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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:

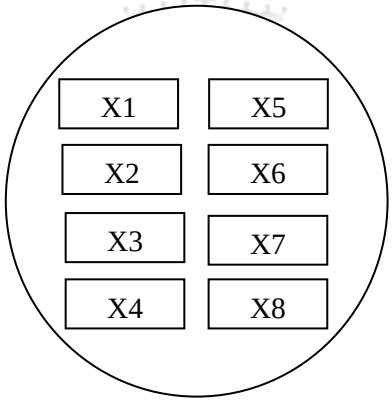


Figure No. 1: The application area to the rabbit's back is done by scrambling the position of X1-X8

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:

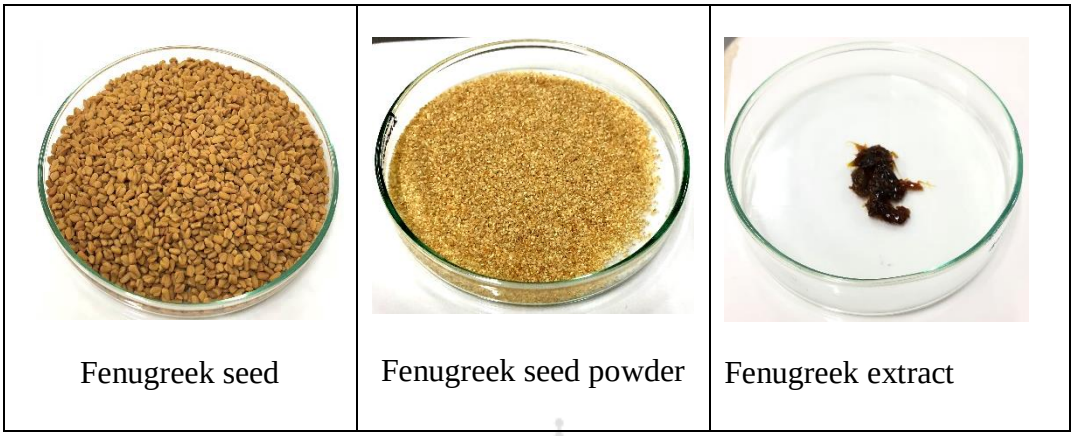
Apply a hair tonic preparation (0.1 ml) and cover with plaster for 24 hours. After 24 hours lift the patch and clean the area then evaluated for redness and swelling. Then the skin is evaluated again at the 48th and 72nd hours after the patch is opened.

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.

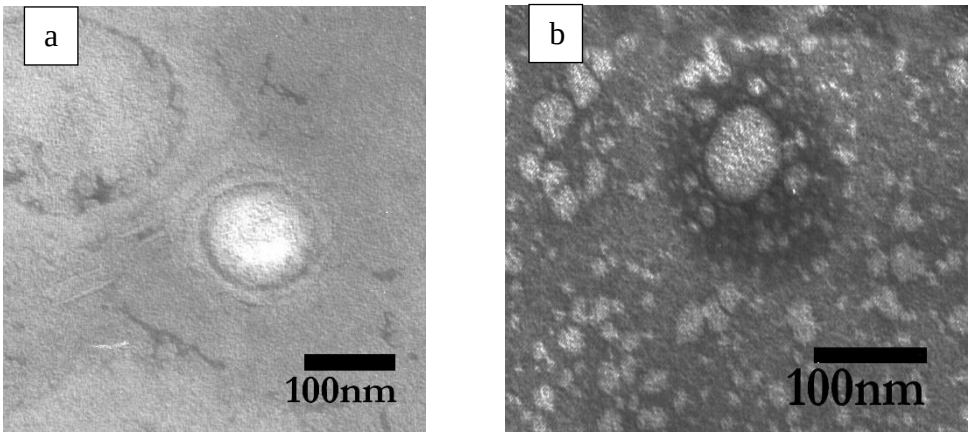


Figure No. 4: TEM results of formula A[a)] and formula C[b)]

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).

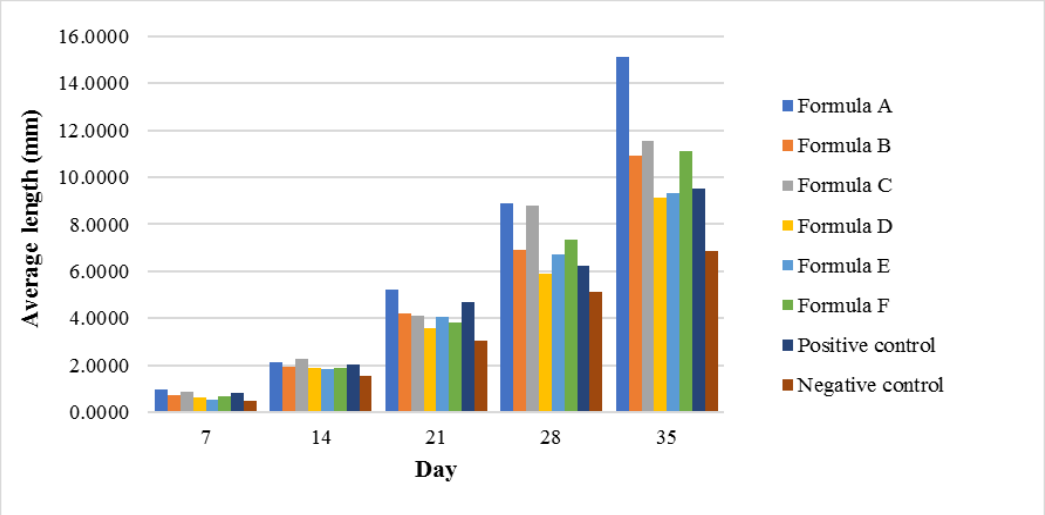


Figure No. 5: Hair tonic activity test against the average hair length on 35 days

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).

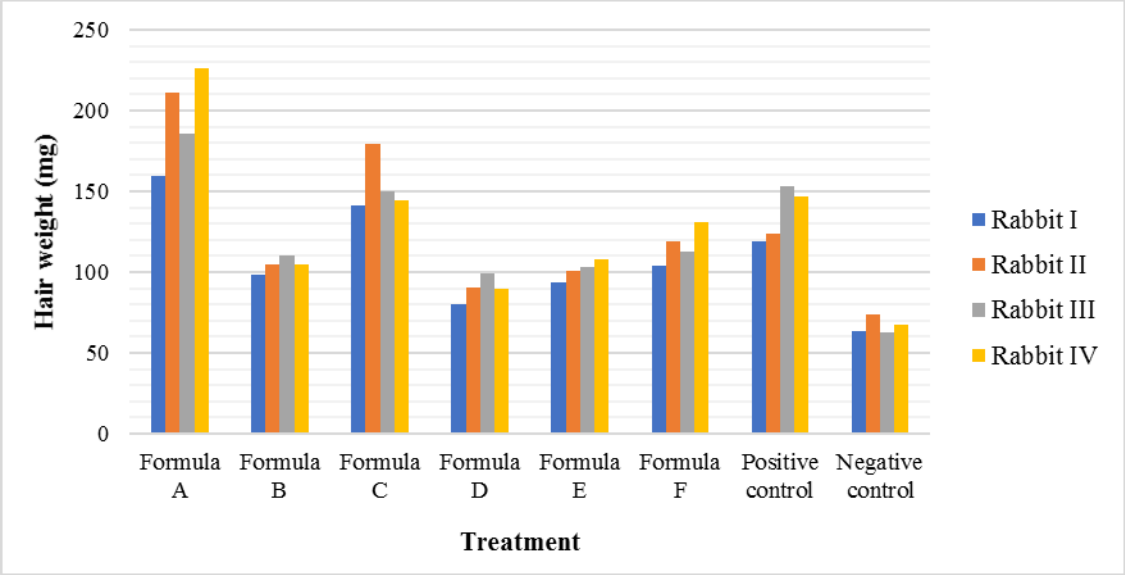


Figure No. 6: Hair tonic activity test against the average hair weight

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.

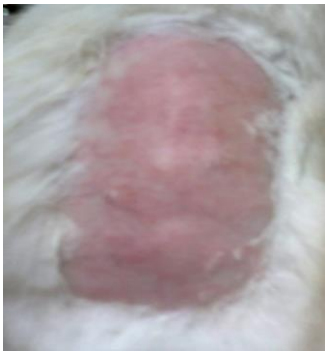
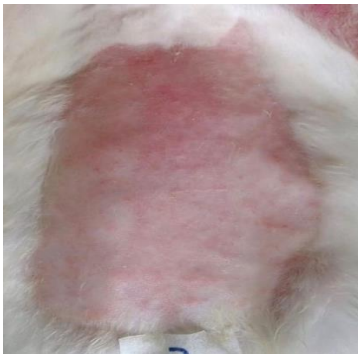
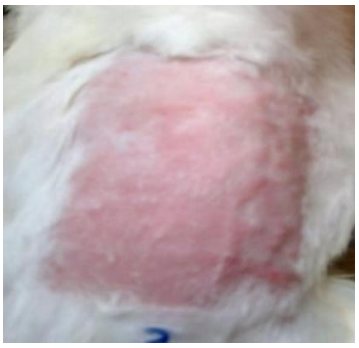
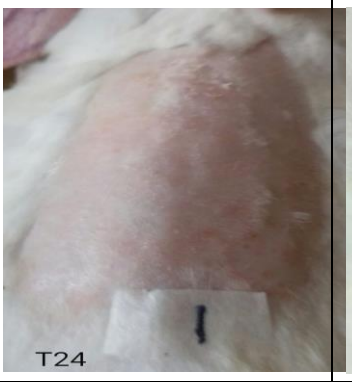
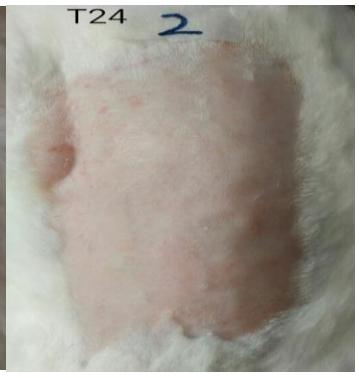
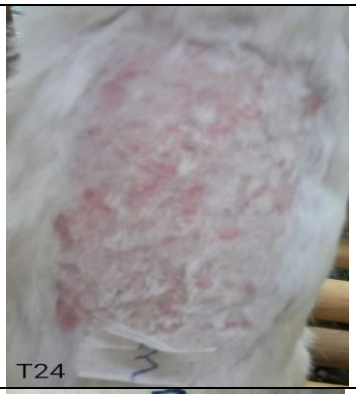
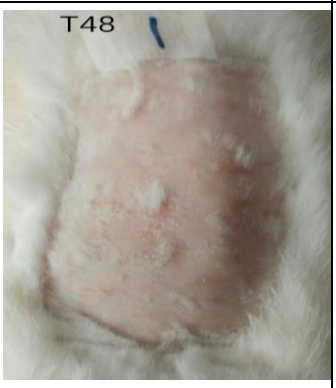
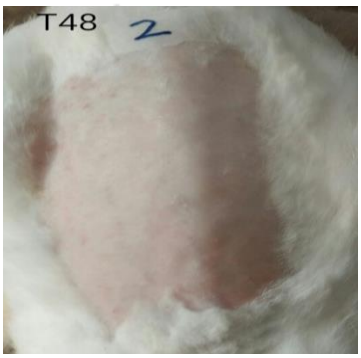
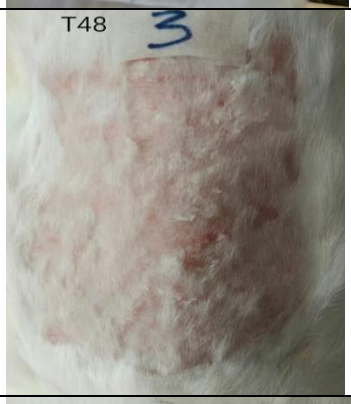
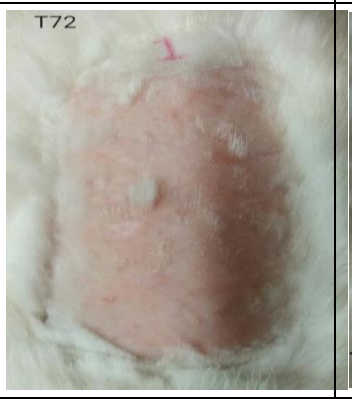
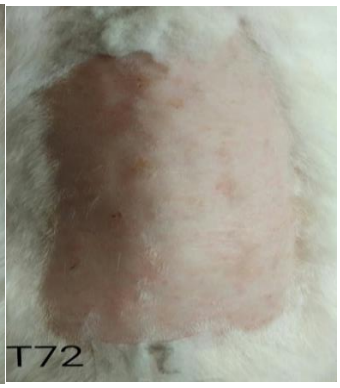
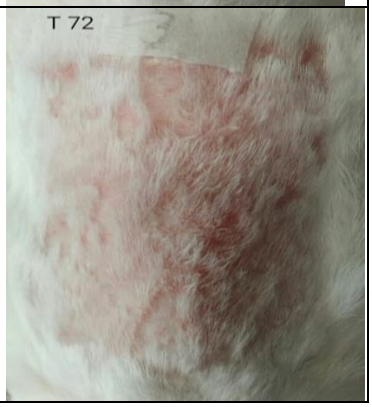
	Rabbit No.1	Rabbit No.2	Rabbit No.3
0 hours			
24 hours			
48 hours			
72 hours			

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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