



Development of an Ethnopharmacognosy-Based Traditional Herbal Hair Growth Formula from East Nusa Tenggara: A Multiparametric *In Vivo* Evaluation

Burhan Ma'arif^{1*}, Seow L. Jing², Abdul Wafi³, Novia Maulina¹, Erlangga S. Subiyakto¹, Maximus M. Taek⁴

¹Department of Pharmacy, Faculty of Medicine and Health Science, Universitas Islam Negeri Maulana Malik Ibrahim Malang, Malang, East Java, Indonesia

²Pharmacy Programme, Universiti Kuala Lumpur Royal College of Medicine Perak, Ipoh, Perak, Malaysia

³National Research and Innovation Agency (BRIN), Gunungkidul, Special Region of Yogyakarta, Indonesia

⁴Department of Chemistry, Faculty of Mathematics and Natural Sciences, Widya Mandira Catholic University, Kupang, East Nusa Tenggara, Indonesia

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ABSTRACT

Chemotherapy-induced alopecia is a visible and psychologically distressing adverse effect of anticancer therapy, supporting the search for safer and culturally relevant hair-growth interventions. This study evaluated an East Nusa Tenggara ethnomedicinal herbal formulation containing *Aleurites moluccanus*, *Hibiscus rosa-sinensis*, *Ficus benjamina*, *Polyscias scutellaria*, and *Hibiscus tiliaceus* in a croton oil-induced inflammatory alopecia model. Two independent male mouse cohorts were used to assess clinical hair growth score, hair length, hair weight, and hair follicle thickness, with 5% minoxidil and vehicle-treated groups as comparators. The formulation produced higher clinical hair growth scores than 5% minoxidil from days 3 to 7, although its day-8 score was lower than that of minoxidil (2.17 ± 0.24 versus 2.83 ± 0.24), and produced the highest day-28 hair length at 6.3 ± 0.8 mm. Welch analysis of variance showed a significant difference in day-28 hair length among groups ($p = 0.001$), with greater values than minoxidil and untreated normal control. Hair weight increased earlier during the third week, although endpoint values were comparable with minoxidil and vehicle-containing controls. Histological analysis showed the highest hair follicle thickness in the formulation group at 47.04 ± 7.64 μ m, with a significant overall treatment effect ($p = 0.013$) and significant differences from all comparator groups after adjustment. These findings support further development of this ethnomedicinal formulation as a plant-based topical preparation for alopecia management.

Keywords: Ethnomedicine, Chemotherapy-induced alopecia, Clinical hair growth score, Hair length, Hair weight, Hair follicle thickness.

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Introduction

Cancer remains one of the leading causes of mortality worldwide. According to Global Cancer Statistics 2022, approximately 20 million new cancer cases and 9.7 million cancer-related deaths were estimated globally, with a five-year prevalence of approximately 53.5 million cases. In Indonesia, an estimated 408,661 new cancer cases and 242,988 cancer-related deaths were recorded during the same period.¹ Chemotherapy remains a major component of cancer treatment because of its potent cytotoxic activity against rapidly proliferating malignant cells. However, its limited selectivity may also damage normal tissues with high proliferative activity, resulting in adverse effects such as bone marrow suppression, gastrointestinal toxicity, and immune dysfunction.² Among these adverse effects, chemotherapy-induced alopecia (CIA) is one of the most visible and psychologically distressing dermatological consequences of systemic anticancer therapy, with substantial effects on patients' body image, emotional well-being, and quality of life.³

*Corresponding author. Email: burhan.maarif@farmasi.uin-malang.ac.id
Tel : +62 813-3555-5725

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Chemotherapy-induced alopecia (CIA) is among the most visible and psychologically distressing adverse effects of systemic anticancer therapy. The sudden and extensive loss of hair may negatively affect body image, self-esteem, emotional well-being, and social functioning, with the burden being particularly pronounced among women receiving chemotherapy.^{3,4} Minoxidil is commonly used to promote hair regrowth and may accelerate recovery following chemotherapy; however, current evidence does not demonstrate consistent prevention of hair loss during cytotoxic treatment.³ Moreover, topical minoxidil requires repeated and prolonged application and may cause scalp irritation, pruritus, erythema, contact dermatitis, and unwanted facial or body hair growth, which can reduce treatment acceptability and adherence.⁵ These limitations underscore the need to explore safer, affordable, and culturally relevant alternatives for supporting hair regrowth.

Indonesia possesses extensive biocultural diversity and a long-standing tradition of plant-based medicine, with ethnomedicinal knowledge preserved and transmitted across generations in many local communities.⁶ In East Nusa Tenggara, this heritage includes a traditional hair-growth formulation composed of *Aleurites moluccanus*, *Hibiscus rosa-sinensis*, *Ficus benjamina*, *Polyscias scutellaria*, and *Hibiscus tiliaceus*. These plants contain flavonoids, phenolic compounds, anthocyanins, quercetin and kaempferol derivatives, and lipid constituents that may exert antioxidant and anti-inflammatory effects within the hair-follicle microenvironment.⁷ Related topical evidence has shown that candlenut oil-containing spray formulations can stimulate hair growth, supporting the relevance of *A. moluccanus* as a formulation component.⁸ Plant-derived flavonoids and other polyphenolic compounds have also been reported to promote dermal papilla cell activity and hair-follicle regeneration by modulating Wnt/ β -catenin signaling, a central pathway involved in anagen induction and hair-shaft formation.^{9,10}



Therefore, scientific evaluation of this ethnomedicinal formulation may facilitate the translation of local knowledge into an affordable, standardized, and culturally grounded hair-growth intervention.

The herbal formulation (HF) comprises five ethnomedicinal ingredients traditionally used in East Nusa Tenggara for scalp care and hair growth, including *Aleurites moluccanus*, *Hibiscus rosa-sinensis*, *Ficus benjamina*, *Polyscias scutellaria*, and *Hibiscus tiliaceus*. The plant-derived components were incorporated into virgin coconut oil as the topical carrier and contain flavonoids, phenolic compounds, and lipid constituents with potential antioxidant and hair follicle-supporting activities. Based on previous optimization using the Taguchi–Orthogonal Stimulus Response Method and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay as the response variable, each 10 mL formulation contained 0.75 mL of *A. moluccanus* oil, and 5, 5, 5, and 10 mg of *H. rosa-sinensis*, *F. benjamina*, *P. scutellaria*, and *H. tiliaceus* extracts, respectively. Subsequent evaluation in a croton oil-induced alopecia model showed that the optimized formulation produced a hair density of $49.45 \pm 4.70\%$, compared with $35.37 \pm 5.33\%$ in the 5% minoxidil-treated group, indicating a greater improvement in hair follicle density under inflammatory conditions.⁷

This study extends previous investigations of the East Nusa Tenggara ethnomedicinal hair-growth formulation by providing a more comprehensive *in vivo* assessment of its biological effects. The formulation was evaluated using complementary outcome measures, including the clinical hair growth score (CHGS), hair length (HL), hair weight (HW), and hair follicle thickness (HFT), in a croton oil-induced alopecia model. The novelty of this study lies in the integration of complementary outcomes obtained from two independent *in vivo* cohorts, combining longitudinal visual assessment through CHGS with quantitative and histomorphometric evaluations of HL, HW, and HFT. This multiparametric approach was designed to characterize the onset and progression of visible hair growth, hair-shaft elongation, biomass formation, and structural changes in the hair follicle. The findings are expected to strengthen the preclinical evidence supporting this traditional HF and to provide a scientific basis for its further development as a culturally grounded, plant-based hair growth cosmeceutical with potential relevance to alopecia management.

Materials and Methods

Materials

All chemicals and reagents used in this study were of analytical grade. Ethanol 70%, Tween 80, and distilled water were obtained from SmartLab (Indonesia). Croton oil (Sigma-Aldrich) was diluted to 5% (v/v) in acetone and used to induce inflammatory alopecia, while topical minoxidil 5% (Regrou Forte, Indonesia) served as the positive control. Virgin coconut oil (VCO; Bali Coconut, Indonesia; Certificate No. SIG.LHP.XII.2021.060955351) was used as the formulation vehicle. For histological procedures, acepromazine maleate (Castran) and xylazine 2% (Xyla) were used for anesthesia, while 0.9% sodium chloride solution, 10% neutral-buffered formalin, and hematoxylin–eosin staining reagents were used for tissue washing, fixation, and histological staining, respectively. The principal instruments included a drying oven (Mettler, Germany), rotary evaporator (Büchi, Switzerland), moisture content analyzer (Mettler Toledo, Switzerland), analytical balance, mechanical grinder, electric hair clipper, Vernier caliper (Tekiro, Indonesia), and light microscope (Eclipse Ei; Nikon, Japan) equipped with an Optilab digital camera. Hair-follicle morphometry was quantified using ImageJ version 1.54 for Windows (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) software. All measuring instruments were calibrated or verified for proper performance before use.

Plant Materials

Four plant materials were used viz. leaves of *H. rosa-sinensis*, aerial roots of *F. benjamina*, leaves of *P. scutellaria*, and leaves of *H. tiliaceus*. The materials were collected from four sampling sites: *H. rosa-sinensis* (6°29'42.78" S, 106°50'48.96" E), *F. benjamina* (6°29'45.87" S, 106°51'02.04" E), *P. scutellaria* (6°36'35.93" S, 106°46'21.68" E), and *H. tiliaceus* (7°40'44.80" S, 108°37'56.67" E), washed, oven-dried at 50 °C to constant weight, milled, and stored in

airtight containers until extraction. Botanical identity was documented by UPT Laboratorium Herbal Materia Medica Batu, East Java Provincial Health Office, Indonesia, under determination certificate Nos. 000.9.3/1132/102.20/2025 (*H.rosa-sinensis*), 000.9.3/1134/102.20/2025 (*F. benjamina*), 000.9.3/1135/102.20/2025 (*P. scutellaria*), and 000.9.3/1133/102.20/2025 (*H. tiliaceus*). The plant determination numbers were authorized/signed/authenticated by Sabrina Martha, SKM., M.Sc. (Head of the Section for Development of Medicinal and Traditional Medicinal Plants). *A. moluccanus* was used as commercially obtained candlenut oil and was not subjected to simplisia preparation or extraction. Its identity and quality were verified using the supplier's Certificate of Analysis.

Extract Preparation

The dried materials of *H. rosa-sinensis*, *F. benjamina*, *P. scutellaria*, and *H. tiliaceus* were separately milled into coarse powders and extracted by maceration with 70% ethanol. A plant material-to-solvent ratio of 1:10 (w/v) was used, except for *F. benjamina* aerial roots, which were extracted at 1:15 (w/v) to facilitate solvent penetration and mass transfer within the denser fibrous matrix.¹¹ Maceration was performed at room temperature for 72 h with periodic stirring. The mixtures were filtered through Whatman No. 1 filter paper, and the filtrates were concentrated under reduced pressure using a rotary evaporator at 50 °C until viscous extracts were obtained. Each extract was further dried to constant weight, and the extraction yield was calculated as the percentage ratio of dried extract weight to the initial dry plant material weight. The extracts were stored in airtight, light-protected containers until formulation.

Formulation of the Herbal Preparation

The herbal preparation was formulated according to the optimal ratio previously determined using the Taguchi–Orthogonal Stimulus Response Method.⁷ Each 10 mL formulation contained 0.75 mL of commercial *A. moluccanus* oil and ethanolic extracts of *H. rosa-sinensis*, *F. benjamina*, *P. scutellaria*, and *H. tiliaceus* at 5, 5, 5, and 10 mg, respectively. The extracts and candlenut oil were dispersed in virgin coconut oil containing 5% Tween 80, and the final volume was adjusted to 10 mL with virgin coconut oil. The mixture was homogenized at room temperature until a visually uniform preparation was obtained. The formulation was stored in an airtight, light-protected container at room temperature until *in vivo* administration.

Ethical Approval

All animal handling and experimental procedures complied with institutional ethical guidelines and were approved by the Health Research Ethics Committee, Faculty of Health Sciences, Universitas Muhammadiyah Malang, Indonesia (Approval No. E.4.d/201/KEPK/FIKES-UMM/XI/2025).

Experimental Animals and Study Design

A total of 63 healthy male mice (*Mus musculus*), aged 2–3 months and weighing 20–30 g, were used in two independent experimental cohorts. The animals were maintained under standard laboratory conditions with a 12 h light/dark cycle, controlled room temperature (22–28 °C), adequate ventilation, and unrestricted access to standard feed and water, followed by a seven-day acclimatization period. The first cohort comprised 28 mice allocated into four groups (n = 7 per group): positive control (croton oil plus 5% minoxidil), negative control (croton oil plus VCO), normal control (no croton oil induction or topical treatment), and HF (croton oil plus herbal formulation). This cohort was used for CHGS assessment. The second cohort initially comprised 35 mice allocated into five groups (n = 7 per group): negative control (croton oil plus VCO), positive control (croton oil plus 5% minoxidil), normal control I (no croton oil induction or topical treatment), normal control II (VCO containing 5% Tween 80 without croton oil induction), and HF (croton oil plus herbal formulation). One mouse in each group died during the experimental period, resulting in six evaluable mice per group for HL, HW, and HFT assessments. The final sample size of six mice per group met the minimum requirement of Federer's formula. After acclimatization, the dorsal hair was shaved over an area of approximately 2 × 2 cm. Mice assigned to the induced groups (negative



control, positive control, and HF) received topical 5% croton oil to produce localized inflammation and alopecia, whereas the normal control groups were not induced. Normal control II received virgin coconut oil containing 5% Tween 80 without croton oil induction. The assigned topical preparations were applied at 0.2 mL per application twice daily with an 8-h interval for 28 consecutive days. The 5% croton oil induction, 0.2 mL application volume, twice-daily regimen, 28-day treatment duration, 5% minoxidil positive control, and VCO-based control conditions were selected to reproduce the regimen used in the previous *in vivo* evaluation of the same optimized formulation.⁷ The 5% minoxidil concentration is also consistent with its established topical use for hair-growth treatment.⁵

In vivo Assessment of Clinical Hair Growth Score (CHGS)

Clinical Hair Growth Score (CHGS) was assessed exclusively in the first experimental cohort comprising 28 mice. Hair regrowth was assessed at predefined time points using CHGS based on changes in dorsal skin pigmentation, onset of the anagen phase, and visible hair coverage. Scores were independently assigned by three blinded evaluators according to the criteria presented in Table 1.¹² For each evaluator, R1, R2, and R3 represented the mean score of the seven mice within each group at each time point. The final average and standard deviation (SD) were calculated from the three evaluator-specific means and compared among groups.

Table 1: Scoring of Hair Growth and Criteria for Grading.¹²

No.	Observation	Hair growth score
1	No hair growth, pink skin	0
2	Skin color changes from pink to gray/light gray without visible hair growth	0.5
3	Skin color changes from gray/light gray to dark gray/black without visible hair growth, indicating the onset of anagen	1
4	Sparse hair growth	1.5
5	Diffuse short hair growth	2
6	Moderate hair growth	2.5
7	Dense, normal coat hair	3

Hair Length (HL) and Hair Weight (HW) Assessment

Hair length (HL) and hair weight (HW) were assessed in the second experimental cohort, with six evaluable mice per group. The test preparations were applied topically to the designated dorsal area at 0.2 mL per application twice daily for 28 consecutive days, with an approximately 8-h interval between applications. HL and HW were assessed on days 7, 14, 21, and 28. At each time point, 10 hairs were randomly plucked from the treated area, including the roots. Each hair was placed on a black background and measured using a Vernier caliper, and the mean value was recorded as the HL for each animal. The same 10 hairs were subsequently weighed collectively using a milligram-sensitive analytical balance, and their total weight was recorded as the HW. Hair length measurement was adapted from a previously described murine hair-growth assessment.¹³ Hair weight was likewise quantified gravimetrically using an analytical balance, consistent with the general approach in that study, with the methodological modification that the same 10 sampled hairs were weighed collectively in the present longitudinal assessment.¹³ Day-28 HL data were analyzed using Welch analysis of variance (ANOVA) followed by Games-Howell post hoc testing, whereas day-28 HW data were analyzed using the Kruskal-Wallis test followed by pairwise Mann-Whitney tests with Holm adjustment.

Histological Preparation and Hair Follicle Thickness (HFT) Assessment

At the end of the 28-day treatment period, dorsal skin samples from the six surviving mice per group in the second experimental cohort were collected for HFT assessment. After completion of all treatments, dorsal skin samples were collected and fixed in 10% neutral-buffered formalin for 24 h. The tissues were subsequently dehydrated through graded alcohol, cleared, infiltrated with paraffin, and embedded in paraffin blocks. Sections measuring approximately 5–7 μ m in thickness were prepared using a semi-automated rotary microtome (RMT-SA358, Infitec Co., Ltd., China), mounted on glass slides, dried, stained with hematoxylin and eosin, and coverslipped. The histological sections were examined using a light microscope (Eclipse Ei; Nikon Corporation, Tokyo, Japan) at 400 $\times$ magnification, and digital images of clearly visible hair follicles were analyzed using ImageJ. HFT was determined by drawing a line perpendicular to the basal layer of each follicle. Twenty follicles were measured per section, and the mean value was recorded as the HFT for each sample. This histomorphometric procedure was adapted from a murine hair-growth study using hematoxylin-and-eosin-stained sections, ImageJ analysis, and 20 follicle-diameter measurements.¹⁴ Day-28 HFT data were analyzed using one-way ANOVA followed by least significant difference (LSD) pairwise comparisons with Holm adjustment.

Statistical Analysis

Data were expressed as mean $\pm$ standard deviation (SD). For CHGS, seven mice per group were evaluated by three independent blinded evaluators, and the reported mean $\pm$ SD was calculated from the three evaluator-specific group means. For HL, HW, and HFT, six mice per group were analyzed; HL and HW measurements used 10 hairs per mouse at each assessment, and HFT was calculated from 20 follicles per mouse. Clinical hair growth score data were summarized descriptively. Day-28 hair length data were analyzed using Welch one-way ANOVA followed by Games-Howell post hoc tests. Day-28 hair weight data were analyzed using the Kruskal-Wallis test, followed by pairwise Mann-Whitney U tests with Holm adjustment. Day-28 hair follicle thickness data were analyzed using one-way ANOVA followed by least significant difference pairwise comparisons with Holm adjustment. The effect size for hair follicle thickness was expressed as eta squared (η^2). Statistical significance was defined as $p < 0.05$, and Holm-adjusted p -values were used for multiple pairwise comparisons. Statistical analyses were performed using IBM SPSS Statistics for Windows, version 27.0.1 (IBM Corp., Armonk, NY, USA).

Results and Discussion

Extraction Yield

The extraction yields of *H. rosa-sinensis*, *F. benjamina*, *P. scutellaria*, and *H. tiliaceus* were 22.64%, 12.69%, 43.15%, and 16.63%, respectively in Table 2.

The variation in yield may reflect differences in plant matrix characteristics and the proportion of constituents soluble in 70% ethanol, which can extract a broad range of polar and moderately polar phytochemicals, including phenolics and flavonoids.^{15,16} However, extraction yield represents the total recovered material and does not directly indicate bioactive compound content or biological activity.

Table 2: Extraction yields of the plant materials

Extract	Extraction yield (%)
<i>H. rosa-sinensis</i>	22.64
<i>F. benjamina</i>	12.69
<i>P. scutellaria</i>	43.15
<i>H. tiliaceus</i>	16.63

Clinical Hair Growth Score (CHGS)

The longitudinal CHGS profiles revealed distinct temporal patterns of hair regrowth among the experimental groups (Table 3).



Table 3: Clinical Assessment of Hair Growth Following the Administration of the Traditional Herbal Mixture in Croton Oil-Induced Alopecia Mice

Days	Group	R1	R2	R3	Mean $\pm$ SD
1	Positive control	0.00	0.00	0.00	0.00 $\pm$ 0.00
	Negative control	0.00	0.00	0.00	0.00 $\pm$ 0.00
	Normal control	0.00	0.00	0.00	0.00 $\pm$ 0.00
	HF	0.00	0.00	0.00	0.00 $\pm$ 0.00
2	Positive control	0.50	0.50	0.50	0.50 $\pm$ 0.00
	Negative control	0.00	0.00	0.00	0.00 $\pm$ 0.00
	Normal control	0.00	0.00	0.00	0.00 $\pm$ 0.00
	HF	0.50	0.50	0.50	0.50 $\pm$ 0.00
3	Positive control	0.50	0.50	1.00	0.67 $\pm$ 0.24
	Negative control	0.50	0.50	0.50	0.50 $\pm$ 0.00
	Normal control	0.00	0.00	0.00	0.00 $\pm$ 0.00
	HF	1.00	1.00	1.00	1.00 $\pm$ 0.00
4	Positive control	1.00	1.00	1.00	1.00 $\pm$ 0.00
	Negative control	0.50	0.50	0.50	0.50 $\pm$ 0.00
	Normal control	0.00	0.00	0.00	0.00 $\pm$ 0.00
	HF	1.00	1.50	1.50	1.33 $\pm$ 0.24
5	Positive control	1.50	1.00	1.00	1.17 $\pm$ 0.24
	Negative control	0.50	0.50	0.50	0.50 $\pm$ 0.00
	Normal control	0.00	0.00	0.00	0.00 $\pm$ 0.00
	HF	1.50	2.00	2.00	1.83 $\pm$ 0.24
6	Positive control	2.00	1.50	1.50	1.67 $\pm$ 0.24
	Negative control	1.00	1.00	1.00	1.00 $\pm$ 0.00
	Normal control	0.50	0.50	0.50	0.50 $\pm$ 0.00
	HF	2.00	2.00	2.00	2.00 $\pm$ 0.00
7	Positive control	1.00	2.00	2.00	1.67 $\pm$ 0.47
	Negative control	1.00	1.00	1.00	1.00 $\pm$ 0.00
	Normal control	0.50	0.50	0.50	0.50 $\pm$ 0.00
	HF	2.00	2.50	2.50	2.33 $\pm$ 0.24
8	Positive control	3.00	2.50	3.00	2.83 $\pm$ 0.24
	Negative control	1.50	1.00	1.50	1.33 $\pm$ 0.24
	Normal control	0.50	1.00	0.50	0.67 $\pm$ 0.24
	HF	2.00	2.00	2.50	2.17 $\pm$ 0.24

All groups had a CHGS of 0.00 on day 1, indicating the absence of visible hair growth following shaving and alopecia induction. On day 2, both HF and the positive control reached a mean score of 0.50, whereas no visible changes were observed in the negative and normal controls. From days 3 to 7, HF consistently produced higher CHGS values than the positive control, reaching 1.00 $\pm$ 0.00, 1.33 $\pm$ 0.24, 1.83 $\pm$ 0.24, 2.00 $\pm$ 0.00, and 2.33 $\pm$ 0.24, respectively. In comparison, the positive control reached 0.67 $\pm$ 0.24, 1.00 $\pm$ 0.00, 1.17 $\pm$ 0.24, 1.67 $\pm$ 0.24, and 1.67 $\pm$ 0.47 over the same observation period. These findings indicate that HF accelerated the early visual progression from skin pigmentation changes to sparse and diffuse hair emergence. On day 8, the response pattern changed, with the positive control achieving the highest CHGS of 2.83 $\pm$ 0.24, followed by HF at 2.17 $\pm$ 0.24, the negative control at 1.33 $\pm$ 0.24, and the normal control at 0.67 $\pm$ 0.24.

Thus, HF was characterized by an earlier onset and more rapid progression during days 3–7, whereas the positive control produced a greater final response. The photographic observations supported these trends, as HF-treated skin showed more evident pigmentation and early hair emergence by day 4, while the positive control produced denser visible coverage by day 8 (Figure 1). The modest decrease in HF score from day 7 to day 8 is unlikely to represent true biological regression within 24 h and may instead reflect the ordinal nature of CHGS and variability related to hair orientation, illumination, animal positioning, or differentiation between adjacent scoring categories.

The early response following HF treatment may be related to its capacity to improve the follicular microenvironment after croton oil-induced injury.

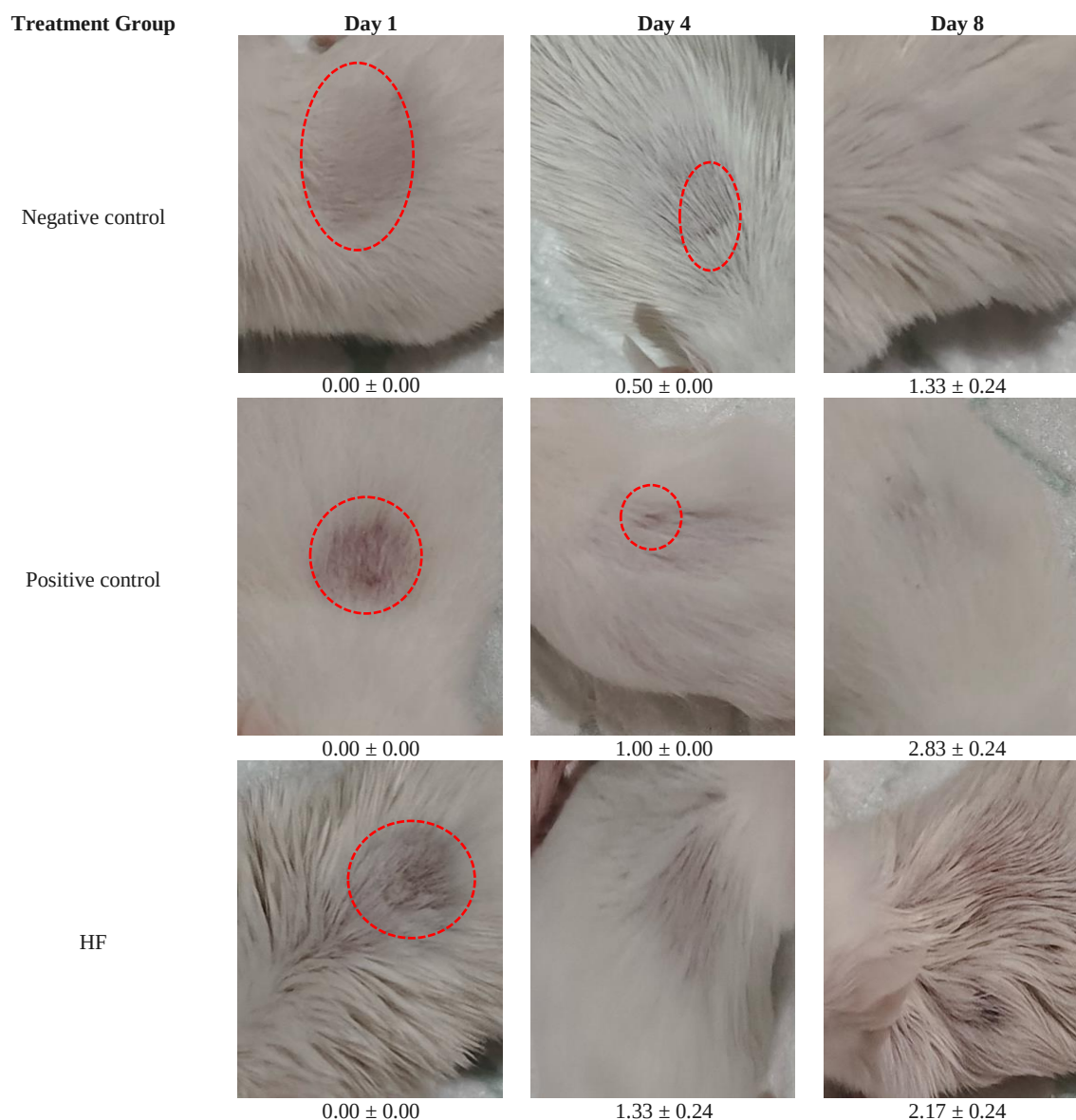


Figure 1: Visual Progression of Dorsal Hair Regrowth Following the Administration of the Traditional Herbal Mixture and Comparator Treatments from Day 1 to Day 8

Croton oil contains phorbol esters that activate protein kinase C and induce acute cutaneous inflammation characterized by edema, vascular alterations, inflammatory-cell recruitment, and increased production of inflammatory mediators.^{17,18} Persistent inflammation and excessive reactive oxygen species can impair dermal papilla cell viability, reduce their hair-inductive capacity, and disturb epithelial–mesenchymal communication required for normal hair cycling.^{19,20} Therefore, the antioxidant activity previously demonstrated for HF may facilitate earlier recovery from the inflammatory and oxidative conditions generated by croton oil.⁷

Restoration of the follicular microenvironment may subsequently favour activation of Wnt/ β -catenin signaling, which regulates dermal papilla activity, hair follicle stem-cell activation, matrix-cell proliferation, and entry into the anagen phase. Experimental activation of Wnt/ β -catenin in dermal papilla cells or hair follicle stem cells has consistently been associated with increased cell proliferation, earlier anagen induction, hair-shaft elongation, and accelerated hair regrowth in animal and cellular models.^{21–23} Several plant-derived compounds have also promoted hair growth through β -catenin stabilization and increased expression of downstream anagen-related targets, supporting the biological plausibility that phytochemical-rich formulations may influence this pathway.^{10,24}

Hair Length (HL)

Hair length (HL) increased progressively in all experimental groups throughout the 28-day treatment period, although the magnitude and temporal pattern of elongation differed markedly among treatments (Table 4). On day 7, the highest HL was observed in the VCO-treated normal control group at 3.1 ± 1.6 mm, followed by HF at 2.9 ± 0.6 mm, whereas the negative control, minoxidil, and untreated normal control groups reached 1.7 ± 1.0 , 2.1 ± 0.5 , and 2.1 ± 1.1 mm, respectively. By day 14, HF and the VCO-treated normal control group reached an identical mean HL of 3.5 mm. A pronounced separation occurred between days 14 and 21, when HL in the HF group increased by 2.3 mm to 5.8 ± 0.9 mm, substantially exceeding the increments observed in the negative control, minoxidil, untreated normal control, and VCO-treated normal control groups. At day 28, HF produced the highest HL at 6.3 ± 0.8 mm, followed by the VCO-treated normal control at 5.9 ± 0.9 mm, the negative control at 5.4 ± 0.4 mm, minoxidil at 4.3 ± 0.5 mm, and the untreated normal control at 3.9 ± 1.3 mm. This trajectory demonstrates that HF generated its strongest elongation response during the third week of treatment and maintained the highest endpoint value. Welch ANOVA showed a significant difference in day-28 HL among the experimental groups ($p = 0.001$). As presented in Table 5, Games-Howell analysis demonstrated that HF produced significantly greater

HL than 5% minoxidil, with a mean difference of 2.0 mm ($p = 0.006$), and the untreated normal control, with a mean difference of 2.4 mm ($p = 0.032$). No significant differences were observed between HF and the croton oil plus VCO negative control ($p = 0.219$) or the non-induced VCO control ($p = 0.871$). These findings indicate that HF enhanced endpoint hair elongation relative to minoxidil and spontaneous growth, although the comparable responses in the VCO-containing groups suggest a substantial contribution from the formulation vehicle.

Hair length (HL) reflects the cumulative activity of anagen follicles, particularly matrix keratinocyte proliferation, differentiation, and keratinized hair-shaft production under the regulatory influence of dermal papilla cells.²⁵ Canonical Wnt signaling stabilizes β -catenin, promotes its nuclear translocation, and activates T-cell factor/lymphoid enhancer factor (TCF/LEF)-dependent genes involved in hair follicle stem-cell activation, matrix-cell proliferation, telogen-to-anagen transition, and hair-shaft elongation.^{21,23} The marked increase in HL in the HF group between days 14 and 21 is therefore consistent with enhanced recruitment and maintenance of follicles in active anagen. This interpretation is supported by the previously reported radical-scavenging activity and increased follicular density of the same

formulation.⁷ Oxidative stress and inflammation may impair dermal papilla viability, suppress hair-inductive signaling, and disrupt Wnt/ β -catenin activity, thereby limiting sustained hair-shaft elongation.^{19,20} The lower day-28 HL in the minoxidil group may reflect differences in pharmacological action within an inflammatory alopecia model. Minoxidil requires sulfotransferase-mediated conversion to minoxidil sulfate and primarily promotes adenosine triphosphate (ATP)-sensitive potassium-channel opening, vascular endothelial growth factor (VEGF) expression, vascular support, and anagen prolongation, whereas HF combines antioxidant, anti-inflammatory, and hair-inductive activities within the follicular microenvironment.^{5,26} However, the non-induced VCO control reached 5.9 ± 0.9 mm and the croton oil plus VCO control reached 5.4 ± 0.4 mm, compared with 6.3 ± 0.8 mm in the HF group. Thus, the HL response likely resulted from the combined effects of the herbal constituents and VCO. Overall, the longitudinal pattern summarized in Table 4, together with the pairwise comparisons in Table 5, demonstrates that HF produced the highest day-28 HL and a pronounced acceleration of hair elongation during the third treatment week.

Table 4: Longitudinal Assessment of Hair Length Following the Administration of the Traditional Herbal Mixture and Comparator Treatments

Day	Negative control	Positive control	Normal control I	Normal control II	HF
7	1.7 ± 1.0	2.1 ± 0.5	2.1 ± 1.1	3.1 ± 1.6	2.9 ± 0.6
14	3.1 ± 1.4	2.4 ± 0.7	3.1 ± 1.3	3.5 ± 1.2	3.5 ± 0.9
21	4.2 ± 1.2	3.4 ± 0.6	3.5 ± 1.5	3.6 ± 0.4	5.8 ± 0.9
28	5.4 ± 0.4	4.3 ± 0.5	3.9 ± 1.3	5.9 ± 0.9	6.3 ± 0.8

Note: Data are presented as mean $\pm$ SD in millimeters, $n = 6$ mice per group. Negative control: croton oil plus VCO; positive control: croton oil plus 5% minoxidil; normal control I: no induction or treatment; normal control II: VCO containing 5% Tween 80 without croton oil induction; HF: croton oil plus herbal formulation.

Table 5: Games–Howell Pairwise Comparisons of Hair Length on Day 28 Following the Administration of the Traditional Herbal Mixture and Comparator Treatments

Group comparison	p-value	Interpretation
Negative control vs. HF	0.219	Not significant
Positive control vs. HF	0.006	Significant
Normal control I vs. HF	0.032	Significant
Normal control II vs. HF	0.871	Not significant

Hair Weight (HW)

Hair weight (HW) increased progressively during the 28-day treatment period, although the timing and magnitude of the response differed among groups (Table 6). On day 7, HF and the positive control reached 0.00217 ± 0.0011 g and 0.0021 ± 0.0016 g, respectively, whereas the negative control, normal control I, and normal control II reached 0.0016 ± 0.0005 g, 0.0016 ± 0.0008 g, and 0.0018 ± 0.0008 g, respectively. HF reached 0.0023 ± 0.0008 g on day 14 and increased to 0.0036 ± 0.0008 g on day 21. By day 28, HF reached 0.0038 ± 0.0004 g, followed by normal control II at 0.0036 ± 0.0012 g, the negative control at 0.0035 ± 0.0008 g, the positive control at 0.0031 ± 0.0004 g, and normal control I at 0.0028 ± 0.0004 g. Thus, HF produced an earlier increase in HW during the third treatment week, although the endpoint values converged among HF, the positive control, and the VCO-containing controls.

The day-28 HW data were non-normally distributed and were therefore analyzed using the Kruskal–Wallis test. A significant overall difference was observed among the experimental groups ($H = 9.581$, $df = 4$, $p = 0.048$). As presented in Table 7, pairwise comparisons showed differences between HF and the positive control ($p = 0.027$), and between HF and normal control I ($p = 0.006$), whereas HF did not differ from the negative control or normal control II (both $p = 0.247$). After Holm adjustment for multiple comparisons, the difference between HF and normal control I remained significant (adjusted $p = 0.024$), while the difference between HF and the positive control was no longer significant (adjusted $p = 0.081$). These findings indicate that HF increased hair biomass relative to untreated physiological growth, but

its endpoint effect was comparable to the positive control and the VCO-containing groups.

Because a fixed number of 10 hairs was weighed at each assessment, HW represents the collective biomass of the sampled hair shafts rather than follicular density. This parameter integrates shaft length, diameter, keratin content, and structural integrity. Hair-shaft biomass is generated during anagen through sustained proliferation and differentiation of matrix keratinocytes under the regulatory influence of dermal papilla cells.²⁵ Activation of Wnt/ β -catenin signaling promotes hair follicle stem-cell activation, matrix-cell proliferation, and anagen-associated shaft production.^{21,23} The increase in HW observed in the HF group on day 21 corresponded with the pronounced increase in HL during the same period, indicating enhanced production of keratinized hair shafts. The earlier HW response in the HF group is consistent with the antioxidant activity and increased follicular density previously demonstrated for the same formulation.⁷ Linoleic acid and related lipid constituents may also stimulate dermal papilla activity through Wnt/ β -catenin-associated signaling, providing a mechanistic contribution from the lipid fraction of the formulation.²³ However, the similarity between HF and the VCO-containing controls indicates that VCO materially contributed to the endpoint response. The repeated whole-milligram values and frequent zero SD values further indicate limited measurement resolution, which may have compressed biological variability and reduced the ability to distinguish closely distributed treatment effects.

Table 6: Longitudinal Assessment of Hair Weight Following the Administration of the Traditional Herbal Mixture and Comparator Treatments

Day	Negative control	Positive control	Normal control I	Normal control II	HF
7	0.0016 ± 0.0005	0.0021 ± 0.0016	0.0016 ± 0.0008	0.0018 ± 0.0008	0.00217 ± 0.0011
14	0.0018 ± 0.0007	0.0023 ± 0.0008	0.0025 ± 0.0008	0.0018 ± 0.0007	0.0023 ± 0.0008
21	0.0025 ± 0.0005	0.0028 ± 0.0004	0.0026 ± 0.0008	0.0028 ± 0.0004	0.0036 ± 0.0008
28	0.0035 ± 0.0008	0.0031 ± 0.0004	0.0028 ± 0.0004	0.0036 ± 0.0012	0.0038 ± 0.0004

Note: Data are presented as mean ± SD in grams, n = 6 mice per group. Negative control: croton oil plus VCO; positive control: croton oil plus 5% minoxidil; normal control I: no induction or topical treatment; normal control II: VCO containing 5% Tween 80 without croton oil induction; HF: croton oil plus herbal formulation.

Table 7: Mann–Whitney Pairwise Comparisons of Hair Weight on Day 28 between the Traditional Herbal Mixture and Comparator Treatments

Comparison	Raw p-value	Holm-adjusted p-value	Interpretation
HF vs. negative control	0.247	0.494	Not significant
HF vs. positive control	0.027	0.081	Not significant
HF vs. normal control I	0.006	0.024	Significant
HF vs. normal control II	0.247	0.494	Not significant

Hair Follicle Thickness (HFT)

Hair follicle thickness (HFT) differed significantly among the experimental groups after 28 days of treatment (Table 8). HF produced the highest HFT at $47.04 \pm 7.64 \mu\text{m}$, followed by normal control II at $38.48 \pm 5.18 \mu\text{m}$, the negative control at $37.07 \pm 8.65 \mu\text{m}$, the minoxidil-treated positive control at $36.30 \pm 4.57 \mu\text{m}$, and normal control I at $33.98 \pm 3.33 \mu\text{m}$. One-way ANOVA confirmed a significant overall treatment effect, $F(4,25) = 3.911$, $p = 0.013$, with an η^2 of 0.385, indicating that approximately 38.5% of the observed variation in HFT

was associated with treatment-group allocation. Pairwise LSD comparisons showed that HF produced greater HFT than the negative control, positive control, normal control I, and normal control II. All four comparisons remained significant after Holm adjustment, with adjusted p-values of 0.020, 0.018, 0.004, and 0.025, respectively (Table 9). The significant difference between HF and normal control II is particularly important because it demonstrates that the structural follicular response was not attributable solely to VCO.

Table 8: Assessment of Hair Follicle Thickness Following 28 Days of Administration of the Traditional Herbal Mixture and Comparator Treatments

Treatment group	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mouse 6	Mean ± SD
Negative control	36.63	47.87	46.10	36.29	28.80	26.72	37.07 ± 8.65
Positive control	41.32	35.72	42.23	34.21	30.57	33.75	36.30 ± 4.57
Normal control I	32.39	30.79	36.27	39.65	32.69	32.08	33.98 ± 3.33
Normal control II	44.87	33.63	44.00	34.48	33.91	39.99	38.48 ± 5.18
HF	56.46	50.02	54.71	39.86	41.17	39.99	47.04 ± 7.64

Note: Data are expressed in micrometers. Each value represents the mean thickness of 20 follicles from one mouse. Group data are presented as mean ± SD, n = 6 mice per group. Negative control: croton oil plus VCO; positive control: croton oil plus 5% minoxidil; normal control I: no induction or topical treatment; normal control II: VCO containing 5% Tween 80 without croton oil induction; HF: croton oil plus herbal formulation.

Table 9: Pairwise Comparisons of Hair Follicle Thickness between the Traditional Herbal Mixture and Comparator Treatments

Comparison	Mean difference (μm)	95% confidence interval	p-value	Holm-adjusted p-value	Interpretation
HF vs. negative control	9.97	2.60 to 17.34	0.010	0.020	Significant
HF vs. positive control	10.74	3.37 to 18.11	0.006	0.018	Significant
HF vs. normal control I	13.06	5.69 to 20.43	0.001	0.004	Significant
HF vs. normal control II	8.56	1.19 to 15.93	0.025	0.025	Significant

Note: Positive mean differences indicate greater HFT in the HF group. Pairwise comparisons were derived from the LSD analysis following a significant one-way ANOVA, $F(4,25) = 3.911$, $p = 0.013$. Holm adjustment was applied across the four comparisons involving HF. Statistical significance was defined as adjusted $p < 0.05$.

Hair follicle thickness (HFT) at the basal follicular region reflects the structural activity of the lower follicle rather than the thickness of the external hair shaft. During anagen, the follicular bulb enlarges as matrix keratinocytes proliferate and differentiate around the dermal papilla, while the lower follicle extends deeper into the dermis and subcutaneous tissue. In contrast, catagen and telogen are characterized by regression of the lower follicle, reduced matrix-cell activity, and a smaller basal diameter. The greater HFT observed in the HF group therefore indicates more extensive anagen-associated follicular enlargement and matrix-cell activity. This interpretation is consistent

with experimental evidence showing that activation of β -catenin signaling in dermal papilla cells drives postnatal hair growth and that stimulation of Wnt-related proteins increases dermal papilla proliferation and follicular growth.^{24,27} The representative hematoxylin and eosin sections should consequently show broader basal follicles and more prominent follicular bulbs in the HF group than in the comparator groups (Figure 2).

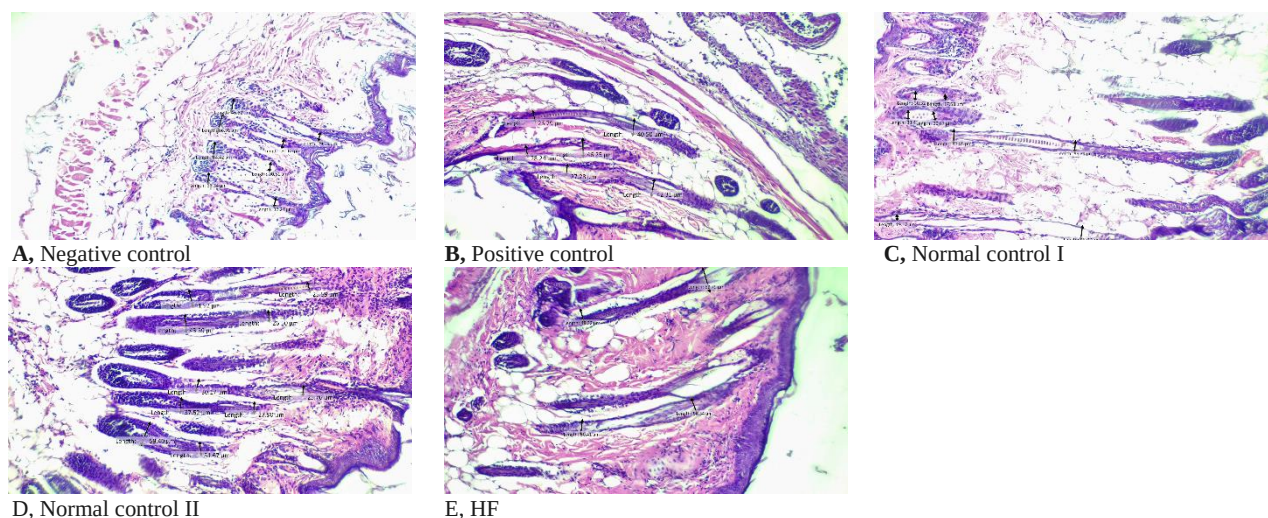


Figure 2: Representative Histological Sections of Dorsal Hair Follicles Following 28 Days of Administration of the Traditional Herbal Mixture and Comparator Treatments

Croton oil produces acute cutaneous injury characterized by tissue edema, altered vascular permeability, inflammatory-cell recruitment, and remodeling-related protein expression. These changes disrupt the perifollicular microenvironment and can suppress the proliferative capacity of dermal papilla and matrix cells.¹⁸ The greater HFT produced by HF indicates that the formulation counteracted this inflammatory disruption and restored lower-follicle growth more effectively than either VCO or minoxidil alone. Polyphenolic compounds can reduce oxidative injury in dermal papilla cells while restoring β -catenin transcriptional activity and anagen-related gene expression.¹⁹ This mechanism is consistent with the strong radical-scavenging activity previously reported for the optimized formula and with its ability to increase histomorphometric follicular density in the same croton oil-based model.⁷

The herbal formulation (HF) exceeded 5% minoxidil by 10.74 μ m in mean HFT, with an adjusted p-value of 0.018. Minoxidil requires sulfotransferase-mediated bioactivation within the outer root sheath, and variation in follicular sulfotransferase 1A1 (SULT1A1) activity can influence treatment response.^{28,29} In this inflammatory model, HF provided broader pharmacological coverage through simultaneous follicular stimulation, oxidative protection, and modulation of the damaged skin microenvironment. The HFT findings therefore provide the strongest structural evidence of an HF-specific effect because, unlike HL and HW, HF remained significantly superior to the VCO-treated normal control. Together with the earlier CHGS response and the increases in HL and HW during the third treatment week, the enlarged basal follicles demonstrate that HF promoted coordinated visual, shaft-level, and histomorphometric hair-growth responses.

Conclusion

The East Nusa Tenggara ethnomedicinal herbal formulation promoted hair regrowth in the croton oil-induced alopecia model, as demonstrated by earlier CHGS improvement, greater HL and HW progression, and the highest HFT among all groups. The significant increase in HFT compared with minoxidil and VCO-containing controls indicates a follicular effect attributable to the complete herbal formulation. These findings support its further development as a plant-based topical

preparation for alopecia management. Further studies should standardize chemical markers, distinguish the contributions of the individual herbal components and vehicle, confirm the proposed molecular mechanisms, and evaluate dermal safety, formulation stability, and clinical efficacy.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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