [19]. Saponin is a chemical compound mostly found in licorice extracts that can stimulate hair growth in alopecia (balding) cases caused by hormones or genetics. Saponin can form bubbles, meaning it can clean dirt from the skin and can also act as a counterirritant. Saponin can increase peripheral blood circulation, thereby increasing hair growth [20]. Moreover, the phytoestrogen compounds (isoflavone daidzin, genistein, and glycitein) contained in licorice are the active ingredients in preventing hair loss by blocking the formation of DHT (a DHT blocker), and the β-sitosterol compound acts as a hair regrowth promoter [9].

Based on the results obtained from this research, the licorice extract hair tonic accelerated the active phase of hair growth, prolonged the active phase, and stimulated hair follicles, leading to larger strands of rabbit hair. Based on the stability test, which studied the solutions' color, odor, and pH stability, the hair tonic solutions showed good physical stability when stored at low (4±2°C), room (25±2°C), and high (40±2°C) temperatures. The safety test, which examined the skin irritation of the hair tonic solutions containing 10% licorice ethanol extract on 20 volunteers, showed that the solutions were safe for skin. However, the eye irritation test showed that the 2.5% licorice ethanol extract hair tonic solutions were irritative to the eyes.

CONCLUSION

Licorice ethanol extract hair tonic solutions in concentrations of 2.5%, 5%, and 10% had hair growth activity similar to that of the positive control (minoxidil) according to the hair length and hair thickness parameters. The 5% and 10% licorice ethanol extract hair tonic solutions had better hair weight and density than that of the positive control. However, the 5% hair tonic solution had worse hair density results than the positive control. The concentration of glycyrrhizic acid contained in the licorice ethanol extract used as an active ingredient in the hair tonic solutions was 1456.65 mg/g or 5.665%. The hair tonic solutions containing 2.5%, 5%, and 10% licorice ethanol extract showed physical and chemical stability when stored at low (4±2 °C), room (28±2°C), and high (40±2°C) temperatures for 12 weeks. The HET-CAM and patch safety test showed that the 10% licorice ethanol extract hair tonic solution was safe for topical use. However, the 2.5% licorice ethanol extract hair tonic solution was irritative to the eye.

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Inhibition of 5α-Reductase, IL-6 Secretion, and Oxidation Process of *Equisetum debile* Roxb. ex Vaucher Extract as Functional Food and Nutraceuticals Ingredients

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Abstract: This study aims to investigate the biological activities related to hair loss of *Equisetum debile* extracts, including 5α-reductase inhibition, interleukin-6 (IL-6) secretion reduction, and anti-oxidation. *E. debile* extracts were obtained by maceration in various solvents. Crude extract (CE) was obtained by maceration in 95% ethanol. Chlorophyll-free extract (CF) was the CE which of the chlorophyll has been removed by electrocoagulation. Hexane extract (HE), ethyl acetate extract (EA), and ethanolic extract (ET) were fraction extracts obtained from maceration in hexane, ethyl acetate, and 95% ethanol, respectively. The extracts were investigated for inhibitory activity against 5α-reductase and IL-6 secretion. Total phenolic contents (TPC) were investigated and antioxidant activities were determined by means of 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2′-diphenyl-1-picrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP) assays. The inhibition of lipid peroxidation was determined by the ferric thiocyanate method. The cytotoxicity of the extracts on dermal papilla cells and irritation test by hen's egg test chorioallantoic membrane assay were also investigated. All extracts could inhibit 5α-reductase and decrease IL-6 secretion in lipopolysaccharide-stimulated macrophage. The antioxidant activity of *E. debile* extracts was directly related to their TPC. ET which contained the highest TPC (68.8 ± 6.7 mg GA/g) showed the highest equivalent concentration (EC_1) of 289.1 ± 26.4 mM $FeSO_4$/g, TEAC of 156.6 ± 34.6 mM Trolox/g, and 20.0 ± 6.0% DPPH inhibition. However, EA exhibited the highest inhibition against lipid peroxidation (57.2 ± 0.4%). In addition, EA showed no cytotoxicity on dermal papilla cell line and no irritation on chorioallantoic membrane of hen's eggs. In conclusion, EA was suggested as the most attractive ingredients for functional food and nutraceuticals because of the high inhibitory activity against 5α-reductase, IL-6 secretion, and lipid peroxidation inhibition.

Keywords: *Equisetum debile*; chorioallantoic membrane assay; 5α-reductase; interleukin-6; lipid peroxidation

1. Introduction

Androgenetic alopecia (common baldness) is recognized increasingly as a physically and psychologically harmful medical condition in which the pathogenesis is far from complete elucidation [1]. Generally, it is caused by aberrant hair follicle cycling and miniaturization of hair follicles, which depends on the presence of the androgenic hormones, including testosterone and dihydrotestosterone (DHT) [1–3]. In the human body, DHT is an enzymatic product converted from testosterone by the role of 5α-reductase. Since DHT is more active than testosterone, blocking the conversion of testosterone to DHT would reduce the androgenic effect. Thus, anti-androgenic drugs, which inhibit 5α-reductase or bind between DHT and androgen receptor, may be useful for protection from androgenetic alopecia [4].

The hair follicle is a cutaneous organ that remodels itself during cyclical periods of active hair growth (anagen), apoptosis-driven involution (catagen), hair shedding (exogen), and relative rest (telogen) [5]. Beside the androgenic hormones, the miniaturization of hair follicle might be explained by a shorter anagen cycle [6]. The hair follicle size and the duration of anagen phase indicate the length and the size of hair shaft, respectively [3]. The normal duration of anagen is around 2–6 years on average, and then it will turn to a short transitory period of catagen, in which the follicle will undergo apoptosis [7]. Therefore, one of the goals for treating androgenetic alopecia is to prolong the anagen [3].

Several cytokines are involved in the hair growth cycle, including interleukin-6 (IL-6). It has been reported that IL-6 is much more upregulated in balding dermal papilla cells comparing to non-balding dermal papilla cells [8]. Moreover, recombinant human IL-6 could inhibit the hair shaft elongation and suppressed proliferation of matrix cells in cultured human hair follicles, which lead to the premature onset of catagen during anagen phase [8]. Therefore, IL-6 might be a crucial inducer of hair loss in androgenetic alopecia.

Free radicals, which are highly reactive molecules with unpaired electrons that can directly damage various cellular components, might be another factor affecting the hair loss in androgenetic alopecia. Since the oxidation process leads to progressive damage of cellular structures, the ageing phenotype of hair manifests as a decrease in hair production [9]. It has been reported that lipid peroxides on hair follicles led to the early onset of the catagen in murine hair cycles [10]. Therefore, antioxidant compounds might be used to prolong the anagen phase and reduce the hair loss.

Nowadays, the potassium channel opener minoxidil and the dihydrotestosterone synthesis inhibitor finasteride have been used for the treatment of androgenetic alopecia [1]. However, these chemicals might cause some adverse reactions. Some patients using minoxidil encounter with fast or irregular heartbeat, rapid weight gain, bloating, flushing, redness of skin, swelling of feet or lower legs, etc., whereas, finasteride can cause cold sweats, confusion, dizziness, faintness, loss in sexual ability, etc. Therefore, there is an interest in finding new compounds for the treatment of androgenetic alopecia, especially from natural sources.

Equisetum Linn. is one of the most oldest living genera of vascular plant which comprises about twenty-five species [11]. The most investigated species was *Equisetum arvense* L. which has been widely used in traditional medicines for the treatment of hair loss [12]. The mixture of *E. arvense* shoot extract and mustard oil has been used as a hair tonic [13], whereas the mixture of *E. arvense* extract and other herbal extracts, such as bilberry, *Ginkgo biloba*, and saw palmetto, has been used as supplements for maintain a healthy hair follicle [14]. Additionally, the superior reduction of telogen effluvium duration in patients treated with herbal drug containing *E. arvense* extract (seven weeks) comparing to minoxidil solution (seven weeks) has also been reported [15].

Beside *E. arvense*, there are several species of *Equisetum* that have not been well studied. *Equisetum debile* Roxb. ex Vaucher (horsetail), a plant in the family Equisetaceae, is native to tropical South Asia [16]. It is widely distributed throughout the highland area of Thailand, especially 500 m above sea level. It has been used in folklore remedies by local highland people as diuretic, wound treatment, muscle relaxant, hair growth stimulant, and as anti-hair loss treatment. The decoction of *E. debile* has also been used for the hair strengthening [17]. Since *E. debile* and *E. arvense* are in the same family of Equisetaceae, they might have similar phytochemical compounds and biological activity. Some biological activities of *E. debile* extracts have been reported, such as antioxidant and antibacterial activity [18]. However, the phytochemical and anti-hair loss activities, such as the inhibitory activity against 5α-reductase and IL-6, have not yet been reported. Therefore, the aims of the present study were to investigate the anti-hair loss activities of fractionated *E. debile* extracts, including in vitro 5α-reductase inhibition, IL-6 secretion reduction, and antioxidant activity. Moreover, the irritation of the extracts on the chorioallantoic membrane of hen's eggs was firstly reported in the present study.

2. Materials and Methods

2.1. Plant Materials

E. debile (horsetail) was collected from the highland area of Chiang Mai, Thailand, in January 2016. It was authenticated by Highland Research and Development Institute and its voucher specimen number 023221 was deposited in the Herbarium of the Faculty of Pharmacy, Chiang Mai University, Thailand. The aerial part of *E. debile* was washed and dried in the oven at a temperature of 40 °C. The dried plant material was ground into powder.

2.2. Chemical Materials

Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH), Folin–Ciocalteu reagent, 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,4,6 tripyridyl-s-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, 2,2′-diphenyl-1-picrylhydrazyl (DPPH), lipopolysaccharide (LPS), linoleic acid, 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) and 3-(4,5-dimethylthiazolyl-2) -2,5-diphenyl tetrazolium bromide (MTT), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium persulfate, potassium dihydrogen phosphate (KH_2PO_4), potassium chloride, sodium carbonate, sodium hydroxide, sodium acetate, sodium chloride, ferric chloride, ferrous sulfate, ferrous chloride, magnesium sulfate, and ammonium thiocyanate were purchased from Fisher Chemicals (Loughborough, UK). Tris base was purchased from Fisher Chem Alert (Fair Lawn, NJ, USA). RPMI-1640, Dulbecco modified eagle medium (DMEM), Penicillin/Streptomycin, L-glutamine, and trypan blue were purchased from Invitrogen™ (Grand Island, NY, USA). Hydrochloric acid and acetic acid were AR grade obtained from Merck (Darmstadt, Germany). Methanol, ethanol, hexane, ethyl acetate, dimethyl sulfoxide (DMSO), dichloromethane were AR grade and were purchased from Labscan (Dublin, Ireland). Sodium dodecyl sulfate (SDS) was purchased from EMD Millipore Corporation (Billerica, MA, USA).

2.3. Plant Extraction

2.3.1. Crude Ethanolic Extraction

The dried plant powder was macerated in 95% ethanol with some agitations for 72 h. The maceration was done triplicately, all the filtrates were pooled together and the solvent was removed under vacuum using rotary evaporator until dryness. The crude ethanolic extract (CE) was obtained and kept at 4 °C until further use.

2.3.2. Chlorophyll-Free Extraction

CE was dissolved in 95% ethanol. Chlorophyll was then removed from the ethanolic solution by electrocoagulation using electrocoagulation reactor with aluminum probes for 3 h. The coagulated chlorophyll was removed by filtration through Whatman No. 1 filter paper. The solvent was then removed under vacuum using rotary evaporator until dryness. The chlorophyll-free extract (CF) was kept at 4 °C until further use.

2.3.3. Fractionated Solvent Extraction

The dried plant powder was macerated in hexane with some agitations for 72 h. The maceration was done in triplicate and all filtrates were pooled together. The solvent was removed under vacuum using rotary evaporator until dryness and the fraction hexane extract (HE) was obtained. The plant residue was then fractionally macerated in ethyl acetate using the same method and the fraction ethyl acetate extract (EA) was obtained. Finally, the plant residue was fractionally macerated again in 95% ethanol using the above method and the fraction ethanolic extract (ET) was obtained. All dried extracts were kept at 4 °C until further use.

2.4. Determination of 5α-Reductase Inhibition

Anti-androgenic activity via the steroid 5α-reductase inhibition mechanism was evaluated using a label-free enzymatic inhibitory assay. The enzymatic activity was determined by analyzing the DHT formation after an enzymatic reaction using liquid chromatography–mass spectrometry (LC-MS) [19].

2.4.1. Enzymatic Preparation

The enzyme homogenate from androgen-dependent prostate cancer cell, LNCaP cells (CRL-1740™ from American Type Culture Collection (ATCC), Manassas, VA, USA), was used as a source of 5α-reductase [20]. LNCaP cells expressing human steroid 5α-reductase were cultured in a 175 cm^2 culture flasks at 37 °C under 5% CO_2 humidified atmosphere. The medium was RPMI-1640 supplemented with 10% (*v/v*) fetal bovine serum and 100 U/mL penicillin G and 100 µg/mL streptomycin (Gibco, Paisley, Scotland). At ≥80% cell confluence, the medium was discarded, the cells washed with Tris-HCl buffer pH 7.4, and then scraped off, centrifuged at 1900× *g* for 10 min. Lysis buffer pH 7.4 was added to the cell pellet to give a cell suspension $\geq 9 \times 10^7$ cells/mL. This was homogenised on ice using a sonication probe with 10 s pulse on, 10 s off for 1 min 40% amplitude (Sonics Vibracell™ VCX130 probe V18, Newtown, CT, USA). After that, glycerol (Invitrogen, Carlsbad, CA, USA) was added to be 20% (*v/v*) and the homogenate stored at −80 °C until use. In this step, glycerol is necessary for enzyme homogenate since it would act as a cryoprotectant to protect damaging protein structure from the ice crystal during the storage under −80 °C. The homogenate protein content was measured using Pierce bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA), according to the manufacturer's instructions and using bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) as standard. The calibration curve of the BSA was plotted against the OD595 in the range of 10–500 µg/mL.

2.4.2. Enzymatic Inhibitory Assay

The test samples were dissolved in DMSO and aliquots of these added to the assay solution. Assays were performed in 96-deep-well plates (Agilent Technologies, Santa Clara, CA, USA) covered by well-cap mats (Thermo Scientific, Waltham, MA, USA). The total volume of enzymatic reaction mixture was 200 µL, composed of test substance, 34.7 µM testosterone and 1 mM NADPH in Tris buffer pH 7.4. The reaction was started by adding 200 µL of homogenate enzyme (75 µg total protein) and incubated at 37 °C for 60 min. The reaction was stopped by adding 300 µL of hydroxylamine (10 mg/mL in 80% (*v/v*) ethanol) and incubating at 60 °C for 60 min for derivatization process. Then, the 96-well plate was centrifuged at 1700× *g* for 10 min using microplate centrifugation, and the supernatants

transferred to another 96-well plate ready for injection into the LC-MS. Two control samples were used which were C1 and C2. Both controls contained the complete reaction mixture as described above but C1 was stopped before enzymatic incubation, whereas, C2 was stopped after 60 min of incubation. In the test sample, 10 µL of *E. debile* extract dissolved in DMSO was added instead of Tris-HCl buffer pH 7.4. However, in the blank, DMSO was used instead of Tris-HCl buffer pH 7.4. The DHT production was measured using LCMS. The extracted ion chromatogram (EIC) of derivatizedDHT (*m/z* $[M + H]^+$, 306.2428), the area under curve was used to calculate enzymatic inhibition:

$$\%\text{Steroid } 5\alpha\text{-reductase inhibition} = [1 - (\text{Sample} - \text{C1})/(\text{C2} - \text{C1})] \times 100 \quad (1)$$

The standard steroid 5α-reductase inhibitor, finasteride (Sigma-Aldrich, St. Louis, MO, USA) was used as positive control (95 ± 2.2% inhibition at 1.5 µg/mL, triplicated).

2.4.3. LC-MS Method for the Measurement of DHT

The Agilent 1260 Infinity Series HPLC system with an auto-sampler accommodating either two 108-vial trays or two 96-well plates (Agilent Technologies, Santa Clara, CA, USA) was used. The analytical reversed phase column was a Phenomenex Luna® C18 (2) (150 mm × 4.6 mm, 5 µm) with a guard column (Phenomenex C18, 4 mm × 3 mm, 5 µm). The HPLC was connected with an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS (Agilent Technologies, Santa Clara, CA, USA), equipped with a dual electrospray ionization (ESI) in positive mode and *m/z* range 100–1200. Nitrogen was the nebulizing gas at 30 psi, and the drying gas (10 L/min; 350 °C). The mobile phase was 0.1% (*v/v*) formic acid in purified water (solvent A) and 0.1% (*v/v*) formic acid in acetonitrile (LC-MS grade, ACI Labscan, Bangkok, Thailand) as solvent B. The gradient program was used as follows; the initial mobile phase was 60% solvent B and 40% solvent A; solvent B was linearly increased up to 80% over 8 min then held constant for 4 min. Each run was followed by a 2 min post-run. The total run-time analysis was therefore 14 min with the column temperature controlled at 35 °C. The flow rate was 0.5 mL min^{-1} and the injection volume was 20 µL. Mass data were analyzed using Agilent Mass Hunter Qualitative Analysis software version B06.00.

2.5. Determination of IL-6 Secretion Inhibition in LPS-Stimulated Macrophages

2.5.1. Macrophage Culture

Lipopolysaccharide (LPS) stimulated RAW 264.7 (mouse macrophage) cells were used to examine the effect of *E. debile* extracts on the inflammatory process. Dexamethasone, a well-known anti-inflammatory drug, was used as a positive control. The cell culture was performed following the method used in the previous study of Mueller et al. with slight modifications [21]. Briefly, RAW 264.7 cells were seeded at a density of 2×10^6 cells per well in DMEM in 24 well plates, and incubated at 37 °C, 5% CO_2 and 90% humidity for 24 h. On the following day, 1 µL of test compound in ethanolic solution were added, and further incubated under the same condition for 2 h. After that LPS was added to a final concentration of 1 µg/mL and further incubated in the same condition for 24 h. On the third day, the media was removed and centrifuged at 13,500× *g* for 10 min to remove cells. Supernatant was aliquoted and analyzed by ELISA. Cells which were not treated with LPS served as a negative control and cells incubated with ethanol and LPS served as a positive control, of which the secreted cytokines was defined as 100%. The IL-6 concentration in the cell supernatants (100 µL) was determined by ELISA according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). All incubation steps were performed at room temperature. The optical density at 450 nm, corrected by the reference wavelength 570 nm, was measured with a Genios Pro microplate reader (Tecan, Crailsheim, Germany).

2.5.2. Determination of the Cell Viability by MTT Assay

Simultaneous with the ELISA, the viability of LPS-stimulated cells was assessed by a MTT assay, based on the mitochondrial-dependent reduction of MTT to formazan. After removing the supernatant for ELISA analysis, MTT was added to the cells, and the cells were incubated for at 37 °C, 5% CO_2 and 90% humidity for 2 h. The supernatant was then removed, and the cells were lysed with lysis buffer (10% (*w/v*) SDS in 0.01 N HCl). The optical density at 570 nm, corrected by the reference wavelength 690 nm, was measured using a Genios Pro microplate reader.

2.5.3. Calculation of the IL-6 Secretion

The calculated concentrations of cytokines were normalized to MTT values to reduce any variation from differences in cell density. For a positive control, cells were treated with only LPS and the resulting amount of secreted cytokines was defined as 100%. The results from the experimental compounds were then calculated as a percent of this value. The entire inflammation assay, starting with cell seeding and LPS-induction, was performed in triplicate in three time independent experiment.

2.6. Determination of Total Phenolic Contents by Folin–Ciocalteu Method

Total phenolic contents of each extracts were determined by Folin–Ciocalteu method with some modifications [22]. Briefly, 20 µL of the sample solution in DMSO with the concentration of 1 mg/mL was mixed with 180 µL of 1:10 diluted Folin–Ciocalteu reagent and kept in room temperature for 4 min. Then 80 µL of saturated sodium carbonate solution (~0.7 M) was added and kept in room temperature for another 2 h. The absorbance was measured at 750 nm by using a multimode detector (Beckman Coulter DTX880, Fullerton, CA, USA). Gallic acid was used as a standard and the total phenolic contents were expressed as mg/g gallic acid equivalents (GAE). Total phenolic content was calculated using the following equation:

$$\text{Total phenolic content (mg GA/g)} = [(a - b) - 0.021]/0.0057 \quad (2)$$

where a is an absorbance of sample solution with the present of Folin–Ciocalteu reagent and b is an absorbance of sample solution without the present of Folin–Ciocalteu reagent. The entire experiment was done in triplicate.

2.7. Determination of Antioxidant Activity

2.7.1. 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) Assay

Each extract was tested for its ABTS radical cation ($ABTS^{\bullet+}$) scavenging activity by ABTS assay with some modifications [23]. Briefly, $ABTS^{\bullet+}$ was previously prepared by mixing 7 mM ABTS with 2.45 mM potassium persulfate ($K_2S_2O_8$) and kept in the dark at room temperature for 16 h. On the experiment day, 20 µL of the sample solution in DMSO with the concentration of 1 mg/mL was mixed with 180 µL of 1:20 diluted $ABTS^{\bullet+}$ solution and kept in room temperature for 5 min. The absorbance was measured at 750 nm by using a multimode detector (Beckman Coulter DTX880, Fullerton, CA, USA). Trolox was used as a standard and the $ABTS^{\bullet+}$ scavenging activity was expressed as Trolox equivalent antioxidant capacity (TEAC) which was calculated using the following equation:

$$\text{TEAC (mM Trolox/g)} = [(a - b) - 0.7573]/ - 0.0145 \quad (3)$$

where a is an absorbance of sample solution with the present of $ABTS^{\bullet+}$ solution and b is an absorbance of sample solution without the present of $ABTS^{\bullet+}$ solution. All experiments were done in triplicate.

2.7.2. 2,2′-diphenyl-1-picrylhydrazyl (DPPH) Assay

Each extract was tested for their radical scavenging activity against stable DPPH by DPPH assay with some modifications [24]. Briefly, 20 µL of the sample solution in DMSO with the concentration of 1 mg/mL was mixed with 180 µL of 167 µM DPPH solution and kept in the dark at room temperature for 30 min. The absorbance was measured at 520 nm by using a multimode detector (Beckman Coulter DTX880, Fullerton, CA, USA). The scavenging effect was calculated using the following equation:

$$\% \text{ scavenging effect} = \{1 - [(a - b)/(c - d)]\} \times 100, \quad (4)$$

where a is an absorbance of 20 µL of ethanol and 180 µL of 167 µM DPPH mixture, b is an absorbance of 200 µL of ethanol, c is an absorbance of 20 µL of sample solution and 180 µL of 167 µM DPPH mixture, and d is an absorbance of 20 µL of sample solution and 180 µL of ethanol mixture. All experiments were done in triplicate.

2.7.3. Ferric Reducing Antioxidant Power (FRAP) Assay

Each extract was tested for its reducing power by FRAP assay with some modifications [25]. Briefly, 20 µL of the sample solution in DMSO with the concentration of 1 mg/mL was mixed with 180 µL of freshly prepared FRAP solution, which contains 0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6 tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and 20 mM ferric chloride (10:1:1), and kept in room temperature for 5 min. The absorbance was measured at 595 nm by using a multimode detector (Beckman Coulter DTX880, Fullerton, CA, USA). Ferrous sulfate ($FeSO_4$) was used as a standard and the ferric ions reducing power were expressed as equivalent capacity (EC_1) which represented the amount of $FeSO_4$ equivalents per mg of the sample. EC_1 was calculated using the following equation:

$$EC_1 \text{ (mg } FeSO_4/g) = [(a - b) - 0.0211]/0.0027 \quad (5)$$

where a is an absorbance of sample solution with the present of FRAP solution and b is an absorbance of sample solution without the present of FRAP solution. All experiments were done in triplicate.

2.7.4. Inhibition of the Lipid Peroxidation by the Ferric Thiocyanate Method

Each extract was tested for its inhibition against lipid peroxidation by thiocyanate method with some modifications [26]. Briefly, solutions containing 50 µL of the sample solution in DMSO with the concentration of 1 mg/mL, 50 µL of 50% linoleic acid in DMSO, 50 µL of 10% aqueous solution of ammonium thiocyanate (NH_4SCN), and 50 µL of 2 mM ferrous chloride ($FeCl_2$) solution, were incubated at 37 °C for 1 h. The absorbance was measured at 500 nm by using a multimode detector (Beckman Coulter DTX880, Fullerton, CA, USA). The inhibitory activity was calculated using the following equation:

$$\% \text{ inhibition} = \{1 - [(a - b)/(c - d)]\} \times 100, \quad (6)$$

when a is an absorbance of linoleic acid, NH_4SCN, and $FeCl_2$ mixture, b is an absorbance of the solvents, c is an absorbance of sample solution, linoleic acid, NH_4SCN, and $FeCl_2$ mixture, and d is an absorbance of sample solution and the solvents. The entire experiment was done in triplicate.

2.8. Cytotoxicity of E. debile Extracts on Dermal Papilla Cells

2.8.1. Dermal Papilla Cells Culture

Dermal papilla cells were purchased from Promo cell; Bio-med (Bangkok, Thailand). Frozen cells were thawed under water bath at 37 °C. The cells were suspended into follicle dermal papilla cell growth medium (PromoCell GmbH) to which was added fetal bovine serum (4% *v/v*), bovine pituitary extract (0.4% *v/v*), basic fibroblast growth factor (1 ng/mL) (PromoCell GmbH, Heidelberg, Germany).

Cells were incubated under 37 °C, 5% CO_2 with 95% of relative humidity. The cells were sub-cultured when they reached 80–90% confluence.

2.8.2. Cytotoxicity and Cell Proliferation Testing

Ten thousands of dermal papilla cells per wells were incubated in 96 wells plate for 24 h under 37 °C and 5% of CO_2. The cells were treated by the plant extracts dissolved in ethanol with various concentrations ranging from 1 to 500 μg/mL. After that, cells were re-incubated for another 24 h. MTT assay was used for determining cell viability. Fifty microliter of 1 mg/mL of MTT solution was added into each well and incubated for 3 h. Formazan crystal was produced by living cell and was then dissolved in DMSO. Absorbance was determined at 515 nm by using microplate reader. Cytotoxicity and cell proliferation were determined by comparing with controlled cells [27].

2.9. Irritation Test by Hen's Egg Test Chorioallantoic Membrane (HET-CAM) Assay

The irritation study was performed using hen's egg test chorioallantoic membrane (HET-CAM) assay with slight modifications [28,29]. This experiment was one of the convenience and famous irritation studies since the ethical approval did not need to be applied when the age of animal's embryo was less than half of the total incubation period. The hen eggs were obtained after fertilization from Faculty of Agriculture, Chiang Mai University. All eggs were incubated for 7 days in the hatching chamber with 37.5 ± 0.5 °C, humidity 55 ± 7%.

For preparation of the CAM, the air chamber of the egg was indicated by flooding the eggs with light. The egg shell was opened with an electric drill and the white egg membrane that appeared was removed. The samples dissolved in jojoba oil were exposed to the CAM, and the specific alterations of the membrane and its blood vessel network were examined as hemorrhage, lysis, and coagulation. The hemorrhage was observed as the bleeding out from blood vessels of the vascularized CAM. The lysis was indicated by a disappearance of small blood vessels on the CAM as a consequence either of bleeding, dystonia of these fine vessels, or real disintegration. The coagulation included either intravascular coagulation (thrombosis) or extravascular coagulation of proteins on the CAM, which normally increases the CAM opacity. The time of first occurrence of the three above-mentioned endpoints were registered within a maximum period of 5 min (300 s). From these data, an irritation index (IS) was calculated using the following equation:

$$IS = [(301 - t(h))/300 \times 5] + [(301 - t(l))/300 \times 7] + [(301 - t(c))/300 \times 9], \quad (6)$$

where $t(h)$ is the time (s) when the first vascular hemorrhage was detected, $t(l)$ is the time (s) when first vascular lysis was detected, and $t(c)$ is the time (s) when the first vascular coagulation was detected. The irritation score (*IS)* was then evaluated as follows: 0.0–0.9, no irritation; 1.0–4.9, mild irritation; 5.0–8.9, moderate irritation; and 9–21, severe irritation [30]. The blood vessel networks were observed again after 60 min to see the long term irritation. The pictures of the CAM were then captured under the microscope by Lumix digital camera (Panasonic, Beijing, China).

2.10. Statistical Analysis

All data were presented as a mean ± standard deviation (S.D.). Individual differences were evaluated by *t* test or one-way ANOVA followed by post-hoc tests. In all cases, * denotes $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.001$ indicated statistical significance.

3. Results

3.1. E. debile Extracts

Five *E. debile* extracts, CE, CF, HE, EA, and ET, were obtained by maceration. All extracts showed almost the same external appearances, i.e. they were dark green, sticky, and semisolid. CE showed

the highest yield (16.1%) and CF showed lower yield (7.3%) because of the chlorophyll remove. The fractionated extracts show small amount of the yield. HE and EA showed the comparable yields which were 3.6% and 4.2%, respectively. The smallest yield was obtained in ET (1.2%) since the nonpolar and semi-polar compounds had been previously extracted by hexane and ethyl acetate.

3.2. 5α-Reductase Inhibition

The inhibitory activity against 5α-reductase of *E. debile* extracts are shown in Figure 1. EA possessed the significantly highest 5α-reductase inhibition among five extracts. Palmitic acid and phytosterols, which has been reported as a major component of the plant in the family of Equisetaceae [31,32], might be responsible for the 5α-reductase inhibitory activity of EA [33,34].

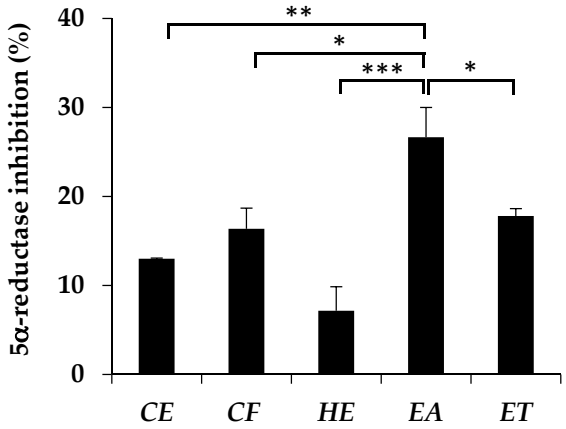


Figure 1. Inhibitory activity against 5α-reductase of crude extract (CE), chlorophyll-free extract (CF), fraction hexane extract (HE), fraction ethyl acetate extract (EA), and fraction ethanolic extract (ET) at the concentration of 0.1 mg/mL. Data are the mean value ± S.D. of three independent experiments (* denotes $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.001$).

3.3. IL-6 Secretion Inhibition in LPS-Stimulated Macrophages

The IL-6 secretion levels were investigated in the RAW 264.7 cell line from which the cell viability after exposure to the *E. debile* extracts is shown in Figure 2. It was noted that the extracts had no toxicity on the cells because no significant difference was observed between the cell viability after exposure to each extracts for 24 h and the control cells which were not exposed to any extract ($p > 0.05$). Moreover, nearly 100% of cell viability was observed at the tested concentrations (50 and 100 μg/mL) of all extracts.

The inhibitory activity against IL-6 secretion after treatment with various *E. debile* extracts are shown in Figure 3. There was no significant reduction of IL-6 secretion at low concentration (50 μg/mL) of all extracts but the obvious reductions were detected at high concentration (100 μg/mL) when compared to the cell control stimulated with LPS alone.

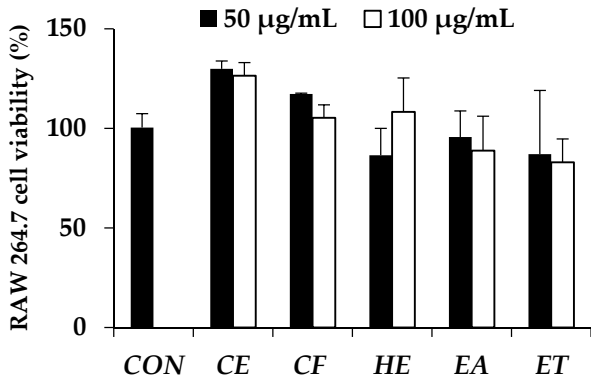


Figure 2. Cell viability of RAW 264.7 cell line after treatment with no extract (CON), crude extract (CE), chlorophyll-free extract (CF), fraction hexane extract (HE), fraction ethyl acetate extract (EA), and fraction ethanolic extract (ET) for 24 h. Data are the mean value ± S.D. of three independent experiments.

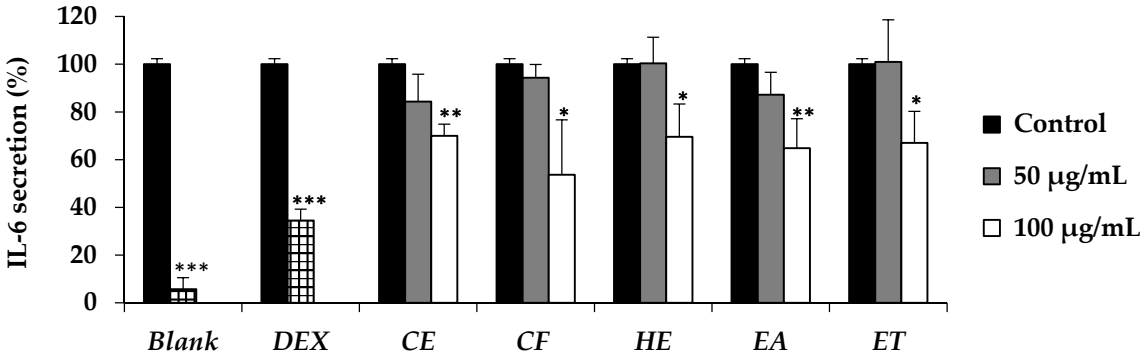


Figure 3. IL-6 secretion by the RAW 264.7 cell line without LPS treatment (Blank) and after being treated with LPS followed by 10 μM dexamethasone (DEX), crude extract (CE), chlorophyll-free extract (CF), fraction hexane extract (HE), fraction ethyl acetate extract (EA), and fraction ethanolic extract (ET) compared to control. Data are the mean value ± S.D. of three independent experiments. Asterisks denote values that were significantly different from the vehicle control (* denotes $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.001$).

3.4. Total Phenolic Contents and Antioxidant Activity of E. debile Extracts

Total phenolic contents and antioxidant activity of each *E. debile* extracts is shown in Table 1. Individual antioxidants may act via multiple mechanisms. Therefore, no single assay would accurately reflect the antioxidant activity, especially in the plant extracts which contained several components. ET possessed the most potent antioxidant activity among five extracts which was obviously related to its highest total phenolic content, except for the inhibition of lipid peroxidation. The highest lipid peroxidation inhibition was possessed by EA. Besides, CF showed higher antioxidant activity comparing to CE in FRAP and DPPH assay because of the lower amount of chlorophyll. The likely explanation might be due to the production of potentially harmful singlet oxygen from chlorophyll. Chlorophyll was a major source of ROS production since the excitation energy can be transferred from photo-excited chlorophyll pigments to $^3O_2$ that lead to the formation of singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet -}$), and hydrogen peroxide (H_2O_2) [35].

Table 1. Total phenolic contents and antioxidant activity of *E. debile* extracts.

Extracts	GAE (mg GA/g)	EC_1 (mM $FeSO_4$/g)	TEAC (mM Trolox/g)	%Inhibition	
				DPPH	Lipid Peroxidation
CE	26.6 ± 1.7 [a]	89.2 ± 19.5 [a]	12.1 ± 1.0 [a]	6.3 ± 2.5 [a]	25.1 ± 0.6 [a]
CF	35.6 ± 0.5 [b]	129. ± 2.0 [b]	7.2 ± 0.3 [a,b]	8.9 ± 1.2 [a]	12.6 ± 0.9 [b]
HE	5.6 ± 1.1 [c]	30.3 ± 3.1 [a]	2.0 ± 0.0 [b]	ND	16.4 ± 1.1 [c]
EA	30.6 ± 1.2 [a,b]	115.7 ± 39.9 [b]	7.2 ± 3.3 [a,b]	6.3 ± 1.2 [a]	57.2 ± 0.4 [d]
ET	68.8 ± 6.7 [d]	289.1 ± 26.4 [c]	15.7 ± 3.5 [a]	20.0 ± 6.0 [b]	2.4 ± 1.9 [e]

Results expressed as mean ± SD of triplicates. ND: not detected. CE: crude extract, CF: chlorophyll-free extract HE: fraction hexane extract, EA: fraction ethyl acetate extract, ET: fraction ethanolic extract. Superscript letters ([a], [b], [c], [d], and [e]) within the same column indicate significant ($p < 0.05$) differences of means between the groups based on Tukey's HSD one-way ANOVA.

The correlations between total phenolic contents of five *E. debile* extracts and their antioxidant activities from various assays are shown in Figure 4. Linear positive relationships existed between the total phenolic contents and the antioxidant activity of the *E. debile* extracts from DPPH assay ($R^2 = 0.9925$) and ABTS assay ($R^2 = 0.7403$), which measured the radical scavenging activity. Similarly, a linear positive relationship was found in the FRAP assay ($R^2 = 0.9824$), which measured the total reducing capacity of ferric ions. Although the mechanisms of FRAP assay was different from that of ABTS assay, the antioxidant results were comparative because the redox potential of Fe(III)-TPTZ was comparable with that of $ABTS^{\bullet+}$ [36]. Besides, the antioxidant results were in a good agreement with several previous studies which revealed that phenolic compounds had both abilities to scavenge free radicals and prevent generation of reactive oxygen species (ROS) by iron binding [37,38]. However, it was noted that the results from ABTS assay showed less correlation between the total phenolic contents and TEAC value. The likely explanation might be from the favor of water-soluble reactions in ABTS assay [36]. Therefore, the highest TEAC value of the fractionated extracts was found in ET, followed by EA and HE, which were finally extracted by ethanol ($\varepsilon = 24.3$), ethyl acetate ($\varepsilon = 6.02$), and hexane ($\varepsilon = 1.9$), respectively. Likewise, there was no correlation between total phenolic contents and antioxidant activities against lipid peroxidation ($R^2 = 0.1263$) because the phenolic compounds, which were soluble well in water or polar solvents, were not well compatible with the lipid peroxidation test system.

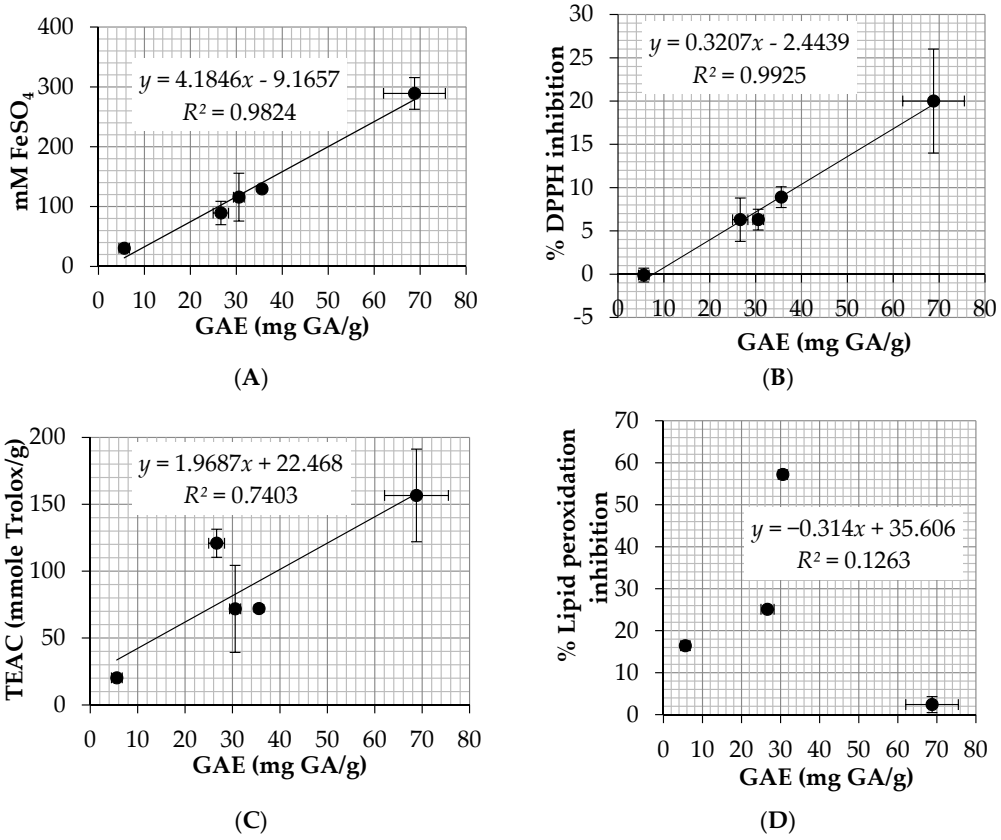


Figure 4. The correlations between total phenolic content and antioxidant activity from: (**A**) ferric reducing antioxidant power (FRAP) assay; (**B**) 2,2′-diphenyl-1-picrylhydrazyl (DPPH) assay; (**C**) 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay; and (**D**) ferric thiocyanate method. Data are the mean value ± S.D. of three independent experiments.

3.5. Cytotoxicity of E. debile Extract on Dermal Papilla Cells

According to the highest inhibitory activity against 5α-reductase and lipid peroxidation, as well as, high IL-6 secretion reduction, EA was the most attractive extract for anti-hair loss. Therefore, the cytotoxicity on dermal papilla cells of EA was investigated to confirm the safety of further uses. The human dermal papilla cell viability after exposure to EA for 24 h is shown in Figure 5. It is noted that EA was very safe since it had no toxic effect on human dermal papilla cells since nearly 100% of cell viability were observed even at high concentration.

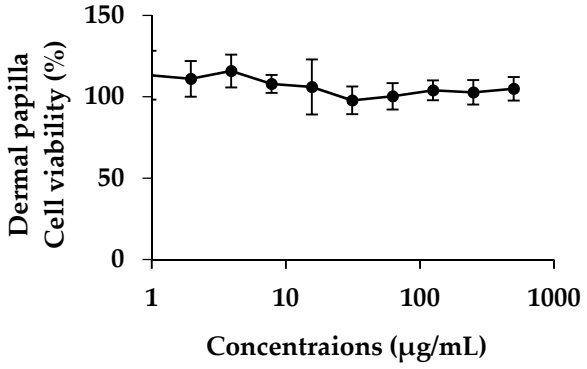


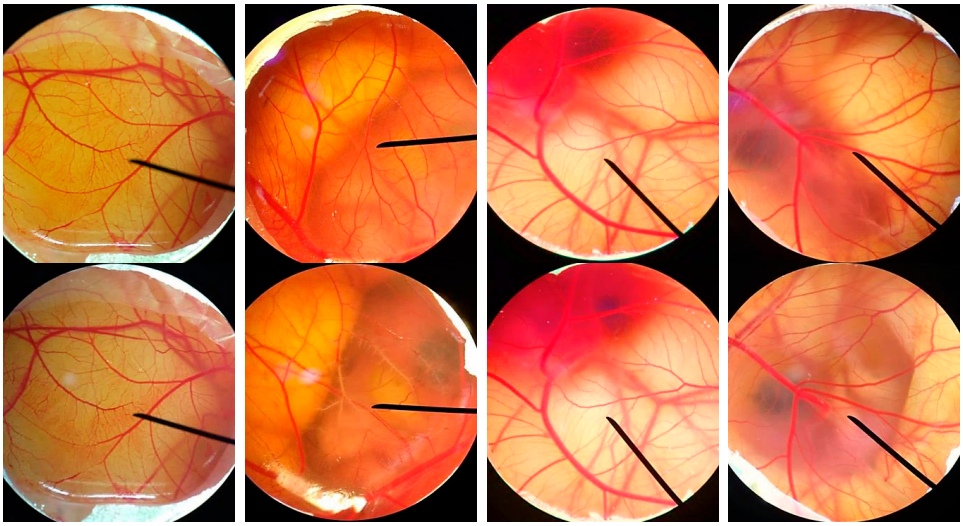
Figure 5. Dose–response curve of dermal papilla cell viability versus concentration of EA. Data are the mean value ± S.D. of three independent experiments.

3.6. Irritation Test by Hen's Egg Test Chorioallantoic Membrane (HET-CAM) Assay

The irritation results of HET-CAM assay are shown in Table 2. No irritation was observed in EA solution, which was the same result as observed in 0.9% (*w*/*v*) NaCl and the vehicle (jojoba oil). In contrast, moderate irritation was observed in 1% (*w*/*v*) SLS which have been known for the cause of scalp and skin irritation. The status of vessels before and after the experiments is shown in Figure 6. Only 1% (*w*/*v*) SLS could produce the lysis. It was noted that no hemorrhage, lysis, and coagulation were detected in the vessel exposed with EA solution after 60 min of exposure. Therefore, it might be concluded that EA was safe and would not cause the skin irritation. Since CAM is the vascularized respiratory membrane including veins, arteries, and capillaries, it has been used as a model for predicting the irritant effect of chemicals on the conjunctiva [39]. Consequently, EA would not cause the eye irritation and have a potential for the development of various anti-hair loss products, including hair tonic and hair spray.

Table 2. IS score and irritation level form hen's egg test chorioallantoic membrane (HET-CAM) assay ($n = 3$).

Samples	IS Score	Irritation Level
EA (0.5% (*w*/*v*) solution in jojoba oil)	0.00	No irritation
Vehicle (jojoba oil)	0.00	No irritation
Negative control (0.9% (*w*/*v*) NaCl)	0.00	No irritation
Positive control (1% (*w*/*v*) SLS)	6.72 ± 0.03	Moderate irritation



(**A**) (**B**) (**C**) (**D**)

Figure 6. Photographs illustrating the effect of: (**A**) negative control (0.9% (*w*/*v*) NaCl); (**B**) positive control (1% (*w*/*v*) SLS); (**C**) vehicle (jojoba oil); and (**D**) EA (0.5% (*w*/*v*) solution in jojoba oil) in hen's egg test chorioallantoic membrane (HET-CAM) assay before applying the sample (upper) and at the end of the experiments of 60 min (lower).

4. Discussion

E. debile extracts have been extracted by maceration using various solvents. CE showed the highest yield since ethanol could extract a wide range of natural compounds from the plants, especially polar compounds. In the present study, electrocoagulation was used to remove chlorophyll from CE and yield CF. The yield was decreased from 16.1% of CE to 7.3% of CF. In addition, *E. debile* was fractionally extracted by using the non-polar solvent (hexane), semi-polar solvent (ethyl acetate), and polar solvent (95% ethanol). Therefore, the non-polar compound would be mostly found in HE, semi-polar compounds would be mostly in EA, and polar compounds would be mostly in ET.

The yield of ET was very small when compared with that of CE since the nonpolar compounds and some semi-polar compounds have been removed.

The extracts were then investigated for anti-hair loss property. There are several biological mechanisms related to hair loss and the present study focused on 5α-reductase, IL-6, and oxidation process.

The presence of DHT, which is converted from testosterone by the role of 5α-reductase, is related to aberrant of hair follicle cycling, miniaturization of hair follicles, and finally hair loss [1–3]. Therefore, the compounds that could inhibit 5α-reductase would be useful for anti-hair loss. The 5α-reductase inhibitory activity of *E. debile* extracts was firstly described in the present study. The results noted that, among the five *E. debile* extracts, EA possessed the significantly highest 5α-reductase inhibition. Although the activity was not as high as finasteride (95 ± 2.2% inhibition at 1.5 μg/mL), EA has a distinctive point as it was from natural source. The presence of palmitic acid as a major component of *E. debile* might be the explanation for the 5α-reductase inhibition [31]. The previous study has been reported that important configurations related to 5α-reductase inhibition included C12–C16 of the fatty acid chains [40]. Therefore, palmitic acid (C16:0) which was saturated C16 fatty acid exhibited the 5α-reductase inhibition [41]. In addition, the inhibitory activity could be more potent if there were the presence of a double bond in the molecule [40]. Additionally, there was a previous study reported that the fraction ethyl acetate extract of *E. debile* contained several phytosterols, such as stigmasterol and daucosterol [32], which could alter the metabolism of testosterone by inhibiting 5α-reductase. However, the evidence from animal studies suggested that a very high dose of phytosterol intake was needed to inhibit 5-alpha-reductase [34].

Beside the role of 5α-reductase and DHT, several cytokines are also related to the hair loss. IL-6 is one of the cytokines which has been more upregulated in balding dermal papilla cells [8]. In addition, IL-6 has been reported to inhibit the hair shaft elongation and suppressed proliferation of matrix human hair follicles cells and finally lead to the hair loss [8]. *E. debile* extracts (CE, CF, HE, EA, and ET) have been reported to reduce the IL-6 secretion in the present study. The active dose was detected at the concentration of 100 μg/mL. The results were in a good accordance with the previous study which reported that n-hexane and ethyl acetate extract of the aerial stems of *E. debile* composed of several phytosterols that could decrease aggregated LDL-induced secretion of IL-6 [32,42]. Since IL-6 has a broad effect on cells of the immune system and those not of the immune system and often displays hormone-like characteristics that affect homeostatic processes [43], *E. debile* extracts that could inhibit the IL-6 secretion might have several health benefits other than anti-hair loss.

Oxidation process is another pathway related to hair loss since free radicals could damage the hair follicle cellular structures and lead to a decrease in hair production [9]. There are several methods to investigate the antioxidant activity of natural compounds, including ABTS, DPPH, FRAP, and lipid peroxidation assay. However, the most relevant method related to hair loss was lipid peroxidation assay since it has been reported that lipid peroxides on hair follicles led to the early onset of the catagen which would lead to the hair loss [10].

Among 5 *E. debile* extracts, EA possessed the highest lipid peroxidation inhibition (57.2 ± 0.4%). EA also showed favorable antioxidant results in other assay with the EC_1 of 115.7 ± 39.9 mM $FeSO_4$/g and TEAC of 7.2 ± 3.3 mM Trolox/g. EA contained high level of total phenolic content (30.6 ± 1.2 mg GA/g) which was more abundant than CE (26.6 ± 1.7 mg GA/g). The explanation might be from the removal of non-polar compounds by the fractionated extraction method since HE which was the nonpolar extract contained very little amount of the phenolic content (5.6 ± 1.1 mg GA/g). The results were in a good agreement with the previous study which reported that a high and significant antioxidant activity was detected in the ethyl acetate fraction when comparing to an aqueous extract (infusion) and the major phenolic compounds responsible for the antioxidant activity were flavan-3-ol, kaempferol, and several phenolic acid derivatives [44]. Moreover, another study reported that quercetin, a flavonoid antioxidant, was isolated from ethyl acetate extract of the aerial stems of *E. debile* [32]. Therefore, quercetin would be one of the compounds that was responsible for the high antioxidant activity of *E. debile* extracts since it is able to scavenge highly

reactive species such as peroxynitrite and the hydroxyl radical [45]. In conclusion, EA was the most attractive extract used for anti-hair loss since it showed the highest inhibitory activity against 5α-reductase, IL-6 secretion, and lipid peroxidation. Besides, the previous studies have been reported that fraction ethyl acetate extract of *E. debile* contained several phytosterols, flavonoids, and phenolic compounds which would be beneficial for health [32,46]. Moreover, EA was not toxic to the human dermal papilla cells and caused no irritation on HET-CAM. Therefore, EA might be used as functional food and nutraceuticals ingredients for anti-hair loss.

5. Conclusions

The present study demonstrated the inhibitory activity against 5α-reductase, IL-6 secretion, and oxidation process of *E. debile* extract. EA exerted the highest inhibition on 5α-reductase (26.6 ± 3.3%) and lipid peroxidation (57.2 ± 0.4%), whereas ET possessed the highest antioxidant activity (EC_1 = 289.1 ± 26.4 mM $FeSO_4$/g, TEAC = 156.6 ± 34.6 mM Trolox/g, and DPPH inhibition = 20.0 ± 6.0%), which was related to its highest amount of phenolic content (68.8 ± 6.7 mg GA/g). The results noted that EA was very safe since it showed no cytotoxicity on dermal papilla cell line and no irritation on chorioallantoic membrane of hen's eggs. Therefore, EA might be an attractive ingredient for functional food and nutraceuticals for anti-hair loss because of the highest inhibitory activity against 5α-reductase, IL-6 secretion, and lipid peroxidation inhibition. However, the pharmacokinetic study of *E. debile* extract would be suggested for the further study.

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Formulation and Evaluation of Herbal Hair Gel Containing Fenugreek Seed Extract for Nourishment and Hair Growth

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ABSTRACT

The present study now a days many people face the major problem related to hair i.e. hair loss. There are many causes of hair loss physiological conditions, emotional or physical stress, nutritional deficiencies, hormonal disorders one of the due to hormone deficiency of estrogen. External administration of the estrogen could changes the hormonal cycle and increase cancer risk some natural alternative estrogen therapy can be found in the various plants containing natural products those having weak estrogen activity like Phyto-estrogen. Herbal drug has less side effects and more effective as comparative to synthetic drug. Phytoestrogen are competing with the estrogen by the binding to the estrogen receptor and produce estrogen effect, Phytoestrogen in the fenugreek seed. Family – Fabaceae Ethanolic extract of (Trigonella foenum-graecum) fenugreek seed prepared for the topical formulation of herbal hair gel formulation by using Carbopol 934 gelling agent, glycerin, pvp, methyl paraben, PEG, Triethanolamine Fenugreek was evaluated for its potency on hair growth activity by in vivo method. In vivo, study 2.5mg of fenugreek extract is used. That is applied on the shaved skin of mice to determine the length of hair and the different cyclic phase of hair follicles like anagen and s phases were will be grow after some time periods. From the study topical use of gel formulation were apply for 30 days .There are use of fenugreek extract containing gel formulation over the shaved skin of mice that shows the significant result by increase the hair growth. The prepared gel was characterized for their physicochemical constants, preliminary phyto-chemical analysis, quantitative analysis, Spread-ability, pH, viscosity, and stability study.

Keywords: Fenugreek Seed Extract, Glycerin, Carbopol 934, Hair Growth

I. INTRODUCTION

The hair has a protective role against the adverse effect of environment, for example temperature and most important role is the aesthetic purpose and if the hair encounters any abnormalities, the confidence of the person will be disturbed or most common abnormality is a depigmentation (gray-hair), dandruff. Now a day's number of people who had suffered from hair loss or hair thinning problem even baldness also is increasing in world wide. Hair loss is a dermatological disorder and this is the major problem, hair loss is the reduction of hair volume.[1, 2] Hair treatment or nourishment is required. To prevent the hair loss or alopecia is a common patient complaint due to psychological and physical distress. By using hair shampoo or conditioner treatment is not possible and not enough to hair growth as well as roots are living cells that need to be nourished in order to stay healthy; therefore, the administration of hair tonic is also required. to treat the such hair loss alopecia hair fall [3]Anti-oxidant present the seeds of

T. foenum graecum have been very used as anti-lice, anti-dandruff activity as well as hair growth and soothing effects produce, Trigonella foenum-graecum, belonging to family Fabaceae, has been used traditionally for various pharmacological effects, such as anti-diabetic, anti-cancer, anti-fungal, anti-pyretic, and antibacterial [4]]. The plant contains active constituents such as alkaloids, flavonoids, steroids, Saponins like in steroidal hormones etc. In that vitamins A, B1, C, and nicotinic acid; and 0.015 % volatile oils contains. [5,6,7].

II. METHODS AND MATERIAL

Mechanism of action :- A competitive inhibitor of type 2 5-alpha reductase' inhibits the transformation of testosterone to dihydrotestosterone (DHT). Androgen-dependent process in hair loss is predominantly due to the binding of DHT to the androgen receptor. The androgen is sex hormones found in the testosterone and androgens are important to hair growth. Fenugreek seed extract increases testosterone level in the body. In that way hair growth is possibly the hormone-receptor complex activates the genes responsible for the gradual change of large terminal follicles to miniaturized follicles. [8]

Formulation

Hair Gel: - A viscous substance styling hair

Gels are the transparent clear dosage form in which drug can be interrupted in aqueous form.

The gels are form cross linking structure relatively newer class of dosage forms created by entrapment of large amounts of aqueous or hydro-alcoholic liquid in a network of colloidal solid particles, Most topical gels are prepared with polymers such as Carbomers which impart an aesthetically pleasing, clear sparkling appearance to the product and a easily washed off the skin with water.

A. Properties of gel:

1. The gel should be sterile or clear.
2. The formulation of gel should not be sticky non greasy.
4. The gelling agent should be inert as well as safe.
5. It should not be reacting with other excipients does not decompose the active ingredients.
6. It should be easily spread on to the skin.
7. Entrapped the drug by swelling property. [9,10].

Fig 1. Hair Gel

Preparation of herbal hair gel formulation.

Six different herbal hair gel formulations were prepared by simple gel formulation preparation method. The gel was prepared by using fenugreek seed extract in which carbopol is used as base or gelling agent and other excipients such as methyl paraben as preservative, glycerin as humectant, poly ethylene glycol (PEG)as penetration enhancer , Carbopol 934 as gelling agent, PVP as hair conditioner and Triethanolamine for adjustment of PH. Add Carbopol 934 two percentages and measured quantity of extracts was dispersed in up to 100 ml of distilled water as solvent and mixed by continuously stirring by using magnetic stirrer at 800 rpm for 1 hour. Glycerin 3 ml was added in to the mixture

under continuous stirring. 5mg PVP, methyl paraben or poly ethylene glycols also add in mixture the mixture was neutralized by drop wise addition of 1ml Triethanolamine. Mixing was continued until a transparent gel was formed. [11]

Table 1. FORMULATION TABLE

Ingredients	F1	F2	F3	F4	F5	F6
Fenugreek extract (g)	2.5	2.5	2.5	2.5	2.5	2.5
Carbopol-934 (mg)	0.5	0.8	1.1	1.4	1.7	2
PVP (mg)	5	5	5	5	5	5
Glycerin (ml)	3	3	3	3	3	3
Methyl paraben (mg)	10	20	10	20	10	20
PEG (ml)	6	6	6	6	6	6
Triethanolamine (ml)	1	1	1	1	1	1
Flavoring agent (q.s)	-	-	-	-	-	-
Water (ml)	Up to 100	Up to 100	Up to 100	Up to 100	Up to 100	Up to 100

A. Preformulation studies:

Physical characteristic of fenugreek extract [12]

Table 2. Preformulation studies

Sr. No.	parameters	Specification as per IP 1996	Result
1	Appearance	Yellowish brown	Yellowish brown
2	Odour	Characteristic	Characteristic
3	Taste	Bitter	Bitter

a) Solubility Study

Table 3. Solubility of fenugreek extract

Sr. No.	Solvents	Result
1	Methanol	soluble
2	water	Soluble
3	Ethanol	soluble
4	Ether	Insoluble
5	chloroform	Insoluble

b) Ash value:

Ash value of pure drug was found to be[13]-

The percentage of total ash was calculated with reference to air dried sample.

= Weight of Empty crucible (A) =16.138 gm
= Weight of Drug taken (B) =1 gm
= Weight of dish +drug (C) =17.137 gm
= Weight of dish + ash [after complete the incineration] (D) =16.219 gm

Calculations:

= Weight of total ash = (D)-(A)

= 16.219-16.138

= 0.081gm

= 1 gm of extracts=weight of total ash

=100 gm of fenugreek seed extracts=?

$$= \frac{100 \times \text{weight of total ash}}{1}$$

$$= \frac{100 \times 0.081}{1}$$

Total Ash Value = 8.1%

c) Acid insoluble:
Total Acid insoluble Ash = 0.358%

d) Loss on drying:
The mean loss on drying was found to be 2.90%

e) Bulk density and tapped density[14]
Bulk density = weight of sample/bulk volume
= 10/22
= 0.454
Tapped density = weight of sample/tapped volume
= 10/17
= 0.5882

f) Hausner ratio[15]

Hausner ratio = Tapped density/Bulk density
= 0.5882/0.454
= 1.2955

III. EVALUATION TEST

a) PH
The pH value of herbal hair gel is determining by the pH meter. The measurement was performed at 1, 30,60, 90 days after preparation to detect any pH changes with time.

b) VISCOSITY
The measurement of viscosity of the prepared herbal hair gel was done by using Brookfield viscometer (model RVTDV II).the reading was taken at 100 rpm using the spindle no.6.

c) APPEARANCE AND HOMOGENECITY
The prepared gels were tested by physical appearance and homogeneity by visual observation of an herbal hair gel formulation. [16].

d) SPREADABILITY: the Spreadability was determined by parallel plate method which is widely used for determining and quantifying the Spreadability of semisolid preparations. Various formulations (1 g) were pressed between two 20 × 20 cm horizontal plates, the upper of which weighed 125 g. The spread diameter was measured after 1 min. Spreadability was calculated using the following formula:

$$S = M \times L / T$$

Where, S = Spreadability, M = Weight in the pan (tied to the upper slide), L = Length moved by the glass slide and T = Time (in sec.) taken to separate the slide completely each other. [16]

DIFFUSION STUDY : - The diffusion study was important to determine the drug release of prepared herbal hair gel formulation. It carried out such way taken Franz tube in which 1gm of herbal hair gel was taken packed on the bottom with cellophane membrane. Membrane is work as skin. The tube surface is deep in to the solution. 250ml phosphate

buffer solution use to absorption media of drug and maintain the PH 7.4 of solution remove the 5 ml sample from the media time to time hours 1, 2,3,4,5,6,7,8 hours and replace with stock solution then determine drug release at 234 λmax of herbal hair gel.[17]

STABILITY STUDY Optimized formulation was subjected to stability as per ICH guidelines at the following conditions (ICH, 2003). It showed No significance change in properties of the optimized formulation & the drug release. Sufficient quantity of herbal hair gel formulation were packed in stability container and kept in a Stability chamber at Samples were kept in stability chamber at following conditions for 3 months- 1. 40± 2°C and 75 ± 5% RH (Accelerated temperature)2. Room temperature Formulations were analyzed at 1, 2 and 3 months for following tests- i) Visual appearance ii) Drug diffusion study [18]

IN VIVO STUDY

Healthy 18 albino rat are purchased from Sainath Agencies Hyderabad (India) and used for hair growth promoting activity. The experimental protocol was approved by Institutional Animal ethics committee, (IAEC), and the care of the animals was carried out as per guideline of the Committee for the Purpose of control and Supervision of Experiments on Animals protocol no.

CPCSEA / CBPL / AH-47 /2018-19 Animals were placed in cages and kept in standard environmental conditions, fed with standard diet ad labium and allowed free access to drinking water. They were acclimated 7 days before entry into subsequent study.
Experimental animal:-

Wistar rats (150-200 gm) of either sex

Diet and water: - Animals had free access to standard pellet diet and water.

The experimental protocol was approved by institutional animal ethical committee (IACE) and laboratory animals were taken care according to the guideline of CPCSEA, ministry of forest and environment, government of India.

The animals are divided in to three groups each group contain 6 animals as following
Group I – was use as Control
Group II – was use as Standard
Group III - was use as Formulation

Skin irritation Studies

The Wistar rat of either sex weighing 150-200 g was used for this test. The intact skin was used. The hairs were removed from the rat 3days before the experiment. The gels containing extracts were used on test animal. Gel base was applied on the back of animal taken as control. The animal was treated daily up 24 hour and finally the treated skin was examined visually for erythema and edema. [19,20]

1. Hair Length -Hair was plucked randomly from the depilated area with the help of electric clipper and measured the hair length with the help vernier caliper and calculated the mean of hair length. [22]

Treatment for hair growth activity *in-vivo* study:

In which research study to determine the formulation of fenugreek seed extract hair gel to improve hair growth on the Wistar albino rat. Eighteen Wistar rats are taken in that study. Those 18 rats are divided in to the three groups each group contain six rats. In which first group use as control group. In which Second group standard product use as 2 % minoxidil to evaluate the hair growth to compare with the standard and in third group 2.5mg

fenugreek extract containing herbal hair gel formulation. Used for hair growth promoting activity or hair growth. Remove hairs by using electric trimmer from the dorsal portion of rat area 3 cm^2 or totally clean skin by using the standard marketed hair remover product veet. This study is 30th day hair growth activity. Shaved area of skin treatment daily applied twice daily to determine the hair growth activity. Observe the increases the hair growth or thickness of hair as well as length. The experimental protocol was approved by the Institutional animal ethics committee. [22.23]

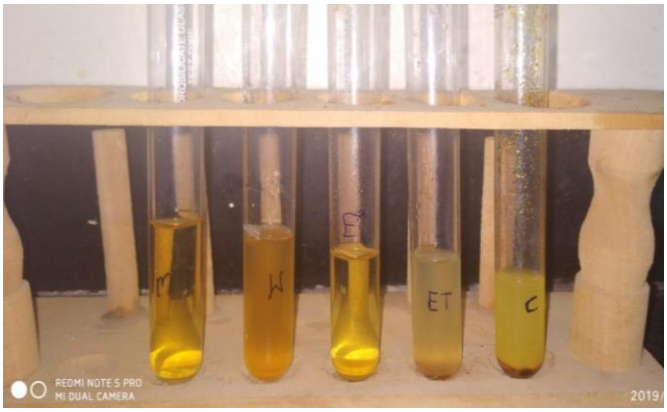


Fig 2.

Interpretation of FTIR Spectra

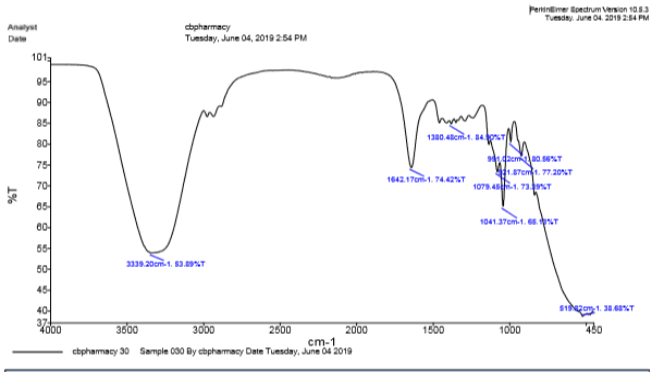


Fig 3. FTIR of Pure Trigonella Foenum Graecum extract

IV. RESULTS AND DISCUSSION

A. Solubility profile:

Solubility of fenugreek seed extract in different was solvents are given below:

Table 4. Interpretation of fenugreek

SR.NO	Functional Group	Standard Frequency	Observed Peak
1	N-H stretching	3350-3310	3339.20
2	C = O stretching	1648-1638	1642.17
3	O-H bending	1390-1310	1380.48
4	C-H,C=O,C-H no stretching	1085-1050	1079.45
5	C-O-O-CO` stretching	1050-1040	1041.37
6	C=C stretching	995-985	991.02

B. Evaluation test of fenugreek herbal hair gel:

Table 5

Sr. No	Formulation	Appearance	pH	Viscosity cps	Homogeneisity	Spradability	consistency
1	F1	Yellowish brown	6.1	12300	Good	No Good	Smooth
2	F2		5.9	17100			
3	F3		6.7	23600		Good	
4	F4		7.2	31831			
5	F5		5.3	42948			
6	F6		6.6	47500			

C. Estimation by UV spectroscopy:

Calibration of fenugreek seed extract

Table 6. Calibration of fenugreek seed Extract

Sr.no	Concentration (ug/ml)	Absorbance (λ max observed at 234.nm
1	2	0.258
2	4	0.356
3	6	0.484
4	8	0.593
5	10	0.682
6	12	0.781

Calibration curve: -

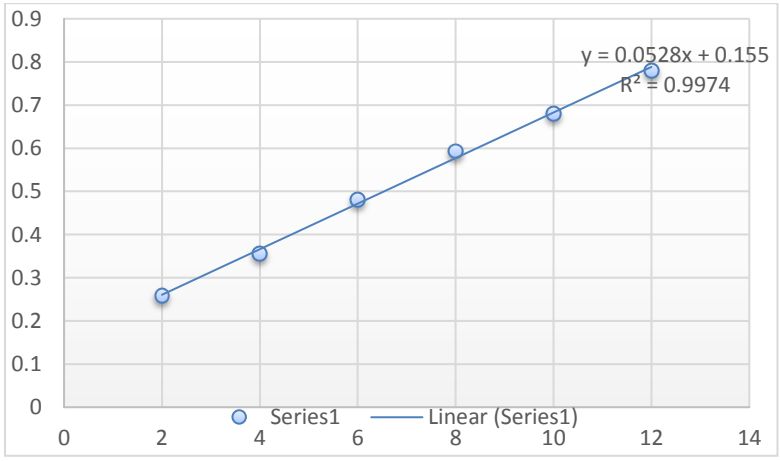


Fig 4. Calibration Curve of fenugreek seed Extract

D. Diffusion Study:

Table 7. Fenugreek Herbal hair gel

Time in hour	% OF DRUG DIFFUSION					
	F1	F2	F3	F4	F5	F6
0	0	0	0	0	0	0
1	11.39	17.96	17.62	20.11	18.69	24.18
2	28.16	26.56	32.18	30.12	27.19	35.76
3	39.74	32.67	44.81	49.18	34.63	48.89
4	43.92	39.84	53.64	56.29	49.28	57.69
5	51.49	48.17	69.37	62.84	58.71	71.23
6	68.32	65.49	76.94	79.43	77.18	84.17
7	71.10	69.21	84.79	89.32	83.20	91.12
8	80.11	77.26	89.44	84.54	80.68	93.14

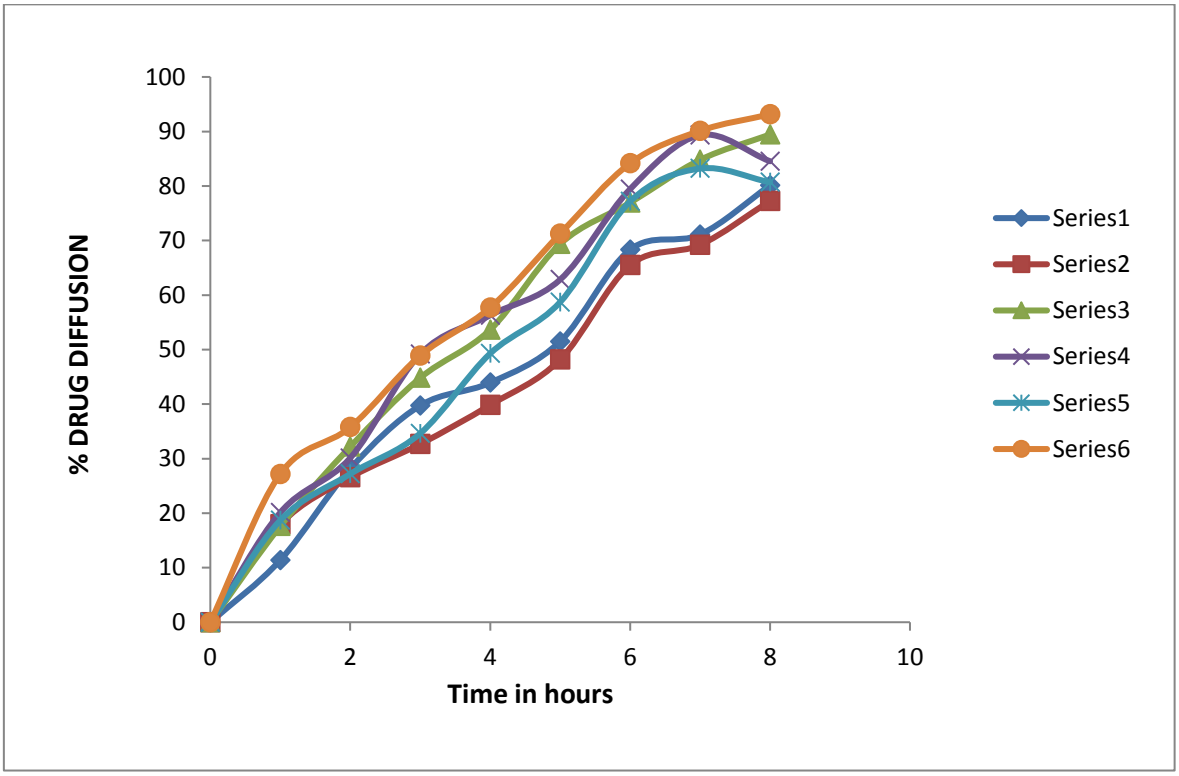


Fig 5. Diffusion study graph

E. Stability study:

Table 8. Stability study

Sr no	Time in days	Physical changes	pH	Spared-ability	viscosity	consistency
1	01	Yellowish Brown	7.2	Good	39100	Smooth
2	15		7.1		42030	
3	30		6.9		45687	
4	45		6.8		47700	
5	60		6.4		51456	
6	75		6.3		55500	
7	90		6.1		56201	

F. Evaluation of Optimize Formula:

Table 9

Sr .No	Time in Hours	% drug release initial	% drug release after 15 days	% drug release after 30 days	% drug release after 45days	% drug release after 60 days	% drug release after 75days	% drug release after 90 days
1	00	00	00	00	00	00	00	00
2	1	24.18	24.08	23.93	23.86	23.72	23.67	23.60
3	2	35.76	35.67	35.59	35.48	35.40	35.31	35.26
4	3	48.89	48.71	48.63	48.56	48.44	48.38	48.21
5	4	57.69	57.58	57.41	57.35	57.29	57.18	57.02
6	5	71.23	71.11	70.90	70.82	70.73	70.61	70.44
7	6	84.17	83.00	83.88	83.73	83.60	83.39	83.22
8	7	91.12	90.88	90.70	90.58	90.49	90.41	90.22

G. IN VIVO STUDY

Skin irritation test:

Does not found or observed any rashes or redness on the skin where applied Herbal hair gel formulation.

Hair length determination

Table 10.

Groups for 10 Day	Formulation	Hair growth in mm Mean±S.D)
Group I	Control	2.2±0.1.5
Group II	2% minoxidil	5.1±0.5
Group III	Herbal hair gel	4.0±0.4

Table 11

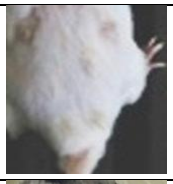
Groups for 20 Day	Formulation	Hair growth in mm (Mean±S.D)
Group I	Control	5.9±0.25
Group II	2% minoxidil	9.7±0.20
Group III	Herbal hair gel	7.5.1±0.35

Table 12

Groups for 30 Day	Formulation	Hair growth in mm (Mean±S.D)
Group I	Control	8.3±0.31
Group II	2% minoxidil	14.3±0.31
Group III	Herbal hair gel	12..4±0.41

H. Observation table:

Table 13

SR. NO	Group I control	Group II minoxidil	Group III formulation
0 Days			
10 Days			
20 Days			
30 Days			

V. DISCUSSIONS

In the present study the work was attempt to carry out the standardization and extraction of active constituents of fenugreek seed extract and it Also formulate and evaluate Fenugreek herbal hair gel. Fenugreek seed extract was evaluated for physico-chemical and phyto-chemical analysis by using different organic solvents. Standardization of fenugreek extract was done with total Ash value, acid insoluble ash loss on drying, Bulk density Hausner ratio etc. Phytochemical analysis was performed on different extracts confirmed the presence of alkaloids, glycosides, triterpenoid, saponins, tannins, flavonoids, etc. The presence of active constituents was also confirmed by the calibration curve of fenugreek seed extract. Formulation of herbal hair gel was done by varying drug concentration of ingredients as F1–F6. Methyl paraben was used as preservative or polyvinyl pyrrolidone as a plasticizer, Triethanolamine use as pH adjuster in of formulation. All formulations were checked for pH, viscosity, Spreadability, homogeneity. The FTIR studies revealed that, the formulated product is a mixture of drug and the polymers used but not the reaction product with the excipients used. From the above result it can be concluded that the prepared herbal hair gel shows significant hair growth activity.

VI. CONCLUSION

The fenugreek seed extract shows the significant effect on hair growth process compared with a 1 ml minoxidil it give results positive optimal concentration of 2.5mg. Fenugreek seed extract on the skin did not cause any irritation. Fenugreek containing flavonoids and triterpenoid possess hair growth promoting activity by increases blood circulation to nourish the hair follicles and there by promoting hair growth as well as testosterone level increases in body. The androgenic alopecia also reduce due to fenugreek contain hormones booster.

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