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TRICHOGENIC PROPERTY OF DALANDAN (CITRUS AURANTIUM) PEEL OIL ON SHAVED ALBINO ICR MICE

[*-**]John Jeamel P. Igpuara
[*]Ceejay P. Patawaran
[*]Rose Ann C. Chico
[*]Stephanie M. Nucup
[***]Pauline Lyzelle G. Francisco

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Corresponding Author: John Jeamel P. Igpuara; **Email:** jjpigpuara.student@ua.edu.ph; doi: 10.46360/globus.met.320242005

Abstract

Hair thinning (baldness) and hair loss (alopecia) are universal problems experienced by individuals across varied demographics. Fruit peels from the citrus genus, like *Citrus aurantium*, known in the Philippines as dalandan, are rich in phytochemicals like limonene. Previous studies revealed that limonene has the potential to affect hair growth in mice. Evaluation of the potential trichogenic (hair growth) property of *C. aurantium* peel involved dividing the ICR mice into six groups. Controls and test samples were applied daily on mice dorsa and were observed for macroscopic and microscopic hair growth study. One-way ANOVA and Tukey's HSD for post hoc comparisons were employed for statistical analysis. Through macroscopic evaluation, longer hairs and quicker hair growth initiation and completion time were observed in groups treated with 5% and 10% peel oil. Meanwhile, the 100% concentration exhibited opposite effects and signs of irritation. Statistical evaluation showed significant differences ($p<0.05$) in both follicle diameter and count in test concentrations particularly with 10% and 100% concentration, while the 5% minoxidil and 5% *C. aurantium* peel oil showed no significant difference in terms of hair length and follicle count ($p=0.18$, 0.08), which indicated likeness in activity. Overall, 10% of peel oil showed the most positive outcome and the greatest activity in both macroscopic and microscopic evaluation. The results show that *C. aurantium* peel oil has the potential as a hair growth promoting agent through its effects on decreasing the initiation and completion time for hair growth and increasing hair length, follicle count, and diameter.

Keywords: C. Aurantium, Peel Oil, Hair Loss, ICR Mice, Trichogenesis.

Introduction

Androgenetic alopecia (AGA) is the most prevalent cause of hair loss (Yan et al., 2023) that is experienced by both sexes with up to 80% of males being affected by this concern the time they turn 80, it occurs when there is a problem with how one's body makes hair, mainly on the head, and is a common concern for millions of people around the world (Gokce et al., 2022). Trichogenesis pertains to the capacity to generate hair follicles and may be used interchangeably with hair growth (Mahjour et al., 2012) and it can serve as an indicator to measure its effectiveness. It is linked to various pathways and compounds which promote the development of hair follicles, and is activated by limonene, a major component of *C. aurantium* plant peels (Ouji et al., 2006; Kang et al., 2022; Abderrezak et al., 2014).

The Filipino populace is celebrated for their naturally dense, dark, and curly hair, which is commonly linked to concepts of beauty and desirability. Hair bears immense cultural importance in Filipino society, transcending mere physical appearance. It symbolizes beauty, individuality, social hierarchy, and self-expression. Women often strive for long, cascading hair, while men use their hairstyles to convey personal style and traditional notions of manliness.

Extracts from the fruits of the Citrus genus have demonstrated remarkable benefits that can make hair smoother and shinier. High amounts of antioxidants (Shim et al., 2019) assist in curing pollution-related damage, which poses potential activity on hair growth. Citrus peels compose nearly half of the fruit mass but are often discarded even though they are high in vitamin B12, vitamin E and contain phenolic compounds that may also aid in hair formation (Tiwari et al., 2021).

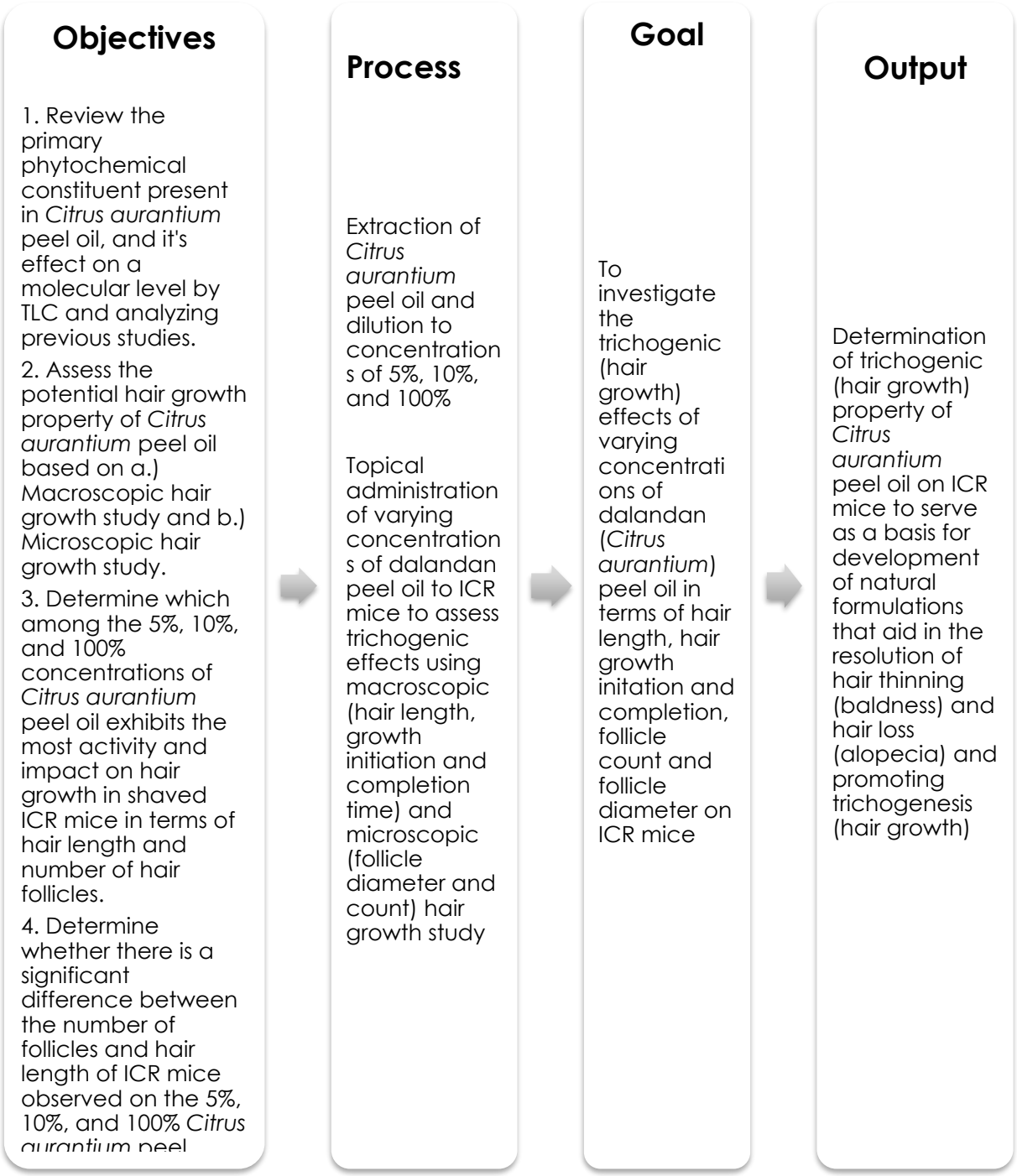
Previous studies have explored various natural compounds and oils for their potential effects on hair growth (Kang et al. 2022; Nguyễn et al. 2022).

*Pharmacy Student, University of the Assumption, Philippines.
** Scholar of the Department of Science and Technology, Philippines.
*** RPh, MSHSA, Faculty of College of Nursing and Pharmacy, University of the Assumption, Philippines.

However, the specific effects of peel oil derived from *C. aurantium* have not been extensively investigated, leaving a notable knowledge gap in the existing literature. While there is some general knowledge about the benefits of citrus fruits and their peels, the distinct properties of Dalandan (*C. aurantium*) peel oil and its impact on hair growth in animal models or human applications remain relatively unexplored.

Conceptual Framework

Figure 1: Conceptual Framework



Statement of the Problem

The study aimed to evaluate the trichogenic property of a topical oil containing *C. aurantium* peel oil on

albino ICR mice. Specifically, it aimed to answer the following questions:

1. What is the primary phytochemical constituent within Citrus *C. aurantium* peel oil that is believed to contribute to its hair growth promotion properties, and how does this component interact with the hair follicles and scalp at a molecular level?
2. Is there a potential hair growth property observed in the topical application of *C. aurantium* peel oil based on its results in:
   2.1 Macroscopic hair growth study and;
   2.2 Microscopic hair growth study?
3. Among the 5%, 10%, and 100% concentrations of *C. aurantium* peel oil, which exhibits the most activity and impact on hair growth in shaved albino ICR mice in terms of hair length and number of hair follicles?
4. Is there a significant difference between the number of follicles and hair length of albino mice observed on the 5%, 10%, and 100% concentrations of *C. aurantium* peel oil when compared to:
   4.1 Negative Control;
   4.2 Positive Control; and
   4.3 Vehicle Control?

Scope and Delimitation

This research investigated the trichogenic property of topically applied Dalandan (*C. aurantium*) peel oil, which may offer safer and more sustainable alternative to synthetic hair growth treatments. It had a specific scope and aimed to provide insights into the potential benefits of *C. aurantium* peel oil on hair growth in albino ICR mice:

1. The trichogenic potential of *C. aurantium* peel oil on albino ICR mice obtained from Pampanga State Agricultural University (PSAU).
2. The activity of *C. aurantium* peel oil against the standard of treatment: positive (minoxidil 5%), negative control (distilled water), and vehicle (jojoba oil) controls.
3. Hair growth as measured by factors such as hair length, the initiation and completion of hair growth, the number of follicles, and their diameters.

There are several limitations, such as:

1. No specific toxicity study was included in the methods.
2. No tests for optimal dosing schedule.
3. Anagen/telogen ratio was not identified.

Methodology

Research Design

True experimental design was employed in the research study. The test plants (*C. aurantium*) peels used in the study were procured from Talisay, Batangas.

Treatment. Animals were divided into six groups of six mice each. Table 1 shows the treatment given to animals of separate groups:

Table 1: Treatment Groups for Shaved Albino ICR Ice

Groups	Treatment Group	Solutions	No. of Mice
T_0	Negative Control	Distilled water only	6
T_C	Positive Control	5% Minoxidil (Regroe®)	6
T_V	Test Vehicle	*Simmondsia chinensis* oil only	6
T_1	Test Sample 1	5% *C. aurantium* peel oil	6
T_2	Test Sample 2	10% *C. aurantium* peel oil	6
T_3	Test Sample 3	100% *C. aurantium* peel oil	6

The treatment used 100 µL of test samples formulated and applied topically by using a micropipette once a day, every day, for 21 days.

Participants/Respondents of the Study (Animals)

Albino ICR mice weighing 35 to 50 g from the Pampanga State Agricultural University (PSAU) were fed a standard diet and were housed at room temperature (23±2ºC) on a normal day-night cycle. The mice's skin was mechanically depilated using a razor and completely denuded with a chemical depilator (Veet®).

In terms of hair growth research, the albino mouse model is one of many mice models employed, providing valuable insights into potential treatments for alopecia (Orăsan & Coneac, 2018; Wang et al., 2023). However, it is imperative to acknowledge substantial disparities in follicle architecture, genetic and hormonal influences, and hair growth cycles between mice and humans.

Instrument/s of the Study (Materials)

C. aurantium peels were thoroughly cleaned and dried in a dry oven at 55°C for several days until entirely dehydrated and pulverized using a mortar and pestle. Room temperature was used for the solvent extraction method using Soxhlet apparatus and n-hexane as the preferred solvent (Syahadat et al., 2021), and the process was repeated to obtain enough oil yield.

Phytochemical analysis of the *C. aurantium* peel oil via Thin Layer Chromatography (TLC) was performed at a Laboratory Animal Research Center (LARC) of the Pampanga State Agricultural University (PSAU) at Magalang, Pampanga.

Data Collection and Analysis

Data were reported as mean ± SEM (Standard Error of the Mean). Statistical data analysis was carried out by one-way ANOVA comparing all test groups versus control, followed by the Tukey-Kramer test using SPSS.

a. *Macroscopic hair growth study*

Macroscopic hair growth was assessed by visually observing two parameters: (a) the time for hair growth initiation, which refers to the minimum duration required to observe noticeable hair growth, and (b) the time for hair growth completion, which is the minimum period needed for new hair to fully cover the exposed skin area (Adhirajan et al., 2001, as cited in Jaybhaye et al., 2010). The time required for hair to initiate and complete growth was documented for each mouse group throughout the study period.

b. *Microscopic hair growth study*

The method of Uno (1991) as cited in Jaybhaye et al. (2010) was followed for the microscopic evaluation of *C. aurantium* oil. After 21 treatment days, all mice subjects were euthanized. Skin biopsies, measuring 1.5 cm², were collected from the denuded area and submerged in 10% formalin for preservation. The specimens were processed at Hi-Precision Diagnostics in San Fernando, Pampanga. Paraffin wax was used to embed the specimen to blocks in preparation for microtomy. Longitudinal skin sections were then cut and dyed applying hematoxylin and eosin. The number of hair follicles and follicle diameter were determined using a microscope and the aid of a veterinarian at PSAU.

Ethical Considerations

Institutional Animal Care and Use Committee (IACUC) clearance was obtained to ensure that procedures are being followed and that the conduct of experiment is ethically appropriate.

Results And Discussion

A. Primary Phytochemical Constituents Impacting Hair Growth

A study by Abderrezak et al. (2014) identified the major components of *C. aurantium* growing in various countries. As reported, limonene is a chemotype of the peel essential oil; its highest yield was in *C. aurantium* growing in Brazil at 93%, and also composes 86.2% of the PEO of *C. aurantium* from Cuba. This suggests the abundance of the said compound in the peels of *C. aurantium* coming from different countries, which may signify presence in *C. aurantium* growing in the Philippines.

The researchers further confirmed the potential presence of limonene after being tested by means of the Thin Layer Chromatography (TLC) method at PSAU, which came positive for oil upon spraying of vanillin-sulfuric acid as shown in Table 4. These results align with the comparative study of Sowmya et al. (2019), where tannins, flavonoids, and oils were detected in the hexane extract of *C. aurantium*.

Table 2: Positive Report on The TLC Method

Spray Reagent	Constituents Tested	Result
Potassium ferricyanide-ferric chloride	Phenol	+
	Tannins	+
	Flavonoids	+
Vanillin-sulfuric acid	Higher Alcohol	+
	Oil	+

***Legend**: Positive result (+); Negative result (+)*

Referencing the study of Mahjour et al. (2012) and Kang et al. (2022), it was revealed that limonene induced an increase in the proliferation of rat dermal papilla cells (rDPC) by modulating the levels of cyclin D1 and p27 and the activation of the Wnt/β-catenin pathway. These findings suggest the potential of limonene in triggering and affecting the hair growth activity of mice.

C. aurantium peels also contained antioxidants like flavonoids, which may have contributed to hair health and subsequently hair growth, this is in line with Maksoud et al. (2021).

B. Macroscopic and Microscopic Hair Growth Study

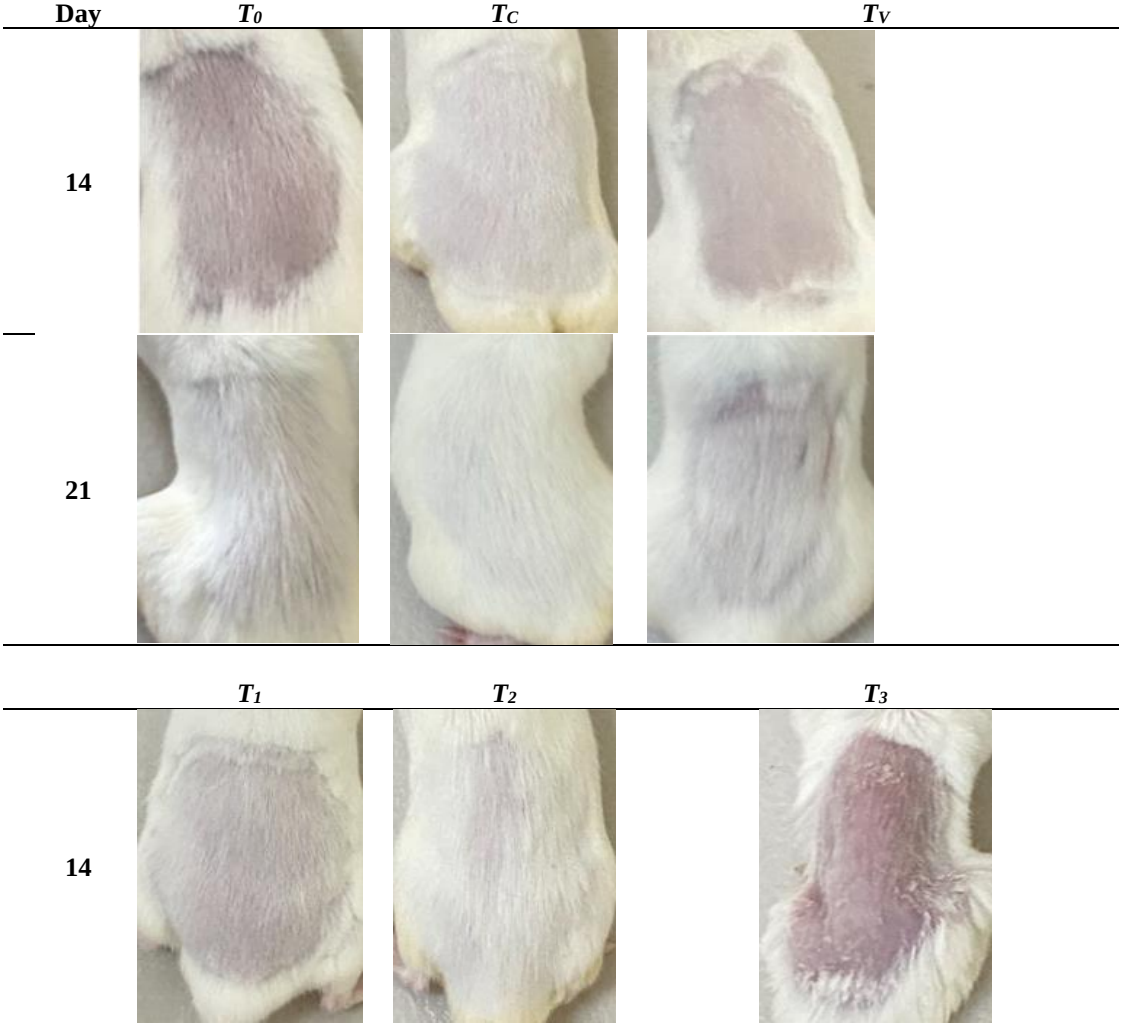
Macroscopic Hair Growth Study. The analysis of hair growth initiation and completion times shows significant differences across treatment groups (Table 3). TC being the standard of treatment demonstrated the fastest initiation, and is consistent with previous studies (Kang et al., 2013). T2 showed strong performance, outperforming both T1 and T3 concentrations. However, T3 and T0 exhibited similar initiation and completion times, suggesting that higher concentrations of *C. aurantium* oil may not affect and even hinder hair growth. Figure 2 serves as visual comparison. The hair growth time was ranked from fastest to slowest, initiation time: $T_C > T_2 > T_1 > T_V > T_0 > T_3$; completion time: $T_C > T_2 > T_1 > T_V > T_3 > T_0$

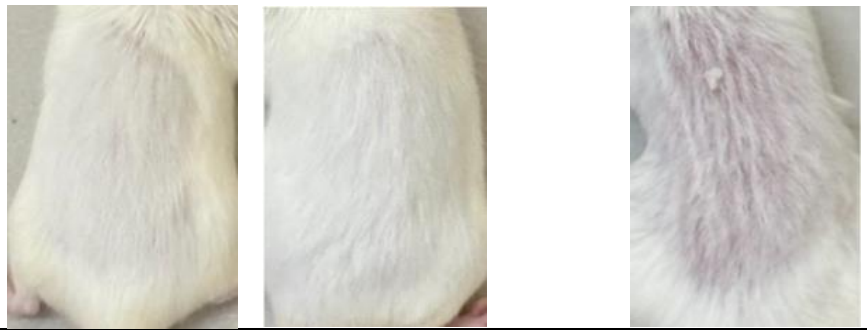
Table 3: Time Taken by Hair for Initiation and Completion of Hair Growth (in Days)

Groups	Treatments	Time (days)	
		Initiation	Completion
T_0	Negative	16±0.26	21±0.39
T_C	Positive (5% Minoxidil - Regroe®)	9±0.26	15±0.48
T_V	Vehicle (Jojoba oil)	15±0.16	20±0.49
T_1	5% *C. aurantium* oil	14±0.28	20±0.20
T_2	10% *C. aurantium* oil	12±0.60	19±0.73
T_3	100% *C. aurantium* oil	16±0.38	20±0.55

All values are mean±std. error of the different treatments, n=6

Figure 2: **Dorsum Photos at Days 14 and 21**





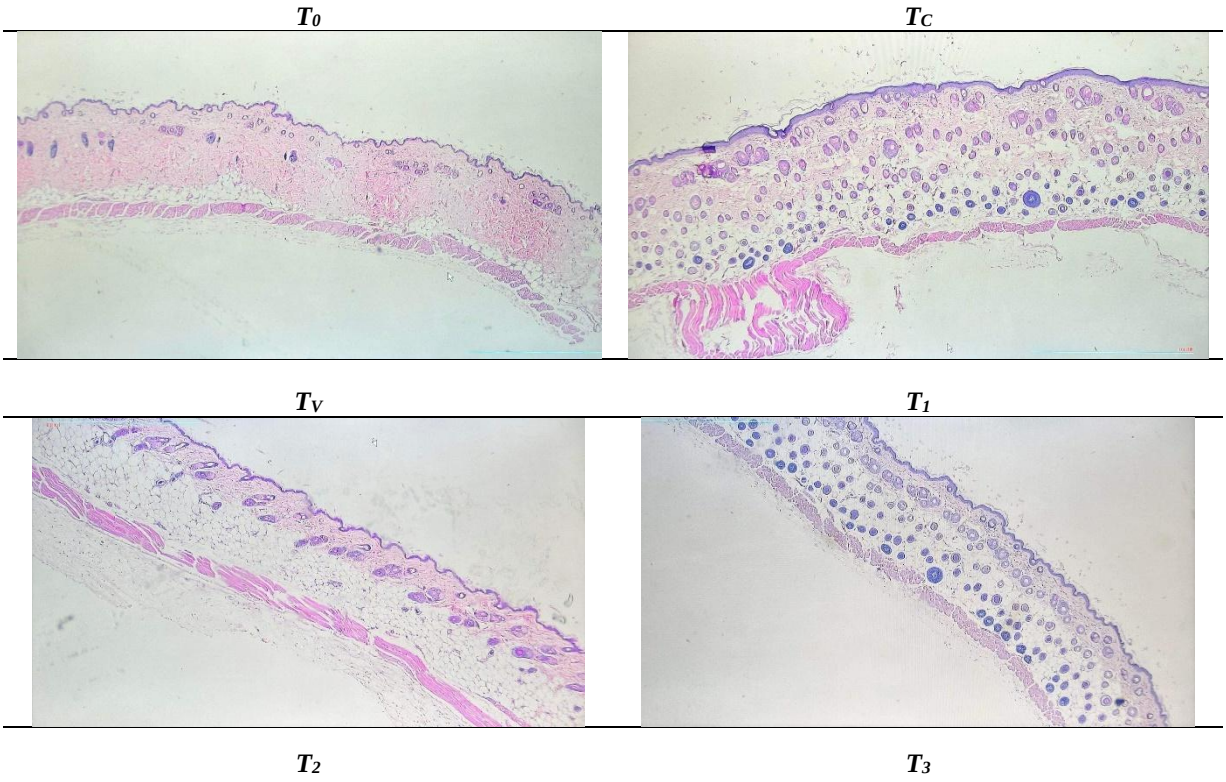
**Each picture is representative of each treatment group*

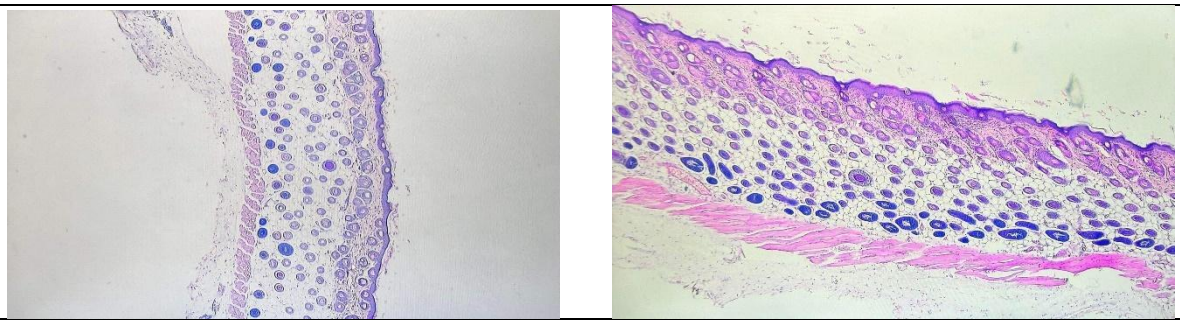
Microscopic Hair Growth Study. The microscopic analysis of hair growth showed notable differences in follicle counts across treatment groups (Table 4). T_0 has the least average number of follicles (194), while T3 showed the highest count at 705 follicles, indicating strong follicle proliferation. Figure 3 is included for visual comparison. From greatest to least number of follicles, test groups are ranked: $T_3 > T_2 > T_C > T_1 > T_V > T_0$

Table 4: Number of Hair Follicles in 1.5 cm^2 Longitudinal Section of Mice Skin

Groups	Treatments	Hair follicles
T_0	Negative	194±43.13
T_C	Positive (5% Minoxidil - Regroe®)	521±51.75
T_V	Vehicle (Jojoba oil)	215±22.66
T_1	5% *C. aurantium* oil	476±41.56
T_2	10% *C. aurantium* oil	580±42.38
T_3	100% *C. aurantium* oil	705±76.40

Figure 3: Longitudinal Section of Mice Skin





**Each picture is representative of each treatment group under scanner magnification*

The study on hair follicle diameter (Table 5) revealed that T2 produced the largest average diameter (164.50 µm), suggesting positive enhancement of hair thickness. T1 also significantly increased follicle diameter (146.58 µm) compared to controls. T3 was effective but slightly less so than 10%. These findings indicate that *C. aurantium* oil, especially at 10%, has a strong potential to improve hair thickness. From greatest to least diameter of follicles, test groups are ranked: $T_2 > T_3 > T_1 > T_C > T_V > T_0$

Table 5: Diameter of Hair Follicle

Groups	Treatments	Diameter (µm)
T_0	Negative	107.58±4.25
T_C	Positive (5% Minoxidil - Regroe®)	132.33±3.12
T_V	Vehicle (Jojoba oil)	124.90±3.81
T_1	5% *C. aurantium* oil	146.58±3.98
T_2	10% *C. aurantium* oil	164.50±3.06
T_3	100% *C. aurantium* oil	153.81±3.38

All values are mean±std. error of the different treatments, n=60

C. Assessing the Performance of the Different Oil Concentrations Against Each Other

Data for hair length measured in Table 6 shows that T2 produced the highest average hair growth (10.29 mm), versus 5% Minoxidil (8.72 mm) and lower concentrations of the oil. T1 also showed effectiveness (9.34 mm), while T3 was less effective (5.58 mm), suggesting that higher concentrations may not enhance efficacy and that again *C. aurantium* oil, especially at 10%, has significant potential as an alternative to standard hair growth treatments. The treatment groups, when ranked from longest to shortest strand, follow this order: $T_2 > T_1 > T_C > T_0 > T_3 > T_V$

Table 6: Length of Hair (in mm)

Groups	Treatments	Day 21
T_0	Negative	7.53±0.11
T_C	Positive (5% Minoxidil - Regroe®)	8.72±0.21
T_V	Vehicle (Jojoba oil)	4.61±0.14
T_1	5% *C. aurantium* oil	9.34±0.26
T_2	10% *C. aurantium* oil	10.29±0.20
T_3	100% *C. aurantium* oil	5.58±0.14

All values are mean±std. error of the different treatments, n=60

D. Efficacy of Different Concentrations of C. aurantium Peel Oil Against Negative, Positive, and Vehicle Controls

Tables 7 shows the p-values of the different treatments when hair length and number of follicles were compared to each other respectively. Interestingly, there is no significant difference only

between 5% minoxidil with 5% *C. aurantium* peel oil. This suggests a similarity in activity on macroscopic and microscopic measures of both treatments and supports the idea that the sample peel oil has effects on hair growth and trichogenesis.

Table 7: Summary of p-Values of Hair Length / Follicle Count in Different Treatments

Groups	T_0	T_C	T_V
T_1	.000* / .000*	.183 / .077	.001* / .001*
T_2	.000* / .000*	.000* / .000*	.000* / .000*
T_3	.000* / .000*	.000* / .000*	.007* / .000*

**p-value < 0.05, hair length/follicle count*

Table 8 shows that there is a statistically significant difference between the hair length as determined by one-way ANOVA (F(5,54) = 142.836, p = .000) and follicle density (F(5,54) = 32.748, p = .000). Based on the foregoing findings, all null hypotheses are rejected. It can, therefore, be concluded that the treatments resulted in statistically significant differences in both hair length and follicle density among the mice, verifying that *C. aurantium*-infused oils are feasible to be utilized and may pose trichogenic activity on ICR mice.

Table 8: Parametric Tests Among Treatments

		Mean Square	F	Sig.	Interpretation
Hair Length	Between Groups	48.956			
	Within Groups	.343	142.836	.000*	Significant
	Total				
Follicle Count	Between Groups	4308.908			
	Within Groups	131.579	32.748	.000*	Significant
	Total				

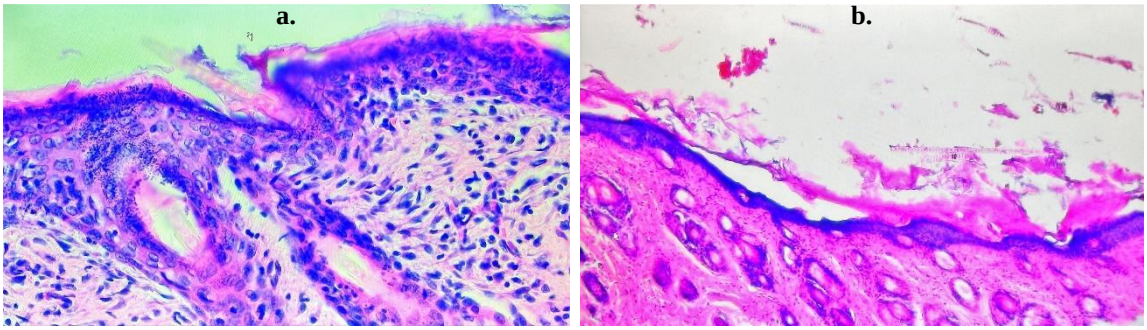
**p-value < 0.05*

E. Incidental Findings

Potential Dermal Toxicity of Pure C. aurantium Peel Oil

The study, in line with its scope and limitations, did not specifically intend to conduct a dermal toxicity study on the sample, but upon histological analysis of the skin samples obtained from mice treated with pure *C. aurantium* peel oil signs of irritation have already been observed and deemed not to be disregarded.

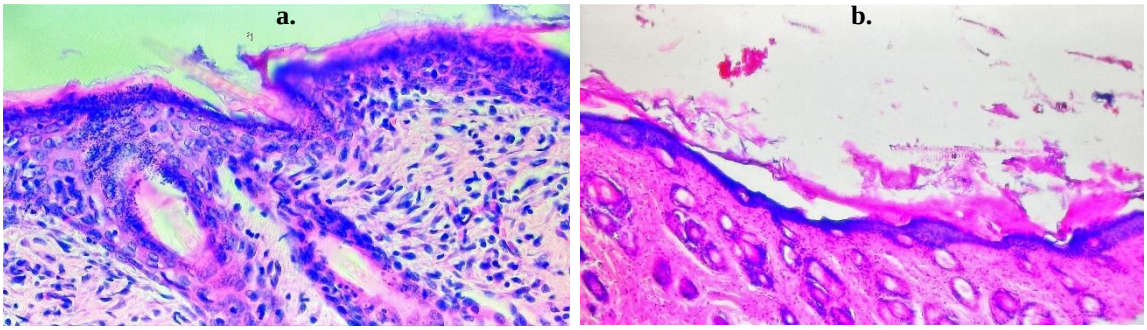
Figure 4: Histology of Pure C. Aurantium Peel Oil vs. Other Test Groups



Legend: ***a.*** *Neutrophilic infiltrates and hypergranulosis;* ***b.*** *Desquamation of mouse skin*

By initial visual inspection, redness, and increased thickness, both of which are significant of irritation, and desquamation, a prominent characteristic of dry skin (Ahmed & Mikail, 2024), have been observed. In terms of histology, skin irritation is typically characterized by dermal inflammation and epidermal hyperkeratosis and hyperplasia as shown in Figure 4 and in line with Mecklenburg et al. (2013).

Figure 5: Signs of Dermal Irritation with 100% Sample



Legend: ***a.*** *Neutrophilic infiltrates and hypergranulosis;* ***b.*** *Desquamation of mouse skin*

Further histological inspection of the skin samples from the mice, as detailed in Figure 5, confirms presence of neutrophilic infiltrates attributed to an increase of pro-inflammatory factors such as cytokines (Cianciulli et al., 2024) leading to skin inflammation. Additionally, mild instances of hypergranulosis, hyperkeratosis, and epidermal hyperplasia are noted. These findings may suggest that higher doses of *C. aurantium* peel cause irritation and contact dermatitis related to dose-dependent dermal toxicity, which is a common occurrence when increasing dosage of medications and various compounds (Fang et al., 2015; Bakar et al., 2019).

Conclusion

Based on the foregoing findings from the results of the study, the following conclusions were derived.

1. Presence of limonene and other phytochemicals like sinapic acid may be linked to hair growth by activating certain pathways and enhancing antioxidant effects due to increased phenols and flavonoids in peel extracts, promoting new hair follicle formation.
2. The hair growth effect of *C. aurantium* peel oil depends on its concentration, with macroscopic and microscopic studies indicating both promoting and inhibiting effects.
3. Among the tested concentrations (5%, 10%, and 100%), the 10% *C. aurantium* peel oil showed the most positive impact on hair length and follicle count in shaved ICR mice.
4. Significant differences were observed between *C. aurantium* peel oil concentrations and the controls, except for the 5% concentration, which showed no significant difference from 5% minoxidil, suggesting similar hair growth activity

Recommendations

Based on the results and conclusions drawn from the study, the following recommendations are suggested:

1. Develop hair growth products using 10% *C. aurantium* peel oil, which showed the highest efficacy in promoting hair growth, surpassing both negative and positive controls.
2. Investigate the molecular mechanisms behind *C. aurantium*'s hair growth properties, focusing on limonene and other key constituents involved in hair follicle generation pathways.
3. Conduct clinical trials to assess the safety and efficacy of 10% *C. aurantium* peel oil in humans, including higher concentrations to determine optimal dosage.
4. Study the anagen/telogen ratio to understand *C. aurantium* peel oil's molecular functions in stimulating hair growth.
5. Pharmacy students can use this study as a reference for research on natural hair growth agents, emphasizing concentration optimization and experimental design.
6. Hair care companies may explore developing products with 10% *C. aurantium* peel oil as a natural alternative to synthetic treatments like minoxidil.
7. Future researchers can examine *C. aurantium*'s effects on other hair loss conditions, different demographics, and related citrus species for potential hair growth applications.

Conflict of Interest

The authors declare that there are no significant competing financial, professional, or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

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