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The Influence of Menthol in Ethanol Extract of Meniran Gel (*Phyllanthus niruri* L.) as a Hair Growth Promoter in Male Wistar Rats

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ABSTRACT

Phyllanthus niruri L. is known to promote hair growth. Menthol is one of the enhancers which promotes drug penetration into the skin. The aim of this research is to determine the potential of menthol in *P. niruri* Ethanol Extracts gel preparation as a hair growth promoter. The test animals were randomized into 4 groups: normal group, negative control (gel without extract and menthol), E1 group (Ethanol Extracts of *P. niruri* 5% gel without menthol) and E2 group (Ethanol Extracts of *P. niruri* 5% gel with menthol). Hair growth activity test was conducted by applying the gel on the rats' back, and hair length was measured on the 7th, 14th and 21st days, while the hair was weighed on the 21st day. The results showed that the E2 group had an average hair length and hair weight of 12.45±3.457 mm and hair weight of 28.53±7.681 mg, greater than the E1 group of 10.67±2.455 mm and 19.87±9.552 mg respectively. Based on these results, it can be concluded that adding menthol in *P. niruri* Ethanol Extract gel preparation can influence hair growth and thickness.

Keywords: *Phyllanthus niruri* L., Gel, *Enhancer* menthol, hair growth

INTRODUCTION

Hair is the outermost part of our body which acts a protective layer. In human, the hair also plays an important role in appearance. Hair loss could be attributed to some factors, such as heredity, malnutrition, vitamin deficiency, hormonal, diseases or medical treatment (chemotherapy), inflammation, as well as physical and emotional stress (Patel *et al.,* 2015). Meniran (*Phyllanthus niruri* L.) is a well known medicinal herb which possess several pharmacological activities, one of them being a hair growth promoter (Patel *et al*., 2015), aside from having immunomodulator, antidiabetic, antihypertension, antibacterial, antiinflammation, antiviral and antioxidant properties (Patel *et al.*, 2011; Adeneye, 2012; Aldi *et al.,* 2014; Da'i *et al.*, 2016). Based on past research, it is known that the 2% petroleum ether extracts of *P. niruri* promote hair growth in albino rats with severe alopecia, by inhibited 5α-Reductase. Lignan and terpenoid are suspected to have this hair regrowing properties (Patel *et al*., 2015). Several compounds such as flavonoid, alkaloid, tannin, and saponin also have the ability to promote hair growth (Patel *et al*., 2015). The phytochemical screening of *P. niruri* ethanol extracts shown the presence of lignan, flavonoid, terpenoid/steroid, tannin and phenol (Bagalkotkar *et al.,* 2006).

Natural ingredients or plant extracts which can be used as a hair growth promoter could be a formulation as a gel, oil or tonic (Patel *et al.,* 2015). Gel humidifies and provide a soothing sensation to the scalp, while it is easy to apply, and to wash. Stratum corneum is the main barrier which prevents penetration of drugs through the scalp, mainly because of its structure. One of the methods to improve skin permeability is to add an enhancer such as menthol. Menthol has shown the ability as an effective enhancer of drug molecules to penetrate through the epidermis, mainly because menthol is able to penetrate and increases lipid layer of the stratum corneum by forming capillary tract (Rajesh *et al.,* 2010).

Based on the study, the researcher formulates a gel from *P. niruri* ethanol extracts with menthol added as an enhancer to promote hair growth. Viscolam is used as the gelling agent. Viscolam is a gelling agent which will not form a layer of the film upon applied to the skin, which will ensure comfortability and smoothness during its application. Parameters to determine hair growth are hair length and weight.

METHODOLOGY

Equipment and Materials

Equipment used in this research were shavers (*Gillette*®), Vernier caliper (*TRICLE BRAND*), digital scale (*Precisa XT 220A*), evaporator (*Buchi R-100*), microscope (*Olympus CX22LED*), oven (*Modena type BO 3633*), hot plate (*SJ Analytics GmbH type D-55122 Mainz*), glassware (*Iwaki Pyrex*®) and pH meter (*HANNA*).

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Table I. Base Optimization Formulation

No.	Materials	Formulation		
		F1(%)	F2(%)	F3(%)
1.	*P. niruri* Herbs Extract	5	5	5
2.	Viscolam MAC 10	6	8	10
3.	DMDM Hydantoin	0,5	0,5	0,5
4.	Sodium Metabisulfite	0,02	0,02	0,02
5.	TEA	1,49	1,675	1,859
6.	Propylene Glycol	5	5	5
7.	Aquadest	Add 100	Add 100	Add 100

Materials used in this research were powdered *P. niruri*, Phyllanthin, Hypophyllanthin, stigmasterol (from Islamuddin's research), quercetin (Sigma Aldric), viscolam MAC 10, DMDM Hydantoin (*Sharon*), Sodium Metabisulfite, triethanolamine (TEA) (*Clorogreen*), propylene glycol, Menthol (Pharmaceutic), Aquadest (local), $FeCl_3$(Pharmaceutic, Brataco), Mg Tape (Various), Concentrated HCl, Chloroform (pro analysis), H_2SO_4, Mayer's Reagent, Dragendorff Reagent, Wagner Reagent, n-hexane (pro analysis), CH_3COOH, KOH, H_2O_2, Benzene (pro analysis), Ethanol, NaCl, Gelatin, Silica Gel (*E Merck*), and GF_{254} TLC plate (*E Merck*).

Preparation of Test Animal

Male Wistar rats (*Rattus norvegicus*) were 2-3 months old, weighing about 150-250 grams. The animals were procured from Bantul, Yogyakarta. This research has passed ethical study, which is approved by Ethical Study Division of the Faculty of Medicine, Tanjungpura University, letter no. 595/UN22.9/DL/2018.

Sample Preparation

The sample, which is powdered *P. niruri* herbs, was obtained from supplier & distributor of herbal medicine "Herbal Anugrah Alam", Yogyakarta, Indonesia.

Extraction of *P. niruri L.* Herbs

About 1.128 g of *P. niruri* powders were macerated using 16,5 L of ethanol for 21 days in room temperature. The extract is sieved using cloth, then evaporated at $\pm 40^0$C

Extract Characterization

Water Soluble Extract

About 1,014±0,005 g of extracts are dissolved in a 25 ml mixture of water and chloroform (1:9) using a closed flask whilst being shaken for the first 6 hours and let to settle for 18 hours. The mixture is sieved, then 5 ml of filtrate is evaporated using tared shallow flat bottom crucible. The residue is then heated at 105°C until it reaches a constant weight. A percentage of water-soluble extract is then calculated, relative to initial extract

Phytochemical Screening

Phytochemical screening is conducted with color tests and thin layer chromatography (TLC). TLC of lignan uses GF_{254} silica gel plate as the stationary phase, with hexane: ethyl acetate (7:3 v/v) mixture as mobile phase. Phyllanthin and Hypophyllanthin are used as a comparative compound. Identification of flavonoid uses chloroform: methanol: water (80: 12: 2 v/v) mixture as mobile phase, quercetin is used as a comparing compound. Terpenoid test uses n-hexane: ethyl acetate (8: 2 v/v) mixture as the mobile phase, while stigmasterol is used as a comparison.

Determination of Total Flavonoid

About 3,27±0,115 mg of the extract is inserted into a 5 ml volumetric flask and is diluted using methanol. 2 ml of the diluted extract is then taken. Add 0,1 ml of 10% aluminum trichloride ($AlCl_3$), 0,1 ml of sodium acetate (1M), and 2,8 ml of distilled water. The mixture is left to rest for 30 minutes in room temperature. Measure absorbance at 434 nm. Quercetin is used as a comparison.

Total Flavonoid content is calculated as below:

$$TFC = \frac{RxD.Fx\ V\ x\ 100}{W}$$

Legends= TFC : *Total Flavonoid Content*; R : *Result*; D.F : *Dilution Factor*; V : *Volume*; W : *Weight.*

Optimization of Gel Base Concentration

Optimization of gel base concentration is conducted by making gel bases with active substances, without the enhancer menthol, which is done by dissolving viscolam MAC 10 at 6, 8 and

Table II. Gel Preparation Formula

No.	Materials	Formulation	
		E1 (%)	**E2 (%)**
1.	*P. niruri* Herbs Extract	5	5
2.	Viscolam MAC 10	10	10
3.	DMDM Hydantoin	0,5	0,5
4.	Sodium Metabisulfite	0,02	0,02
5.	TEA	1,859	1,859
6.	Propylene Glycol	5	5
7.	Menthol	-	1
8.	Aquadest	Add 100	Add 100

Table III. Skin Reaction Scoring

Formation of Erythema	Score
No Erythema	0
Minimum Erythema (nearly indistinguishable) (diameter <25 mm)	1
Clear Erythema (diameter 25,1-30 mm)	2
Moderate to severe erythema (diameter 30,1-35 mm)	3
Severe erythema (exposed tissue), formation of eschar which interferes with erythema scoring (Diameter > 35 mm)	4
Formation of Edema	
No Edema	0
Tiny Edema (Nearly indistinguishable)	1
Light Edema (Area is clearly visualized) (thickness < 1 mm)	2
Moderate Edema (Diameter increased by 1mm)	3
Severe Edema (diameter increased by 1 mm and swells over drug exposure tests.	4

Legends = a : Total erythema and edema score of all sample observation spot on 24, 48 and 72 hours, divided by number of observations; b : Total erythema and edema score on all control observation spot, on 24, 48 and 72 hours, divided by number of observations; c: Number of animals.

10% using aquadest. TEA is added as an alkalizing agent to achieve a pH of 6-7 (Edityaningrum & Heni, 2015). DMDM Hydantoin is then added as a preservative. Extracts which are diluted using propylene glycol and sodium metabisulfite beforehand is added. The extract is then added into a gel base and is stirred until homogenous and 100 ml of water is added, then a spread test is conducted. A Viscolam MAC at 10% is then chosen as it conforms to the requirement or standards.

Gel Formulation and Evaluation

The concentration of Viscolam MAC at 10% passed the requirement, which is then formulated with the addition of Menthol 1% as an enhancer. Evaluations ranging from organoleptic, pH, spreadability, and adhesive capability.

Irritation Study

The test animals are divided into 3 groups, which are formula E1, formula E2, and normal control, with 6 animals within each group. The back of the animal is then shaved on three separate locations. About 0,5g of test substance is then exposed to the back of the animal. Exposure site is then covered with gauze and plaster. In three separate parts of the rats back from each group, one test substances are applied, such as formula E1 or E2. Meanwhile the normal control group receives no gel. The rats are then kept for 24,48 and 72 hours. After 24 hours observation, first test substance (located at top part of the back) is opened and observed. 48 hours observation is conducted by opening the center part of the back, while for 72 hours observation, the lower part of the back is then opened and observed (Darwis, 2008). All animals are checked for possible erythema and edema.

Table IV. Irritation Response Category (Kepala BPOM RI, 2014)

Mean Value	Primary Irritation Index Category
0,0 – 0,4	Not significant
0,5 – 1,9	Slight Irritation
2,0 – 4,9	Moderate Irritation
5,0 – 8,0	Severe Irritation

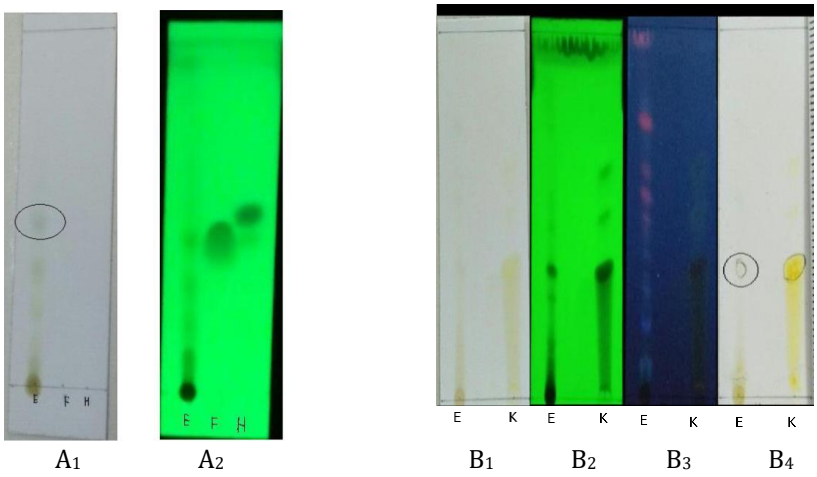


Figure 1. Chromatogram Profile of *P. niruri* Ethanol Extract for Lignan (A1 & A2) and Flavonoid (B1-B4)

Legends :

- TLC of Lignans
  Stationary Phase : GF_{254} Silica Gel; Mobile Phase (A_1&A_2) : n-hexane : ethyl acetate (7:3); E : Ethanol Extract (*P. Niruri* L.); F : Phyllanthin; H : Hypophyllanthin; Detection Method : A_1 Visible Light, A_2 UV 254 nm.
- TLC of Flavonoids
  Stationary Phase : GF_{254} Silica Gel; Mobile Phase (B_1-B_4) : Chloroform: Methanol : Water (80:12:2); E : Extract; K : Quercetin; Detection Method : B_1 (Visible Light), B_2 (UV 254 nm), B_3 (UV 366 nm), B_4 (After $AlCl_3$ 5% spray).

Hair Growth Activity Test

Twenty rats were divided into 4 random groups. The hair on the back of each rat is shaved to form an area of 4x4 cm, then depilatory cream is applied. The shaved rat skin is cleaned using 70% alcohol. At the middle of the rats back, a 2x2 cm rectangle is made. The rats are left for 24 hours. The first day, which sample is applied to the back of the rat is considered as day 0. Test parameters including hair length and weight.

Data Analysis

Analysis of hair length and weight data uses SPSS 22 software. Data is tested for normal distribution and its homogeneity of variance (α>0,05). The data is then tested using One Way ANOVA and LSD post hoc test with a confidence rate of 95%.

RESULTS AND DISCUSSION

Preparation and Extraction

The sample used for this experiment is powdered *P. niruri* herbs. About 284,14 g of extract was obtained, containing 25,18% of rendement.

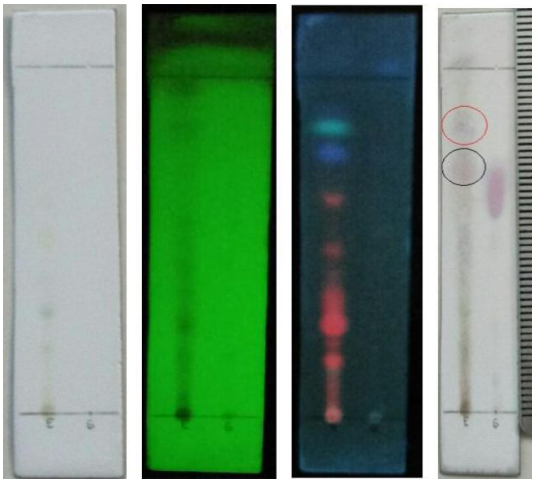
Extract Characterization

Theoretically, the water-soluble content of *P. niruri* is not less than 16% (Depkes RI, 2008). The water-soluble content of *P. niruri* ethanol extract were 32,31±2,92%. Based on the result, it can be concluded that *P. niruri* ethanol extract conforms to the water-soluble standard set by herbal pharmacopeia.

Based on the phytochemical screening result, *P. niruri* ethanol extract contains several secondary metabolites, which were phenol, flavonoids, alkaloids, terpenoid/steroids, tannin, and saponin. The result of TLC which uses spot and comparison compound method shows that *P. niruri* ethanol extract contains lignan, flavonoid, and steroids. Lignan chromatogram profile of *P. niruri* ethanol extract, which uses n-hexane: ethyl acetate (7:3 v/v) mixture as the mobile phase (Figure 1 A). For flavonoids, a polar mixture of chloroform: methanol: water (80:12:2) mobile phase is used. The mobile phase mixture is chosen mainly because of the polar nature of quercetin as a comparison compound, which will elude the sample effectively. Figure 1 B shows a spot after being sprayed with $AlCl_3$ 5% as a visualizer. The spot has a retention factor (Rf) of 0,345, whereas quercetin as the comparison compound also scores the same Rf. This is according to the literature, which states that the Rf value of *P. niruri* herbs extracts and quercetin, with the same solvent and eluent, which is 0,30.

Chromatogram profile for terpenoid/ steroid tests (Figure 3). A nonpolar mixture of n-

hexane: ethyl acetate (8:2) is used as mobile phase. This eluent is chosen as stigmasterol, the comparing compound is nonpolar. After being sprayed by vanillin-H2SO4 in ethanol visualizer, and then heated, a purple spot is seen on the plate, confirming the presence of terpenoid/steroid, which means that the eluent used will be able to elude the sample successfully. The Rf value for spot no. 1 is 0,843, where the Rf value for spot no. 2 is 0,686, and stigmasterol as a comparison compound has an Rf of 0,647. Based on the results, it can be concluded that spot no.2 has stigmasterol, as the Rf value of spot no.1 and the comparison are similar.



Legends = Red Circle : Spot no. 1; Black Circle : Spot no. 2; Stationary Phase : GF_{254} Silica Gel; Mobile phase : n-hexane : ethyl acetate (8:2) v/v; Solvent : n-hexane; Comparing compound : Stigmasterol; Detection : Vanilin-H_2SO_4 10% in ethanol, UV 254 nm and UV 366 nm. A : Chromatogram Profile after elucidation; B : Chromatogram Profile after elucidation and UV 254 nm; C : Chromatogram Profile after elucidation and UV 366 nm; D : Chromatogram Profile after being sprayed by vanilin-H_2SO_4 10%.

Figure 2. Chromatogram Profile of *P. niruri* L. Ethanol Extract for Terpenoid Test

Total Flavonoid Content

For total flavonoid content determination, quercetin is used as the standard. Quercetin is made into 6 concentration series, which are 9, 12, 15, 18, 21 and 24 pp. The equation for quercetin standard curve is y = 0,0358x – 0,0261. Theoretically, total flavonoid content in *P. niruri* is not less than 0,9% calculated as quercetin (Depkes RI, 2008). Determination of total flavonoid in *P. niruri* ethanol extract resulted in 2±0,15% yield. Based on the results, it can be concluded that *P. niruri* achieved the standard set by the herbal pharmacopeia.

Optimization of Gel Base Concentration

Optimization of gel base is conducted to determine the optimum formula for the gel which will be applied to the skin. Physical characteristic observed during the determination for optimum formulation is spreadability, while additional chemical characteristic which can be used is pH. The result of gel spreadability (Table V).

The result of spreadability showed that there were differences in spreadability. Lower viscolam concentration contributed to the higher spreadability of the gel along with the increase in weight. The results of spreadability measurement from the lowest to the highest were gel formula No. F3, F2, and F1, respectively. The F3 gel formula is considered excellent due to its spreadability of 5-7 cm (Garg *et al*., 2002).

Formulation and Evaluation of Gel

The formulation consists of 2 gel formula, which is E1 (without menthol as an enhancer) and E2 (with 1% menthol as an enhancer). The usage of menthol as enhancer is to increase the capability of *P. niruri* ethanol extract gel as a hair growth promoter.

Evaluation of physicochemical properties of the gel formulation can be seen below:

a. *P. niruri* Ethanol extract gel without menthol (E1) is dark brownish in color, has an aroma of the extract, and is semi-solid.
b. *P. niruri* Ethanol extract gel with menthol (E1) is dark brownish in color, has an aroma of the extract and menthol, with a pH of 6,48±0,006 and is semi-solid.

Irritation Study

The result of irritation study in (Table VI) revealed that there was no irritation observed with the use of gel with or without menthol as enhancer.

Hair Growth Activity Study

Calculation of mean hair length for every gel treatment up to 21 days (Figure 1).

Observations conducted at the 7^{th} and 14^{th} day shows hair growth in which the group saw an increase of mean hair length from E1 and E2 formula compared to normal control group. ANOVA tests showed significant differences with the normal control group, meanwhile between E1 and E2 formula group, the differences are not significant. According to the graph, E2 formula has decent mean hair growth than E1 Formula. This means that the addition of menthol as an enhancer

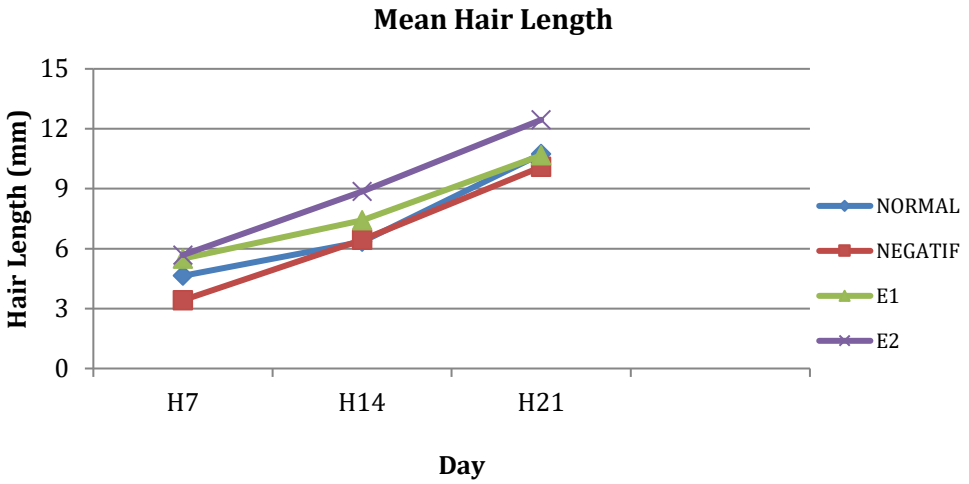


Figure 3. Mean Hair Length after Weekly Treatment

Table V. Average Spreadability

Formula	Diameter (cm)				pH
	Watch glass	50 g	100 g	125 g	
F1	6,85±0,05	7,23±0,104	7,32±0,076	7,37±0,076	6.53±0,017
F2	6,45±0,132	6,87±0,153	7,08±0,115	7,20±0,173	6.53±0,006
F3	5,45±0,05	5,72±0,029	6,07±0,104	6,32±0,076	6.51±0,012

has the ability to improve hair growth, compared to the gel without menthol at the 7th and 14th day.

On the 21st day, mean hair length data for normal control, E1 and E2 formula were 10,74±1,59; 10,67±2,455 and 12,45±3,457 mm respectively. Mean hair growth is also observed from formula E1 and E2 against normal control. The ANOVA tests result shows no significant differences between all treatment groups (α>0,05). However, based on the results, the group treated with formula E2 has a better hair length on average than the group treated using formula E1. This means that adding menthol as an enhancer improves hair growth than the formula without menthol in 21st day.

The capability of hair growth exhibited by *P. niruri* ethanol extract is suspected to be caused by the presence of its flavonoid (quercetin), phenol and terpenoid. Flavonoid (quercetin), phenol and terpenoid are known to inhibit 5α-reductase, in which this enzyme converts testosterone to 5α-DHT (5α-dihydrotestosterone), and 5α-DHT will bind to androgen receptor which contributes in hair loss (Hiipakka, 2002).

The gel formulation with menthol as an enhancer has longer duration of hair growth activity compared to the gel formulation without menthol. This is due to menthol as an enhancer improves diffusion of a substance by disrupting lipid structure and by increasing permeability of the gel (Kamatou *et al.*, 2013). In research which studies the permeability of minoxidil, the use of menthol and clove oil increases the permeation of minoxidil on the test animals via in vitro (Moghimipour *et al.,* 2018). Based on the research by Oh *et al.,* the group which is given menthol (peppermint) shows improved hair growth compared to other treatment groups which formula is enhanced with saline oil, minoxidil 3% and jojoba oil (Oh *et al.,* 2014)

The observation of average hair weight (Figure 2) in normal control, formula E1 and E2 groups resulted in 2,67±1,986; 12±7,472; 19,87±9,552 and 28,53±7,681 mg respectively. Based on the data between gel formula E1 and E2, the test group which is given the gel formulation with menthol added has heavier hair weight compared to the group which rats were given the formula without added menthol. This also shows that the addition of menthol also increases the amount and the volume of the hair.

Heavier hair weight means higher volume of hair in test animals. The significance of hair weight differences is calculated using ANOVA tests, which resulted in significant differences between normal control group against formula E1 and E2, although between E1 and E2, there were no significant differences. It can be concluded that both *P. niruri*

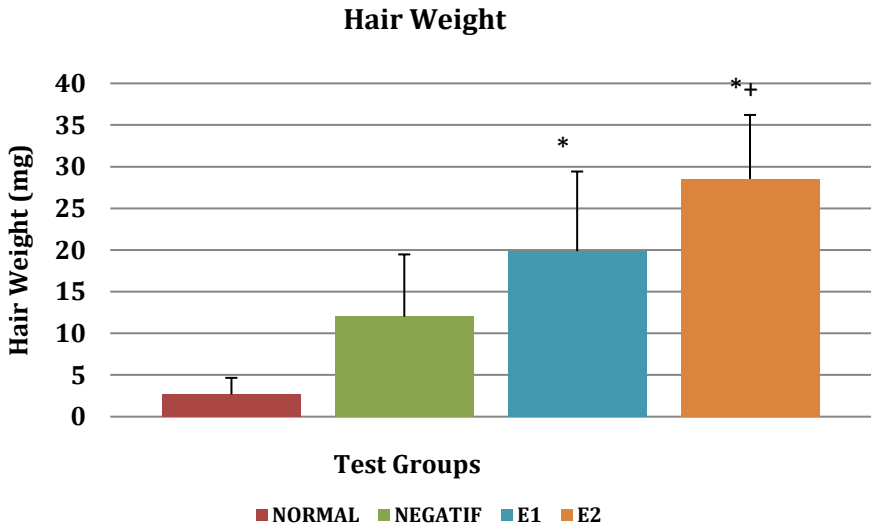


Figure 4. Average Rats Hair Weight on the 21st day

ethanol extract gel formulation has the same hair thickening activities, compared to the normal group.

CONCLUSION

P. niruri ethanol extract gel with added 1% menthol has shown better hair length and weight of the test animals compared to the other gel formulation without menthol, with an average hair length of 12,45±3,457 mm and an average hair weight of 28,53±7,681 mg, whereas the gel formulation without enhancer yielded 10,67±2,455 mm of average hair length and 19,87±9,552 mg of hair weight.

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