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Activity of hair tonic preparation combining *Apium graveolens* L extract, *Tussilago farfara flower extract, Achillea millefolium extract, Cinchona succirubra bark extract* on male New Zealand white rabbits

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ABSTRACT: Numerous hair care products are currently available in the market, including shampoo, conditioner, hair mask, hair serum, and hair tonic. Unlike shampoo, hair tonic remains in contact with the scalp for a longer duration, potentially enhancing its effectiveness. *Apium graveolens* L (EDS) and a combination of *Tussilago Farfara* flowers, *Achillea Millefolium* L leaves, and *Cinchona Succirubra* L stems, marketed as ATG, are known for their hair growth-promoting properties. This study aimed to evaluate the hair growth efficacy, stability, and safety of a hair tonic formulated from EDS and ATG extracts. The prepared extract solution was applied to the shaved backs of male New Zealand rabbits, aged 4 months and weighing 2.5-3 kg, and observations were recorded on days 7, 14, 21, and 28. Parameters measured included hair length and weight. The results indicated that the F1 formulation yielded an average hair length of 9.89 mm and weight of 49.38 mg; F2, 10.36 mm and 49.5 mg; F3, 11.98 mm and 49.5 mg; F4, 17.44 mm and 60.63 mg; F5, 17.27 mm and 52.75 mg; and F6, 19.06 mm and 56.5 mg. Conclusively, the F6 formulation demonstrated the most significant results, with an average hair length of 19.06 mm and weight of 56.5 mg. The hair tonic formulation was found to be non-irritating to the skin and remained stable throughout the study.

KEYWORDS: *Achillea millefolium* L; *Apium graveolens* L.; *Cinchona succirubra* L. hair tonic; New Zealand; *Tussilago farfara*.

INTRODUCTION

Hair plays an important role in human social life. Not everyone has healthy hair, which can be caused by various factors such as systemic diseases, hormonal imbalances, nutritional deficiencies, intoxication, or genetic disorders [1]. Signs of unhealthy hair include hair loss, often necessitating the use of hair tonics.

The active ingredients found in celery leaves include apiin, apigenin, mannitol, inositol, asparagine, glutamine, kalina, linamarose, potassium, and sodium. Apigenin is recognized for its impact on stimulating hair growth by inhibiting Transforming Growth Factor (TGF) β1. TGF β1 is a compound produced by hair follicle cells at the transition from the anagen phase to the catagen phase. Inhibiting TGF β1 is believed to promote hair growth [2].

Tussilago Farfara flower extract [3], Achillea Millefolium L. leaf extract, and Cinchona Succirubra L. stem extract contain contain flavonoid compounds known for enzymatically stimulating hair growth [4]. They contribute to an increased Anagen phase by boosting Oxygen consumption, AMPS accumulation, and elevating Histamine levels [5]. These extracts have enzymatic capabilities to stimulate anaerobic glycolysis, thus promoting hair follicles in the anagen phase and reducing the telogen phase [6]. The combined extracts of *Tussilago Farfara Extract*, *Achillea Millefolium* L. leaf extract, and *Cinchona Succirubra* L. extract are marketed under the brand name ATG.

To enhance hair growth activity, research has been conducted to combine APG and AGT extracts, aiming for their complementary effects to further stimulate hair growth.

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MATERIALS AND METHODS

The materials utilized comprise EDS sourced from traditional markets in Banten, Indonesia and ATG obtained from Vevy Europe spa in Italy; minoxidil 0.2%; Propylene glycol; Tween 80; Phenoxy Ethanol; Disodium EDTA; Methyl Propandiol; Glycerin.

Rotary evaporator; PH meter; Brookfield Viscometer (DV-E); glassware; oven; tools for hair growth tests includes scissors; pet hair razor (Codos Pet Clipper CP-6800); vernier caliper (Absolute Coolant Proof Caliper Series 500, Mitutoyo).

The extraction method used in this research was maceration with 70% ethanol solvent.

Animal experiances

New Zealand White male rabbits aged 4 months and weighing between 2.5-3 kg, were obtained from the SKHB Laboratory Animal Management Unit, Bogor Agricultural Institute (Indonesia). Rabbits are fed twice daily, in the morning and evening. Testing commenced after a 7-day acclimatization period. All experimental protocols and procedures were reviewed and approved by the Animal Ethics Committee of the School of Veterinary Medicine and Biomedicine, IPB University (Approval No. 016/KEH/SKE/II/2023) adhering to the Guide for the Care and Use of Laboratory Animals.

Extract activity test on rabbit hair growth

The rabbits underwent a 7-day acclimatization period to familiarize them with the new environment and treatment procedures. The method involved shaving, dividing the area into four plots measuring 2 x 2 cm, with a 1 cm distance between plots [10], [11]. After shaving and Prior to applying the treatment on the rabbits' backs, the treatment area was cleansed with 70% ethanol for antiseptic purposes [12]. Each experimental formulation was applied to a single rabbit [13]. The treatment plan's layout is illustrated in Picture 1.

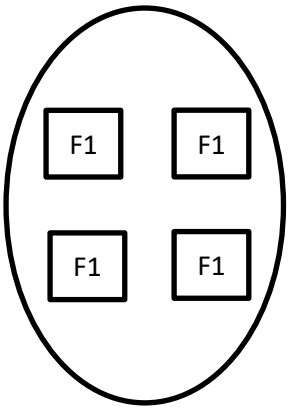

Figure 1. Treatment plan for hair growth test on a rabbit's back.

Information:
F1 : Smeared with hair tonic formulation without any extract (Negative Control)
F2 : Smeared with hair tonic Minoxidil 0.2%
F3 : Smeared with hair tonic containing 7.5% Celery Leaf Extract (EDS).
F4 : Smeared with hair tonic containing a combination of horse's foot flower extract, thousand leaf extract, quinine stem extract (AT) 15%
F5 : Smeared with hair tonic containing EDS 1 : AT 1 extract concentration
F6 : Smear with hair tonic containing EDS 1: AT 0.3 extract concentration

The application was conducted twice daily, in the morning and evening, using a volume of 0.5 mL for a duration of 28 days, starting from day 0. Observations were made at intervals of 7 days by extracting 10 strands of rabbit hair from each section. The length of the hair was measured using a caliper, while the weight of the hair was measured using a balance scale. Measurements were repeated on days 14, 21, and 28. Afterwards, the data obtained was analyzed statistically using the Kruskalls Walls test [19] and Mann Whiteney [20], separately the average hair weight used the Anova test [21], [22].

Hair tonic formulation

The formulations consisted of both single extracts and combinations of extracts. In line with references from previous studies, all individual extracts and their various combinations were tested. Details of the formulations can be found in Table 1.

Table 1. Hair tonic formulation.

No	Material	Amount				
		F1 (%)	F3 (%)	F4 (%)	F5 (%)	F6 (%)
1.	ATG	-	-	15	7.5	2.5
2.	EDS	-	7.5	-	7.5	7.5
3.	Na2EDTA	0.1	0.1	0.1	0.1	0.1
4.	Propylene glycol	10	10	10	10	10
5.	Tween 80	1	1	1	1	1
6.	Phenoxyetahnol	0.8	0.8	0.8	0.8	0.8
7.	Glycerin	5	5	5	5	5
8.	Water	Add 100	Add 100	Add 100	Add 100	Add 100

Information: F1: Deionized water
F2 : EDS 7.5%
F3 : ATG 15%
F4: Combination of EDS 7.5% with ATG 7.5% (1:1)
F5: Combination of EDS 7.5% with ATG 2.5% (1:0.3)

Evaluation of hair tonic preparations [23],[24]

Organoleptic

Hair tonic preparations were observed for organoleptic changes, observations were made visually including changes in color, odor, and clarity, the presence or absence of sediments at room temperature for four weeks.

Relative density

Measurements were taken using a pycnometer at room temperature. An empty pycnometer is weighed and then filled with water, the outside of the pycnometer is dried and weighed. The distilled water is discarded and the pycnometer is dried and then filled with hair tonic preparations that will be measured for relative density and then weighed.

Viscosity

Viscometer measurements of hair tonic preparations were conducted using a Brookfield DV-E viscometer. The hair tonic was placed under the appropriate viscometer spindle and the results were recorded with a stopwatch.

Homogeneity

Homogeneity testing was conducted by applying the preparation to a glass object and observing its homogeneity using a microscope.

pH

The pH test was conducted using a pH meter. The electrode on the pH meter was calibrated with a standard buffer of pH 4 and pH 7. Then the electrode was dipped into the preparation being examined and then waited until a stable value appeared and recorded the pH value that appeared on the screen. The pH of the hair tonic preparation is adjusted to the pH of the scalp, which is in the range of pH 4.5-6.6.

Hair tonic stability test

The purpose of this test is to assess the physical stability of hair tonic preparations at different storage temperatures. Hair tonic preparations were evaluated every 15 days, including organoleptic observations, homogeneity tests, pH tests, viscosity measurements, and relative density tests. It's conducted on preparations stored at 4°C ± 2°C, (25°C ± 2°C) dan suhu 40°C ± 2°C for a period of 3 months..

Hair tonic safety test

Irritation test was conducted on male New Zealand White rabbits in triplo. A 0.5 mL dose was applied to the rabbit's skin. Response assessment was carried out at 1, 24, 48 and 72 hours. If no skin damage occurred, the test can be continued until day 14 to determine reversibility.

RESULTS AND DISCUSSION

Table 2. Rabbit hair length.

Treatment	1st week	2nd week	3rd week	4th week
Positive Control	8.25 ± 1.86	9.63 ± 3.04	7.33 ± 2.36	9.89 ± 1.84
Δp	*0*	*1.38*	*2.30*	*2.56*
Negative Control	5.59 ± 1.37	10.52 ± 4.25	11.5 ± 2.90	10.36 ± 2.98
Δp	*0*	*4.93*	*0.98*	*1.14*
EDS 7.5%	9.58 ± 3.26	17.34 ± 3.80	10.54 ± 5.53	11.98 ± 4.32
Δp	*0*	*7.76*	*6.80*	*1.44*
ATG 15%	6.81 ± 2.77	12.22 ± 4.16	15.88 ± 2.61	17.44 ± 4.81
Δp	*0*	*5.41*	*3.66*	*1.56*
Extract Combination (EDS 7.5%: ATG 7.5%)	10.6 ± 7.01	12.22 ± 4.29	12.57 ± 4.65	17.25 ± 3.19
Δp	*0*	*1.66*	*0.34*	*4.68*
Extract Combination (EDS 7.5%: ATG 2.5%)	10.51 ± 2.32	15.4 ± 3.70	14.22 ± 5.75	19.06 ± 1.00
Δp	*0*	*4.90*	*1.19*	*4.84*

Information:
Δp: Difference in rabbit hair length

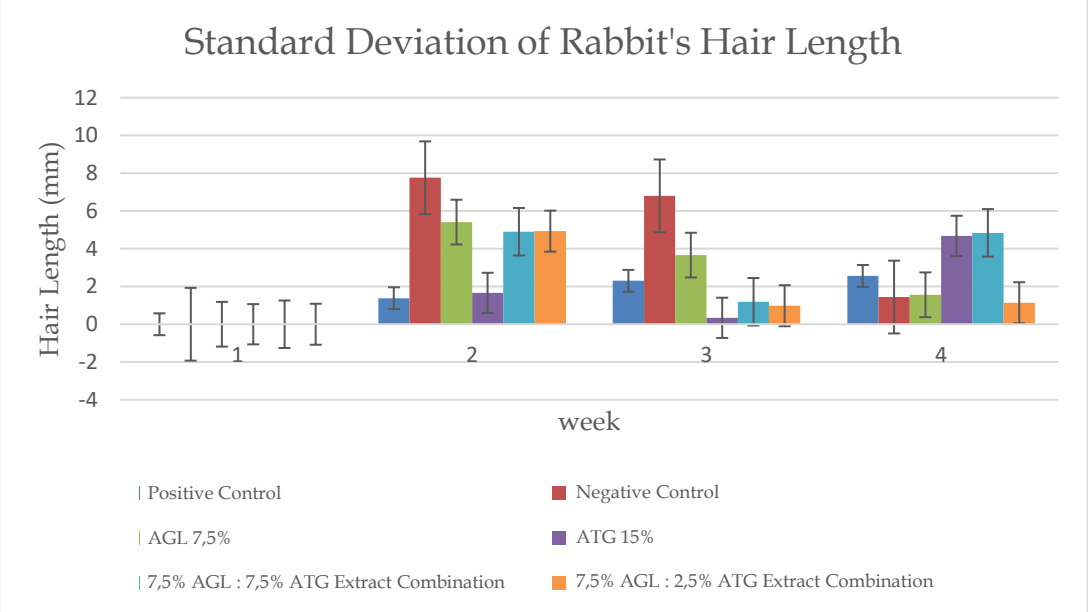

Figure 2. Standard deviation of rabbit's hair length.

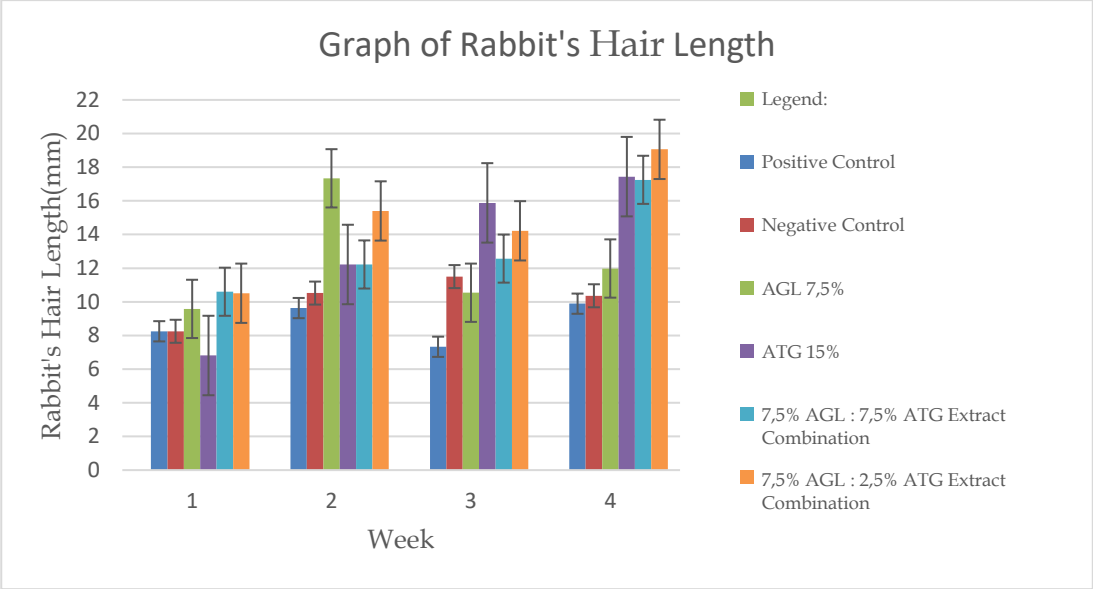

Figure 3. Rabbit hair length graph.

Weekly observations showed varying hair growth rates among different experimental groups: F2 (negative control) averaged 9.49 mm, F1 (positive control) 8.77 mm, F3 (EDS 7.5%) 12.36 mm, F4 (ATG 15%) 13.09 mm, F5 (EDS 7.5% : ATG 7.5%) 13.16 mm, and F6 (EDS 7.5% : ATG 2.5%) 14.80 mm. Comparing the data to the result for normal control, there was a notable increase in average hair growth in the treatment groups. Statistical analysis showed that the data are normally distributed. The data also indicated no significant differences in hair growth among treatment groups in the Kruskal-Wallis test, but the Independent T-test revealed a significant difference between normal controls and F6 ($P < 0.05$). This underscores F6's distinct hair growth activity compared to the normal group, likely due to synergistic effects between ATG and AT extracts. The chosen maceration method was preferred for its simplicity, despite concerns about heat-sensitive flavonoid compounds [7] – [9].

Table 3. Rabbit's hair weight.

Treatment	1st week	2nd week	3rd week	4th week
Positive Control	38.6 ± 26.44	46.38 ± 1.22	44.75 ± 4.24	49.38 ± 5.74
Δp	*0*	*7.78*	*1.63*	*4.63*
Negative Control	34.76 ± 5.21	42 ± 2.65	48.25 ± 1.20	45.75 ± 4.26
Δp	*0*	*7.24*	*6.25*	*2.50*
EDS 7.5%	26.81 ± 5.96	44.88 ± 2.03	40 ± 4.00	49.5 ± 6.20
Δp	0	18.06	4.88	9.50
ATG 15%	45.26 ± 9.79	46.38 ± 3.53	55.38 ± 9.23	60.63 ± 10.21
Δp	0	1.11	9.00	5.25
Extract Combination (EDS 7.5%: ATG 7.5%)	39.71 ± 7.81	45.13 ± 4.65	49.13 ± 2.15	52.75 ± 5.12
Δp	0	5.41	4.00	3.63
Extract Combination (EDS 7.5%: ATG 2.5%)	51.96 ± 24.72	50.38 ± 3.31	46.5 ± 1.94	56.5 ± 4.33
Δp	*0*	*1.59*	*3.88*	*10.00*

Information:
Δp: Differences in rabbit hair length

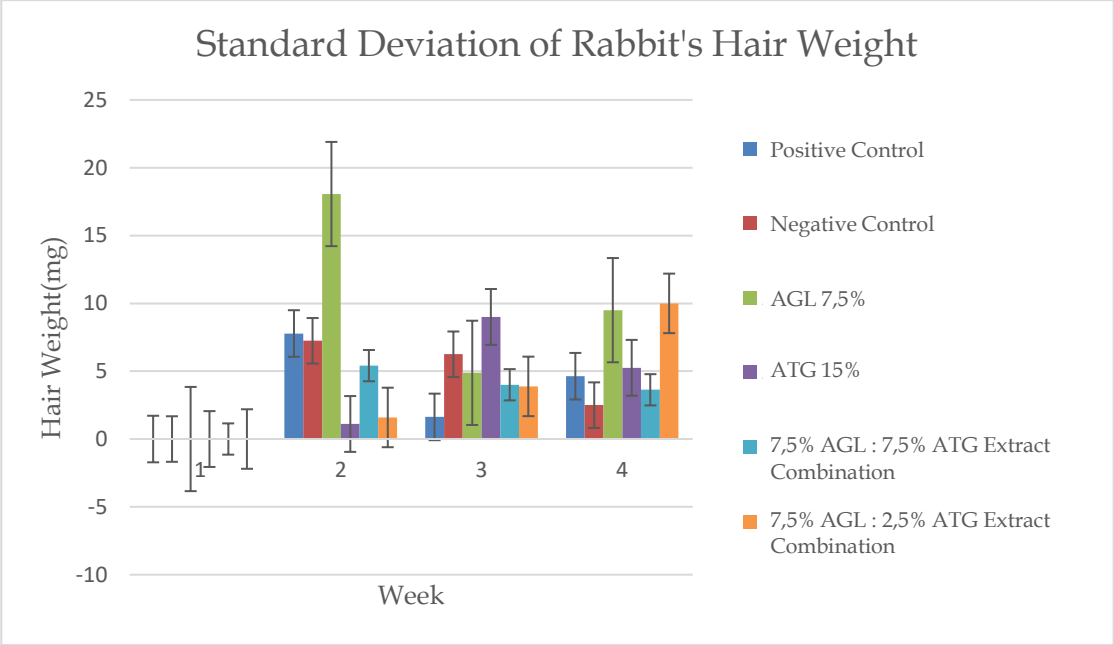

Figure 4. Standard Deviation of rabbit's hair weight.

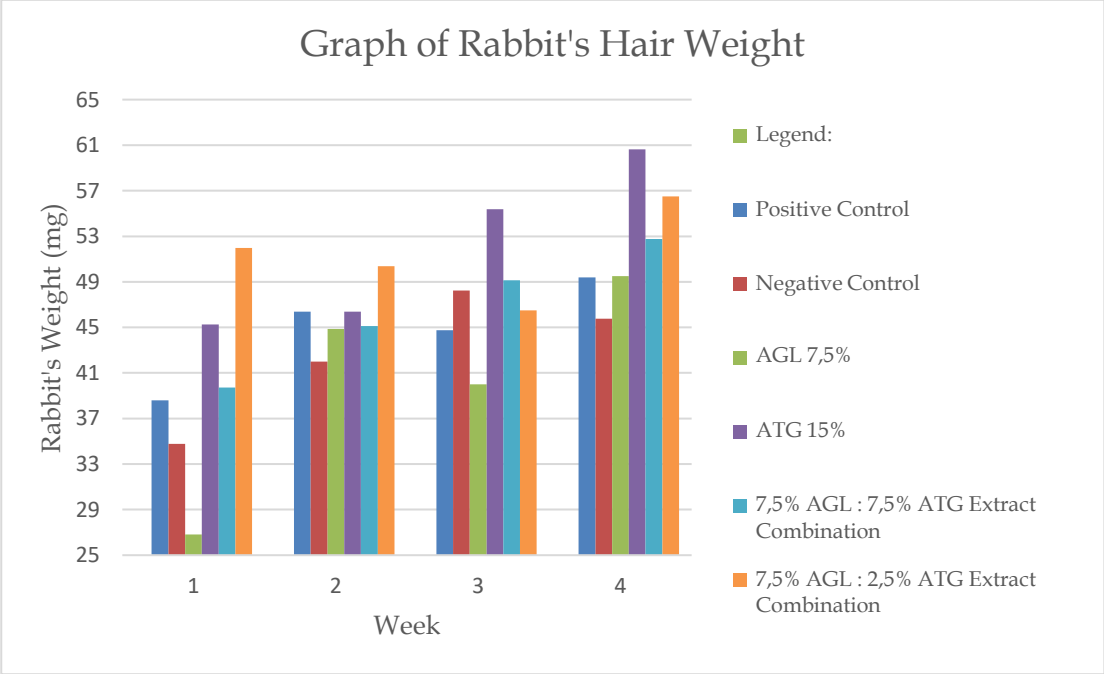

Figure 5. Standard deviation of rabbit's hair weight.

Hair weight growth was observed weekly, with normal controls showing an average of 42.69 mg for F2 (negative control), 44.78 mg for F1 (positive control), 40.30 mg for F3 (EDS 7.5%), 51.91 mg for F4 (ATG 15%), 46.68 mg for F5 (EDS 7.5% : ATG 7.5%), and 51.33 mg for F6 (EDS 7.5% : ATG 2.5%). Comparing the data to the result for normal control, there was a notable increase in average hair growth in the treatment groups. Statistical analysis showed that the data are normally distributed. The data also indicated no significant differences in hair growth among treatment groups in the Kruskal-Wallis test, yet the Independent T-test revealed a significant difference between normal controls and F6 ($P < 0.05$). This suggests that F6 exhibits distinct hair weight growth compared to the normal group. It is important to note that statistical significance,

as determined by the p-value and α value, may not always align perfectly with practical significance, which is reflected in the actual effectiveness observed.

The hair tonic formulation extracts combinations utilizes Na2EDTA as a chelating agent due to concerns about mineral interference from the water source, which could compromise formulation stability [14]. Propylene glycol is added to the formulation as a humectant to prevent liquid shrinkage in hair tonic preparations [15] – [17]. Phenoxyethanol serves as a preservative to inhibit microbial growth, particularly necessary because of the high amount of water content in the formulation [18] while Tween 80 acts as a solubilizer to facilitate dispersion of water-insoluble extracts within the preparation.

EDS contain EGCG and there is stimulates hair follicle cells and promotes hair regrowth by prolonging the anagen phase in vitro and in vivo models. The antioxidative and anti-inflammatory properties of EGCG help protect hair follicles from damage and encourage regeneration ATG. [25] ATG is recognized for its role in enhancing blood flow and nutrient delivery to hair follicles, which may stimulate growth. It works by activating cell regeneration in hair follicles and is often used in combination with other active ingredients for a synergistic effect in promoting hair growth. [26]

Table 4. Table of hair tonic evaluation results.

Characteristics	**Results**				
	F1	F3	F4	F5	F6
Form	Solution	Solution	Solution	Solution	Solution
Color	Clear	Black	Yellowish	Black	Black
Smell	No smell	Aromatic	Aromatic	Aromatic	Aromatic
Flavor	Bitter	Bitter	Bitter	Bitter	Bitter
Clarity	Clear	Clear	Clear	Clear	Clear
Homogeneity	Homogeneous	Homogeneous	Homogeneous	Homogeneous	Homogeneous
Specific gravity	0.9549	0.9435	0.9674	0.9577	0.9867
Viscosity	10.5 cps	11.5 cps	10.5 cps	12.0 cps	12.5 cps

Information : F1: Blanko
F3 : EDS 7.5%
F4 : ATG 15%
F5: Combination of EDS 7.5% with ATG 7.5% (1:1)
F6: Combination of EDS 7.5% with ATG 2.5% (1:0.3)

The table above presents the results of physical observations on hair tonic preparations, both for single extracts and combinations of extracts. Hair tonics containing EDS exhibit a black color and an aromatic smell. Organoleptic evaluations, which include physical observations at low, room, and high temperatures, revealed no changes in odor, homogeneity, or clarity across all formulations. Specific gravity measurements over 90 days at three different temperatures showed minimal variations, indicating that the hair tonic preparations maintain a relatively stable specific gravity regardless of storage conditions. Similarly, viscosity measurements over 90 days at these temperatures exhibited only minor changes, demonstrating that the formulations also maintain relatively stable viscosity. Homogeneity assessments at the three different temperatures indicated inconsistent results across formulations. However, pH measurements over 90 days at various temperatures revealed only slight changes, with values remaining within the skin's pH range. This confirms that the hair tonic preparations have a relatively stable pH under low, room, and high temperature storage conditions.

Table 5. Hair tonic safety test tab.

Group Test	Observation time							
	1 hour		24 hours		48 hours		72 hours	
	Erythema	Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema
1st formulation	0	0	0	0	0	0	0	0
2nd formulation	0.3	0	0.3	0	0	0	0	0
3rd formulation	0	0	0	0	0	0	0	0
4th formulation	0.3	0	0.3	0	0	0	0	0
5th formulation	0.3	0	0.3	0	0.3	0	0	0

Information : F1: Blanko
F3 : Celery Leaf Extract (EDS) 7.5%
F4 : Combined Extract (ATG) 15%
F5: Combination of EDS 7.5% with ATG 7.5% (1:1)
F6: Combination of EDS 7.5% with ATG 2.5% (1:0.3)

The irritation index for the hair tonic, calculated based on observation, falls within a very mild range of 0-0.4. In the hair tonic irritation test, formulations 2, 4, and 5 initially showed very mild irritation, which gradually improved, and by the 48-hour mark, no erythema was observed. The mild reaction is normal because when a new product is applied, the animal's skin may react to the active ingredients or compounds in the hair tonic and also many hair tonics are designed to enhance blood circulation to the scalp. This increase in blood flow can lead to a warming sensation or initial irritation that the animal may experience.

CONCLUSION

The combination of 7.5% APG and 2.5% ATG formulation (F6) showed the highest hair nourishing effect of 19.06 mm for hair length and 56.5 mg of hair weight. The combination formulation of APG and ATG extracts is formulated into a hair tonic preparation that is stable in homogeneity, organoleptic and pH. APG and ATG hair tonic preparations do not irritate rabbit skin.

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