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In-Vivo Study on the Hair Growth-Enhancing Effects of CTUMP's Herb Shampoo

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ABSTRACT

Herbal shampoos are increasingly popular due to their potential to reduce hair loss, stimulate hair growth, and provide a safe, eco-friendly option. This study assessed the *in-vivo* effects of CTUMP's Herb shampoo on promoting hair growth. The day before the experiment, the mice had their back hair shaved (1.5 × 2.5 cm) and were divided into three groups of ten. Groups 1, 2, and 3 received 0.9% NaCl, 5% Minoxidil, and CTUMP's Herb shampoo, respectively. The shaved-off area on the mice's back was treated with 0.5 mL of tested materials for 5 minutes, then rinsed and dried, repeatedly three times a week. Hair growth was observed and photographed on days 7, 14, 21, and 28. Hair growth was determined by measuring its length, weight, growth rate, and histological examination. The group treated with CTUMP's Herb shampoo showed a significantly higher hair length, weight, and growth rate compared to the 0.9% NaCl group ($p < 0.05$). Although it had fewer anagen follicles than the 5% minoxidil group, it still showed 2.6 times more follicles than the NaCl group ($p < 0.05$). CTUMP's Herb shampoo enhances hair condition, promoting faster and denser growth.

Keywords: Hair growth, Hair loss, *In-vivo*, CTUMP's Herb shampoo.

Introduction

Hair loss is characterized by the shedding of hair from the body, often as part of the natural hair growth cycle. However, when hair loss becomes severe and persistent, resulting in issues such as a visible scalp or male-pattern baldness, it is considered abnormal and needs to be addressed.[1] Currently, hair loss is a primary dermatological health concern worldwide, impacting not only an individual's mental well-being and appearance but also their daily social interactions. This condition is widespread among middle-aged people and is also increasingly observed in younger individuals, regardless of gender or age.[2] Various factors contribute to hair loss, including genetics, hormonal imbalances, alopecia areata, scalp infections, fungal infections, lupus erythematosus, exposure to chemical treatments, and deficiencies in essential nutrients (like iron, protein, and vitamin B5). Aging and other factors can also play a role.[3] Furthermore, improper hair care practices, such as frequent curling, straightening, and dyeing, can damage and weaken hair, further exacerbating hair loss. The regular use of shampoos with harsh chemical ingredients, particularly with daily application directly to the hair, also poses a risk. Moreover, common hair loss treatments like minoxidil, finasteride, anthralin, and glucocorticoids can have side effects, including dry scalp, clogged pores, and potential complications like folliculitis, psoriasis, and scalp dermatitis.[4] In response to growing concerns about the harmful effects of chemical-based products, many people are turning to herbal beauty products, including shampoos and hair serums. These natural alternatives are generally safe, gentle, and relatively free of side effects. They effectively combat hair loss, encourage faster hair growth, and are environmentally friendly. Despite the rising demand, relatively few herbal products on the market have proven effective in promoting hair growth. Consequently, the research evaluated the *in-vivo* effectiveness of CTUMP's Herb shampoo on hair growth.

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Materials and Methods

Collection and identification of plant materials

Plant materials including *Citrus grandis* peel, fructus *Gleditsiae australis*, and herba *Ocimum gratissimum* were sourced from Thai Son Herbal Company in Can Tho City (Vietnam) with approximate coordinates at Latitude: 10°04'41.8"N and Longitude: 105°77'90.1"E in March 2022. The plant materials were identified and authenticated by the Department of Pharmacognosy at the Faculty of Pharmacy, Can Tho University of Medicine and Pharmacy, and they met the standards according to the Vietnamese Pharmacopoeia V.[5]

Extraction

The extraction process involved weighing 50 g of *Citrus grandis* peel, 50 g of fructus *Gleditsiae australis*, and 20 g of herba *Ocimum gratissimum* (all ground to particle sizes of 1.0–1.6 mm) into a 2000 mL Erlenmeyer flask. The mixture was extracted using the heat reflux method at a temperature of 80 ± 2°C for 60 minutes, with distilled water as the solvent and a herb-to-solvent ratio of 1:10. The extracts were then combined and concentrated in a water bath (Memmert® WTB 15) at 60 ± 2°C to obtain a liquid extract with a ratio of 1:4.

Formulation of shampoo

The formulation process of CTUMP's Herb shampoo was as follows: lauryl glucoside and cocamidopropyl betaine were mixed with distilled water to form a homogeneous solution (solution 1). HPMC was gradually dissolved in distilled water using a magnetic stirrer set at 300 rpm to obtain a clear solution. Next, citric acid and propylene glycol were added while stirring thoroughly. The concentrated herbal extract was then gradually incorporated, with stirring maintained at 300 rpm to obtain a solution 2. Solution 1 was added to solution 2, and the mixture was homogenized using the magnetic stirrer at 300 rpm for 10 minutes. Finally, the CTUMP's Herb shampoo product was transferred to plastic bottles for labeling [5] (see Figure 1).

0.9% NaCl for injection (Fresenius Kabi, Vietnam) and 5% minoxidil solution (Kirkland, USA) were purchased from a pharmacy in Can Tho city, Vietnam. 10% formalin (Hicytec, Vietnam) and hematoxylin & eosin solutions (Merck KgaA, German) were supplied by the Department of Pathology, Faculty of Medicine, Can Tho University of Medicine and Pharmacy, Vietnam.

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Experimental animals
Male Swiss albino mice (n = 30, 5-6 weeks aged, and 18-22 grams weighed) were healthy and exhibited no deformities or abnormal behavior. These mice were obtained from the Pasteur Institute of Ho Chi Minh City, Vietnam, and maintained at a temperature of 25±3^{0}C under natural lighting conditions. Throughout the experiment, the mice were provided with food and water from the Drug, Cosmetic, and Food Quality Control Center of Can Tho City, Vietnam.
Adult white rabbits, all weighing less than 2 kg and deemed healthy without pregnancy, deformities, or abnormal behaviors, were obtained from the Drug, Cosmetic, and Food Quality Control Center of Can Tho City, Vietnam.
All animal experimentation received the approval of the protocol from the Medical Ethics Council of Can Tho University of Medicine and Pharmacy, Can Tho city, Vietnam (approval number 23.052.GV/HDDD- PCT, Dec 21, 2023).

Evaluation of skin irritation effects
The skin irritation test was conducted following the TCVN 7391-10:2007 standard [6] and OECD 404 guidelines.[7] The procedure involved three healthy, adult rabbits, each weighing less than 2 kg, which were kept separately and acclimated for five days before testing. The rabbits' backs were shaved (10 cm x 15 cm) to apply 0.5 g of 5% Minoxidil and CTUMP Herbs shampoo. The treated area was covered with gauze and held in place with adhesive tape for four hours. After the removal of the tape, the excess formulation was washed off, and the skin was dried. Skin reactions were monitored and recorded at 1, 24, 48, and 72 hours post-cleaning.

Evaluate the effectiveness of hair growth
The day before the experiment, the mice had their back hair shaved (1.5 × 2.5 cm) and were divided into three groups of ten mice each. The following treatment was given to animals of different groups:
Group 1 (negative control): treated with 0.9% NaCl;
Group 2 (positive control): treated with 5% Minoxidil;
Group 3 (test): treated with CTUMP's Herb shampoo;
Mice in groups 1, 2, and 3 were treated with 0.9% NaCl, 5% Minoxidil, and CTUMP's Herb shampoo, respectively, three times a week. Each application consisted of 0.5 mL, which was applied for 5 minutes, then rinsed and dried. The shaved areas were monitored and photographed on days 7, 14, 21, and 28. The effectiveness of hair growth was assessed based on the mice's weight, hair length, hair weight, and degree of hair growth.

Mice's weight
Mice were observed from day 0 to day 28, with weights recorded at specific intervals: day 0 (post-shaving), 7, 14, 21, and 28, using an electronic balance for each mouse in each group. The results were presented as mean weight ± SD.[8]

Hair length and weight in mice
The initiation of hair growth in both the control and test groups was recorded, along with the time required to complete the hair growth process.
Hair length in mice was assessed on days 7, 14, 21, and 28 by pulling 20 hairs from the designated area of each mouse in the group and measuring them with a Stanley electronic caliper. The results were expressed as mean hair length ± SD.[9,10]
Hair weight in mice was measured by weighing each mouse in the group before and after shaving, with data collected on days 0 and 28. The results were expressed as mean weight ± SD.[11,12]

The degree of hair growth
The mice's hair growth patterns were observed for each group, noting whether the growth followed specific patterns, such as head-to-tail, outward from the spine, in random spots, or spreading from a point.
The degree of hair growth was scored using Lee's hair growth scoring,[13] with adjustments for experimental conditions at days 7, 14, 21, and 28, as follows: 0 - no visible hair growth; 1 - thin hair growth, with prominent skin areas exposed; 2 - low hair density, with some patches of skin still exposed; 3 - moderate hair growth, with the skin entirely concealed; 4 - high hair density, resulting in a full and thick fur coat.

Histological study
The histological study was conducted in accordance with the method described by Patel et al.,[14] with necessary modifications. Mice from each group were sacrificed by cervical dislocation on day 29. A 1 × 2 cm piece of back skin was excised, fixed in 10% formalin, and embedded in paraffin. The resulting tissue sections were cut to 4 - 5 µm thickness and 1 cm in length, then stained with hematoxylin & eosin.[15] The samples were examined using an optical microscope E100 (Nikon, Japan) at 4x magnification to count the hair follicles in the anagen and telogen phases, and the anagen/telogen ratio was calculated to evaluate the hair regrowth capability.

Statistical analysis
The data were expressed as the mean ± standard deviation (mean ± SD). The results were processed in Microsoft Excel and analyzed for statistical significance using SPSS 26 (SPSS, Inc., Chicago, IL, USA) through One-Way ANOVA and Student's T-test, with differences considered significant at $P < 0.05$.

Figure 1: CTUMP's Herb shampoo

Results and Discussion

Evaluation of skin irritation effects
Table 1 presented the results of the skin irritation test for the products, including assessments of erythema, scaling, and edema.
The results indicated no signs of irritation, including erythema, scaling, and edema, following the application of CTUMP's Herb and 5% Minoxidil on three rabbits. Both formulations had an average assessment score of 0 (< 0.4). This suggests that CTUMP's Herb and 5% Minoxidil were safe for use in evaluating hair growth effectiveness in mice. Herbal products have been gaining popularity due to their therapeutic benefits and lower risk of side effects. Nonetheless, skin irritation testing was essential to ensure their safety and effectiveness. Testing on rabbits helped evaluate the product's effects and verifies that it does not induce irritation or adverse reactions on human skin.[16]

Evaluate the effectiveness of hair growth
Mice's weight
Table 2 showed the results of the mouse weights recorded on days 0, 7, 14, 21, and 28.
The results indicated that the weights of mice in groups 1, 2, and 3 stayed constant until day 7, after which they began to gradually increase. The weight variations in mice throughout the study period, from day 0 to day 28, were shown in Table 3.

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Table 1: Evaluation of erythema and scaling on healthy rabbit skin

		CTUMP's Herb	5% Minoxidil
Erythema and scaling	No visible erythema	-	-
	Minimal erythema (slightly visible)	-	-
	Noticeable erythema	-	-
	Moderate to severe erythema	-	-
	Severe erythema (deep redness) accompanied by scaling that affects erythema classification	-	-
Edema	No visible edema	-	-
	Minimal edema (slightly visible)	-	-
	Distinct edema (clearly outlined swelling)	-	-
	Moderate edema (skin swollen roughly 1 mm)	-	-
	Severe edema (skin swollen over 1 mm with expansion to surrounding regions)	-	-

Table 2: Effect on **w**eights of mice (n = 10)

Group	Weights of mice (g)				
	Day 0	Day 7	Day 14	Day 21	Day 28
1	21.59 ± 0.73	21.96 ± 1.80	22.80 ± 2.63	25.28 ± 2.26	26.21 ± 3.34
2	21.63 ± 1.27	22.37 ± 1.39	24.40 ± 2.85	23.99 ± 2.90	25.89 ± 2.10
3	21.01 ± 1.45	21.22 ± 1.07	23.18 ± 2.27	24.54 ± 2.02	26.80 ± 3.21

Group 1: treated with 0.9% NaCl; Group 2: treated with 5% Minoxidil; Group 3: treated with CTUMP's Herb shampoo. All the values were expressed as mean ± SD (Standard deviation).

Overall, there was a rise in the mice's weight from day 7 to the end of the experiment; however, this increase was not statistically significant. *In-vivo* models for examining hair loss and regrowth were conducted on a variety of animals, including mice, rats, rabbits, and sheep, but mice were the most commonly employed. Mice had 99% DNA similarity with humans and a hair growth cycle similar to the human cycle. Their short hair growth cycle (around 3 weeks) made them ideal for collecting hair and evaluating hair growth indicators in mice.[17,18]

Table 3: Differences in mouse weight recorded on day 28 versus day 0 (n = 10)

Group	Survival rate	Weight variations (g)
1	100%	4.35 ± 2.73
2	100%	3.95 ± 2.10
3	100%	5.40 ± 3.76

Group 1: treated with 0.9% NaCl; Group 2: treated with 5% Minoxidil; Group 3: treated with CTUMP's Herb shampoo. All the values were expressed as mean ± SD (Standard deviation).

The selection of mice depended on the specific requirements of each experiment. For studies evaluating hair regrowth, C57BL/6 mice were preferred because their dark brown fur allowed for earlier detection of the anagen phase. However, C57BL/6 mice were not commonly used in research in Vietnam due to high maintenance costs, which resulted from differences in climate, diet, and the need for importation. As a more cost-effective alternative, we opted for Swiss albino mice for this study, as they were better adapted to local breeding conditions. This ensured stable health and reduced stress, enhancing the accuracy of experimental results and minimizing unintended bias. [19-22]

Several criteria were used to assess hair growth in mice, including body weight, hair length, hair mass, skin scoring, histological evaluations, immunofluorescence analysis, and follicle count.[23] This study focused on body weight, hair length, hair mass, skin scoring, and histological analysis to investigate and demonstrate the effectiveness of the herbal shampoo formulation in promoting hair growth. After the experiment, all three groups of mice maintained a 100% survival rate, and their body weight increased significantly ($P < 0.05$). While the weight of group 2 dropped on day 21 compared to day 14, it recovered and normalized by day 28, consistent with the other groups. Group 3 (treated with CTUMP's Herb shampoo) exhibited the greatest weight gain. However, the weight differences between the groups were not statistically significant ($P > 0.05$). Research by Lee et al., Ma et al., and Thanh et al. also utilized mouse weight as a parameter and found results consistent with this study.[13,24,25]

Hair length and weight in mice

Results of hair length in mice on days 7, 14, 21, and 28 were presented in Figure 2. During the experiment, the hair length of mice in all three groups gradually increased. Although there was no significant difference in fur length between Group 2 (positive control) and Group 1 (negative control) on day 21, the overall results showed a statistically significant difference among the groups with $P < 0.05$, based on One-way ANOVA and Student's T-test. The measurements of hair weight on day 0 and day 28, after shaving, were presented in Table 4. Mouse hair weight was a reliable quantitative measure for evaluating hair growth. After 28 days, mice treated with 5% Minoxidil (group 2) and CTUMP's Herb shampoo (group 3) exhibited significant increases in hair weight, recorded at 0.93 ± 0.69 and 1.05 ± 0.48, respectively. In contrast, mice treated with 0.9% NaCl (group 1) showed no significant change, highlighting the effectiveness of CTUMP's Herb shampoo in stimulating hair growth.

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Table 4: Effect on hair weight in mice on days 0 and 28

Group	Hair weight (g)	
	Day 0	Day 28
1	0.46 ± 0.27	0.48 ± 0.69
2	0.48 ± 0.37	0.93 ± 0.69[b]
3	0.49 ± 0.26	1.05 ± 0.48[b]

Group 1: treated with 0.9% NaCl; Group 2: treated with 5% Minoxidil; Group 3: treated with CTUMP's Herb shampoo. All the values were expressed as mean ± SD (Standard deviation). b:significant difference compared to the control, p value was <0.05.

The methods used for evaluating hair growth were primarily qualitative and had various limitations, such as macroscopic assessments through visual inspection and microscopic assessments using a dermatoscope. Additionally, straightforward quantitative approaches like measuring hair length and weight were employed. Although these methods could have involved errors due to interpretation or the type of measurement tool used, they generally provided more precise and reliable results than qualitative approaches.[26,27]

The data on hair length and weight suggested that CTUMP's Herb shampoo effectively promoted hair growth, with results comparable to those achieved with 5% Minoxidil. This effectiveness may be attributed to the inclusion of extracts from *Citrus grandis* peel, *Gleditsiae australis*, and *Ocimum gratissimum* in the shampoo, which contain compounds like coumarin, carotene, and essential oils (particularly d-limonene) known to support and stimulate hair growth.[28] These extracts may have facilitated in the transition of hair follicles from the telogen phase to the anagen phase, as noted by Philpott et al. and Chaksupa et al..[29,30]

The degree of hair growth

The results for the degree of hair growth evaluation on days 7, 14, 21, and 28 are shown in Figure 3.

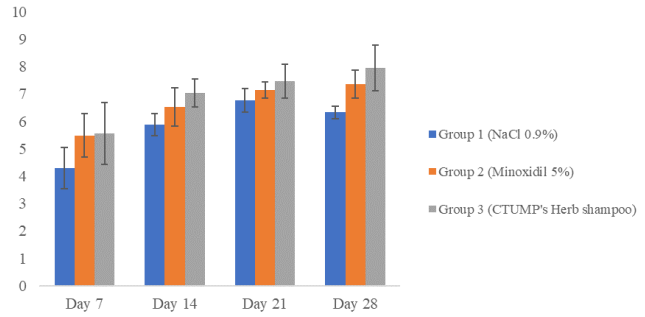

Figure 2: Effect on hair length of mice

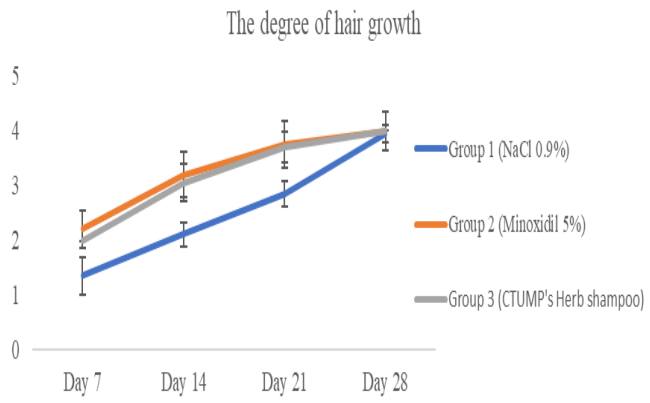

Figure 3: The degree of hair growth in mice

The data indicated that between days 7 and 21, groups 2 and group 3 had higher hair growth scores than group 1, with statistically significant differences ($P < 0.05$). Nonetheless, by day 28, the scores among the three groups showed no significant differences, indicating that the level of hair growth in mice had become comparable across all groups.

Figure 4 illustrated that during the first 21 days, both CTUMP's Herb shampoo and 5% Minoxidil significantly enhanced hair growth, with no notable difference between the treatments. On day 28, hair growth was comparable among all three groups, aligning with the findings of Adhirajan et al..[31] Mice treated with CTUMP's Herb shampoo displayed hair growth patterns extending from the thighs to the back, with increasing density over time.

In *in-vivo* mouse studies, the commonly utilized hair growth scoring systems were those proposed by Lee et al. and Begum et al.,[13,32] both featuring a five-level classification. Lee's system depended on visual assessments of hair growth conditions, whereas Begum's system assessed the percentage of hair coverage on the mice's skin. This study selected Lee's scoring system for its ease of application and greater accuracy, which helped reduce potential subjective errors.

Histological study

Table 5 presented the results showing the number of hair follicles in the anagen and telogen phases in mice, as observed through microscopy on day 29.

Table 5: Effect on hair follicle numbers (n = 5)

Group		Day 29
1	Average anagen follicle number	4.80 ± 0.84
	Average telogen follicle number	5.60 ± 1.52
	Anagen/telogen ratio	0.93 ± 0.37
2	Average anagen follicle number	22.60 ± 2.41
	Average telogen follicle number	8.40 ± 2.07
	Anagen/telogen ratio	2.76 ± 0.38
3	Average anagen follicle number	19.80 ± 2.86
	Average telogen follicle number	8.40 ± 1.52
	Anagen/telogen ratio	2.44 ± 0.66[b]

Group 1: treated with 0.9% NaCl; Group 2: treated with 5% Minoxidil; Group 3: treated with CTUMP's Herb shampoo. All the values were expressed as mean ± SD (Standard deviation). b:significant difference compared to the control, p value was <0.05.

At the end of the study, the group treated with 5% Minoxidil (group 2) had shown a significantly higher number of anagen hair follicles compared to telogen, with fully developed follicles penetrating deep into the dermis and subcutaneous tissue, demonstrating the most robust growth. The group treated with CTUMP's Herb shampoo (group 3) also showed a predominance of anagen follicles, though to a lesser degree than the 5% Minoxidil group (group 2). Although the CTUMP's Herb shampoo group (group 3) had fewer anagen follicles than the Minoxidil group (group 2), it had 2.6 times more than the 0.9% NaCl group (group 1). This suggested that the treatment successfully enhanced hair growth and follicle vitality.

Hyunkyoung Lee's study found that topical minoxidil application increased the number of hair follicles and promoted the transition from telogen to anagen phases.[33] Research by Rahmani et al., Choi et al., Žnidarič et al. reported that minoxidil stimulated epithelial cells near the base of hair follicles and may have expanded blood vessels in the scalp.[34-36] Although the precise mechanism behind CTUMP's Herb remained unknown, research has shown that the shampoo promoted hair follicle growth, with *Citrus grandis* peel, *Gleditsiae australis*, and *Ocimum gratissimum* likely contributing to hair development.

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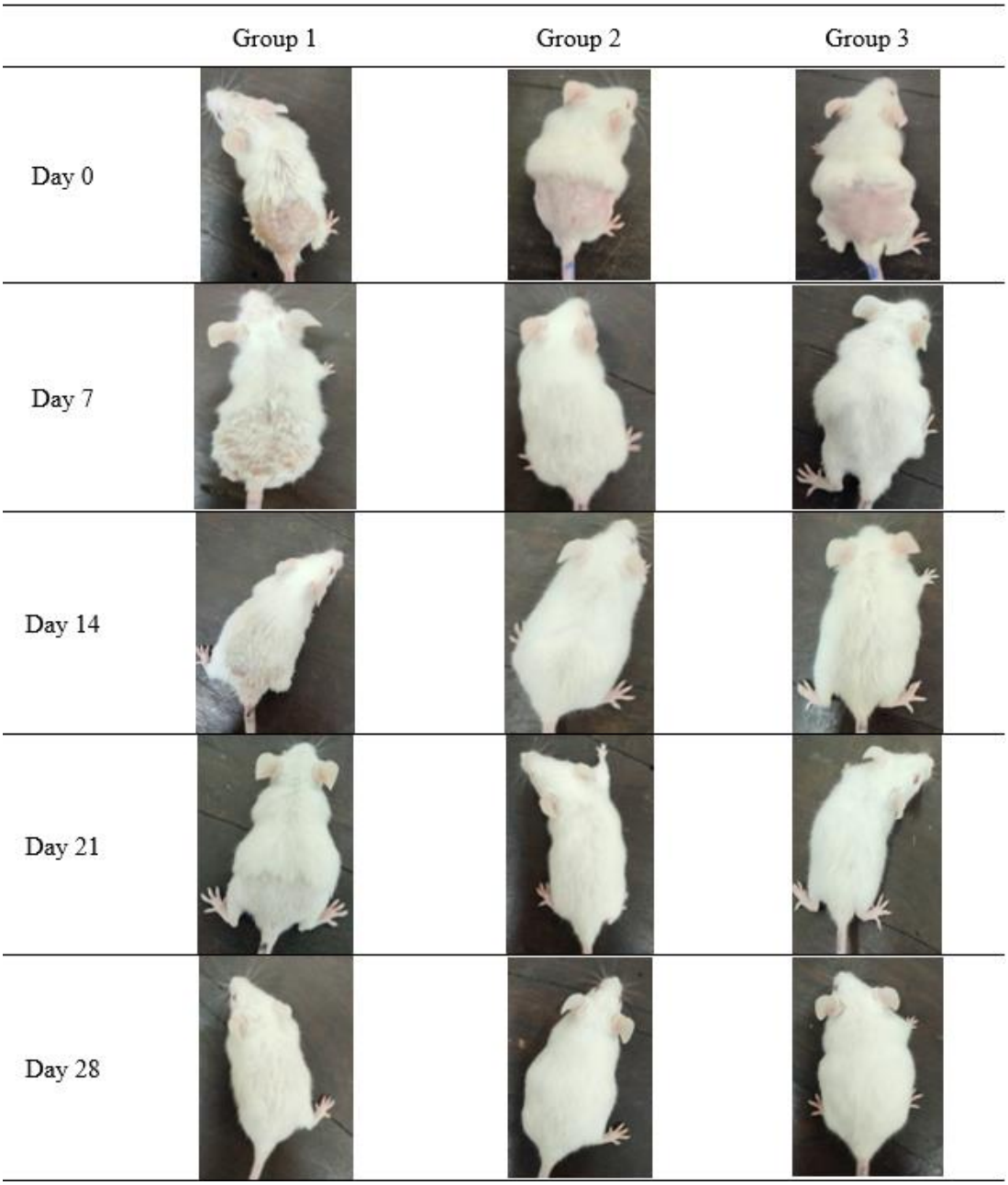

Figure 4: Mice from the three groups at the survey time points
Group 1: treated with 0.9% NaCl; Group 2: treated with 5% Minoxidil; Group 3: treated with CTUMP's Herb shampoo.

Conclusion

CTUMP's Herb shampoo effectively stimulated hair growth in mice. The findings of this study will serve as the foundation for the production and development of products branded by Can Tho University of Medicine and Pharmacy in the future.

Conflict of interest

The authors declare that there is no conflicts of interest.

Authors' Declaration

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

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