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# Study of the hair growth capacity of products derived from rosemary (*Rosmarinus officinalis*), in mice (*Mus musculus*) C57BL6//BIOU

# Estudio de la capacidad de crecimiento capilar de productos derivados del romero (*Rosmarinus officinalis*), en ratones (*Mus musculus*) C57BL6//BIOU

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**Abstract**

*In the present research work, the effect on hair growth of extracts derived from the rosemary plant (Rosmarinus officinalis) individually (essential oil, hydrolate and alcoholic extract) and together (in the form of a shampoo and hair tonic) was studied in male mice of the C57BL6//BIOU line, divided into 5 different groups: distilled water (AD), essential oil (AE), extract (ER), hydrolate (HR) and minoxidil 5% (MXD). Growth was evaluated for 5 weeks, using two methods: a growth scale with photographic record and the measurement of hairs taken by traction. The hair growth produced by these treatments was contrasted with a negative control group (distilled water) and positive control (minoxidil 5%). The growth scale used showed that the mice of the AE, ER and HR groups exhibited considerable hair growth; however, this scale proved to have serious limitations when evaluating mice with melanocytic nevus. On the other hand, the measurement of hair length showed that the AE, ER and HR groups presented a statistically superior growth than the control groups (p-value < 0.05). Finally, it was concluded that the extracts used in this research (essential oil, hydrolate and alcoholic extract) stimulate hair growth to a greater extent than the commercial drug minoxidil.*

**Keywords:** *Rosmarinus officinalis*, alopecia, hair growth, C57BL/6 mice, extract.

**Resumen**

*En el presente trabajo de investigación, se estudió el efecto sobre el crecimiento capilar de extractos derivados de la planta del romero (Rosmarinus officinalis) de manera individual (aceite esencial, hidrolato y extracto alcohólico) y en conjunto (en forma de un champú y tónico capilar) en ratones macho de la línea C57BL6//BIOU, repartidos en 7 grupos distintitos: agua destilada (AD), aceite esencial (AE), extracto (ER), hidrolato (HR) y minoxidil al 5% (MXD). El crecimiento fue evaluado durante 5 semanas, mediante dos métodos: una escala de crecimiento con registro fotográfico y la medición de pelos tomados por tracción. El crecimiento capilar producido por estos tratamientos fue contrastado con un grupo control negativo (agua destilada) y control positivo (minoxidil al 5%). La escala de crecimiento empleada demostró que los ratones de los grupos AE, ER y HR; exhibían un crecimiento capilar considerable, sin embargo, esta escala demostró tener serias limitaciones a la hora de evaluar ratones con nevo melanocítico. Por su parte, la medición de longitud de los pelos, demostró que dichos grupos AE, ER y HR; presentan un crecimiento estadísticamente superior que el de los grupos control (p-valor < 0,05). Finalmente, se concluyó que los extractos empleados en esta investigación (aceite esencial, hidrolato y extracto alcohólico) estimulan el crecimiento capilar en mayor medida que el fármaco comercial minoxidil.*

**Palabra clave:** *Rosmarinus officinalis*, alopecia, crecimiento capilar, ratones C57BL/6, extracto.

## 1 Introduction

For a long time, man has treated diseases with resources available to him, with the Plant Kingdom being the main source of therapeutic treatments. This has been documented in all ancient civilizations. With scientific advancements, the properties of medicinal plants have been proven, making treatments more effective and safer (Barquero, 2007; Caro Marquez et al., 2020; Marrelli, 2021).

The World Health Organization (WHO) recognizes many medicinal and toxic plants in its manual, "WHO Traditional Medicine Strategy 2014–2023." This manual outlines the uses of medicinal plants in natural, safe, effective, and low-cost treatments that are also accessible to the general population and widely accepted by them (Hosseinzadeh et al., 2015; World Health Organization, 2013; Salmerón-Manzano et al., 2020).

With the great therapeutic potential of medicinal plants, it is not surprising that from very early on, people were interested in using them for aesthetic purposes, such as hair care (Abelan et al., 2022; Patel et al., 2015). In the vast majority of ancient cultures, hair represents social status and personal attractiveness. The absence of hair often caused a great negative impact on both men and women (Barve & Dighe, 2016; Caro Marquez et al., 2020).

Many plants have been used on hair for various reasons. It is reasonable to assume that most plants known to man have been tried on hair at some point in history (Barve & Dighe, 2016).

One plant that has sparked the interest of researchers is *Rosmarinus officinalis*, commonly known as rosemary. This plant belongs to the Lamiaceae family (Andrade et al., 2018; de Macedo et al., 2020) and is native to countries on the Mediterranean Sea (Murata et al., 2013). Its use is widely linked to gastronomy (Moore et al., 2016; Nieto et al., 2018).

Several studies show that the extract of *Rosmarinus officinalis* has antiandrogenic properties, which prevent hair loss (de Macedo et al., 2020; Masoud et al., 2020; Murata et al., 2013). In this context, the objective of the present research is justified: to study the hair growth capacity of products derived from rosemary (*Rosmarinus officinalis*) in C57BL6//BIOU mice (*Mus musculus*).

## 2 Theoretical Framework

### 2.1 *Botanical Description of Rosmarinus officinalis*

This is an aromatic, evergreen shrub of the *Lamiaceae* family, growing up to 1.2 m high. It has a straight stem, numerous branches, exfoliating bark, and is finely puberulent. The leaves are sessile, opposite, green, numerous, woolly, obtuse, and glandular. They are 1-3 cm long, nearly cylindrical, and folded inward. The flowers are fragrant, 10-12 mm long, and grow in small terminal clusters. It has a tubular calyx, a two-lipped violet corolla, an elongated style, and an oval fruit divided into four sections (González-Minero et al., 2020; Hammer & Junghanns, 2020).

**Figure 1.** *Rosmarinus officinalis* (rosemary).

### 2.2 *Zoological Description of Mus musculus*

*Mus musculus*, commonly known as the house mouse, is a small rodent belonging to the Muridae family. Adults typically measure between 6.5 to 9.5 centimeters in length, with tails that can be up to 10.5 centimeters long. They weigh from 12 to 30 grams. The fur color varies from light brown to black, often with a lighter or even white underside. Their long tails are either hairless or have very little fur and feature circular rows of annulations (scale-like skin cells) (Macholán et al., 2012).

This species is known for its high reproductive potential, which occurs year-round. However, wild mice may have reproductive seasons that last from April to September. The estrous cycle is 4 to 6 days long, with estrus (the period when the female is receptive to mating) lasting less than a day (Macholán et al., 2012).

Females usually have 5 to 10 litters per year if environmental and nutritional conditions are favorable. Gestation lasts approximately 21 days, and litters can consist of 3 to 12 pups, which are born hairless and blind. Pups are fully covered in fur at 10 days, open their eyes at 14 days, are weaned at 3 weeks, and reach sexual maturity at 5-6 weeks. Their average lifespan in captivity is 2 years, but some individuals have been recorded to live up to 6 years. In contrast, wild mice typically do not live longer than 18 months due to predation (Brust et al., 2015).

**Figure 2.** *Mus musculus (common mous*

## 3 Experimental Procedure

### 3.1 *Preparation of ethanolic extract of rosemary (Rosmarinus officnalis)*

Ten kilograms of plant material belonging to the Rosmarinus officinalis species were collected in the town of Mucuchíes, Rangel municipality, Mérida state, Venezuela. All material was washed with plenty of water to remove any dirt.

The leaves were then separated from the rest of the plant (stems, flowers, and seeds). The leaves were dried in an oven at 40°C for 24 hours, yielding 2,330 kilograms of dried leaves.

The dried leaves underwent continuous Soxhlet extraction, in a ratio of 250 g of plant material to 600 mL of ethanol (98% Merk) at 70°C for 4 continuous hours. A total of 15 extractions were performed, with a consumption of 10.36 L of 70% ethanol.

The resulting extract was concentrated to one-third of its initial volume (3,2 L) in a rotary evaporator at 70°C for 2 hours. The resulting liquid was a slightly thick, greenish-brown liquid with a pleasant odor and pH = 5. It was stored in an amber glass container until further use.

To determine the yield of the extract obtained, 6 flasks were weighed, then 10 mL of the extract was added to each flask (measured with a volumetric pipette) and reweighed. The extract was then dried in an oven at 40°C for 48 hours, and the flasks were reweighed.

### 3.2 *Preparation of 4.8% rosemary essential oil*

Six ml of rosemary essential oil (98% v/v, from QuimicHouse) were mixed with 114 mL of mineral oil. The mixture was stored in a properly labeled amber glass container.

### 3.3 *Preparation of 0.135% rosemary extract*

Six ml of rosemary extract (2.7% w/v) were mixed with 114 mL of distilled water. The mixture was stored in a properly labeled amber glass container.

### 3.4 *Test in C57BL6//BIOU mice*

All animal procedures were evaluated and accepted by the Bioethics Committee of the Bioterium of the University of the Andes (CEBIOULA) under protocol identification "CEBIOULA/131."

Thirty male mice of the C57BL6//BIOU strain, approximately 8 weeks old and weighing an average of 25.7 g, were used. They were given a 1-week adaptation period and maintained under the following housing conditions:

- Cage Type: T1.
- Individuals per Cage: 4.
- Ambient Temperature: (22 ± 1) °C.
- Adaptation Period: 1 week.
- Food and Water: ad libitum (on demand).
- Light/Dark Cycle: 12 hours.

After the adaptation period, the animals were anesthetized with a mixture of ketamine and xylazine, with a dose of 90 mg/kg of ketamine and 10 mg/kg of xylazine, intraperitoneally, obtaining an average anesthesia time of 7-10 minutes, then an area of 4 cm2 (2 x 2 cm) was shaved from the dorsal region of its body (Figure 3), and each animal was identified by small cuts in its ears (Figure 4).

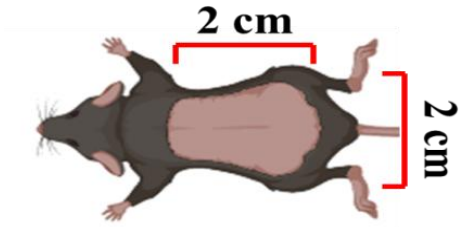


**Figure 3.** Representation of the shaved area on the dorsal region of the mice.

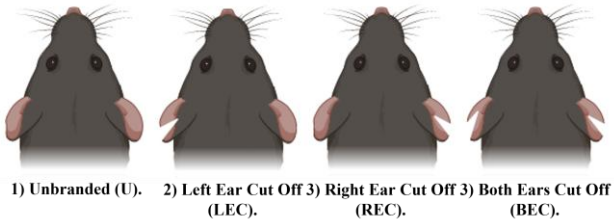


**Figure 4.** Representation of the marking of mice.

After the anesthesia time had passed, the mice were randomly distributed into 5 distinct groups, each made up of 4 individuals, based on the topical application they were given: distilled water (AD), 4.9% rosemary essential oil (AE), 0.135% rosemary extract (ER), rosemary hydrolat (HR) and 5% minoxidil (MDX).

The products were applied daily, with each individual receiving 0.1 mL (measured with a micropipette) of the corresponding product, which was adequately distributed with the help of fingers. In the case of the shampoo, after its application, it was removed with the help of a moist tissue.

### 3.4 *Hair growth assessment*

The evaluation of hair growth in mice was performed using two scales:

Method 1:

A photographic evaluation was conducted at the end of weeks 1, 2, 3, 4, and 5. An observation box was set up, con

sisting of an uncovered box with the following dimensions: 15 cm high, 5 cm wide, and 7 cm deep.

This box had a small opening on its top, which allowed the animal's tail to pass through. The purpose of this box was to suspend the mouse in the air, limiting its movement, thus facilitating photographic capture without the need to anesthetize the individual (Figure 5).

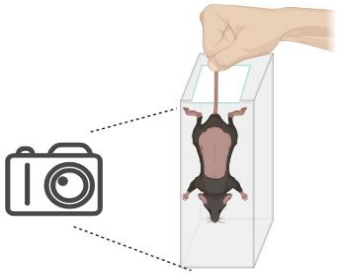

**Figure 5.** Representation of the manufactured observation box.

In this method, the following scoring scale was used:

**Table 1:** Hair growth scoring scale.

| Score | % Growth |
|---|---|
| 1 | (0-20) % |
| 2 | (20-40) % |
| 3 | (40-60) % |
| 4 | (60-80) % |
| 5 | (80-100) % |

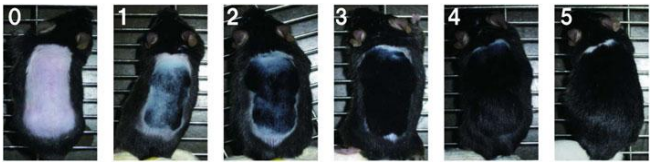


**Figure 6.** Scores for hair growth assessment: 0 = no growth; 1 = less than 20% growth; 2 = 20% to 40% growth; 3 = 40% to 60% growth; 4 = 60% to 80% growth; 5 = 80% to 100% growth (Murata et al., 2013).

Method 2:

At the end of weeks 2, 3, 4, and 5, a tuft of hair was pulled from within the shaved area, ensuring that the hairs were extracted with their respective follicles.

At week 5, a tuft of hair was pulled from within and outside the shaved area for comparison. This procedure was also performed on the control group mice.

The length of these hairs was measured using a 6-diopter magnifying glass (6x magnification) and a digital vernier caliper (DIGITAL CAPILPER brand) with an accuracy of 0.02 mm.

### 3.5 *Statistical analysis*

The data obtained in the study were processed using the statistical package GraphPrism version 8. The Shapiro-Wilk test was used to observe the normal distribution of the data.

The results of the quantitative variables were presented with measures of central tendency in frequencies, percentages, and absolute values using graphs. For differences in mean values, the Student t test was used, accepting significant values below $p < 0.050$.

In this sense, to observe the differences between the mean values among the different groups, a one-way ANOVA test was performed to obtain Fisher's F value.

To obtain the differences and comparative values between each group, a two-way ANOVA test was performed, considering the column factor as the treatment and the row factor as the values obtained between the study weeks.

This analysis was subjected to a post-hoc test using Tukey's method to verify the relationships between means, considering that each measurement was taken at different times. Significant F values above 1 were accepted, as were statistical differences with p values <0.050.

## 4 Results and Discussion

It is important to note that during the initial mouse shaving procedure, three individuals in the AD group (distilled water) were observed to have a melanocytic nevus (mole) covering the entire dorsal region of the animal (Figure 7). This trait was not present in any other mice in the other study groups (CC, AE, ER and MXD).

Initially, it was thought that this characteristic would not impact the hair evaluation of these individuals, and they were not discarded because no other mice of the same lineage and age were available. However, the moles were shown to have a negative effect on the hair growth scale and photographic records at the time of obtaining results. Furthermore, it did not appear to affect the methodology used to measure the length of the plucked hairs.

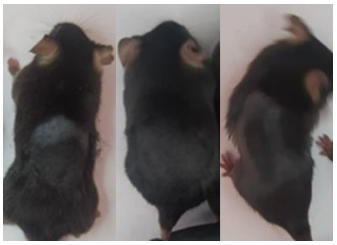

**Figure 7.** AD1, AD2 and AD3 mice presenting melanocytic nevus (mole) in the dorsal region of their body.

4.1 *Photographic record and hair growth scale*

The results shown in Figure 8 illustrate the progression of hair growth over time, from week 0 to week 5. After shaving (week 0), all C57BL6//BIOU mice displayed a pinkish coloration of their skin (with the exception of three individuals in the AD group).

Hair growth stimulation was assessed by observing the darkening of the skin color from bright pink to gray/black, indicative of the transition from the telogen (resting) phase to the anagen (active growth) phase of the hair follicles in the shaved area (Oh et al., 2014).

Throughout the study, no adverse effects due to topical application of the products were observed, such as irritation, scaling, or behavioral signs indicating discomfort in the mice (barbering, excessive grooming, or lack of appetite).

Starting at week 2, the skin of most individuals in the AD, AE, ER and HR groups changed from a pinkish color to a gray/black hue, reflecting significant hair growth in these groups. On the other hand, only half of the individuals in the MXD group exhibited this phenomenon.

At week 4, all mice in the AD, AE and HR groups showed significant hair growth (scoring between 3.5 and 5 on the scale), while the MXD group still had large areas of hairless skin (scoring 2), making this the group with the least hair growth.

At the end of week 5, it can be observed that all mice in the AD, AE and HR groups had recovered virtually all of their hair from the shaved region (scores between 4.75 and 5), while in the MXD group only two mice managed to recover all of their fur, resulting in an average score of 3.75.

Table 5 and Figure 8 show the group average hair growth scores for all groups over the 5 weeks of the trial.

**Table 2:** Group mean on the hair growth scale for the AD, AE, ER, HR and MXD groups, from week 1 to week 5.

| Group | Week 1 | Week 2 | Week 3 | Week 4 | Week 5 |
|---|---|---|---|---|---|
| **AD** | 1 | 2,5 | 3,75 | 4,75 | 5 |
| **AE** | 0,5 | 2,25 | 3,75 | 5 | 5 |
| **ER** | 1 | 1,5 | 2,5 | 4,25 | 5 |
| **HR** | 1,25 | 1,5 | 3,25 | 3,75 | 4,75 |
| **MXD** | 0,25 | 1 | 2 | 2,75 | 3,75 |

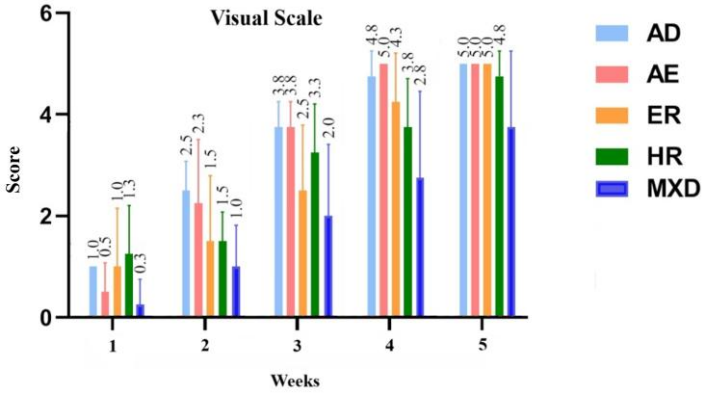


**Figure 8:** Comparison of the effect of hair growth, using the scoring scale, in C57BL6//BIOU mice after topical application of distilled water (AD), 5% essential oil (AE), 5% rosemary extract (ER), rosemary hydrolate (HR) and 5% minoxidil (MXD).

4.2 Measuring hair length

Below, Figure 9 shows a comparison of hair growth between weeks 2 and 5, in the form of column and box graphs.

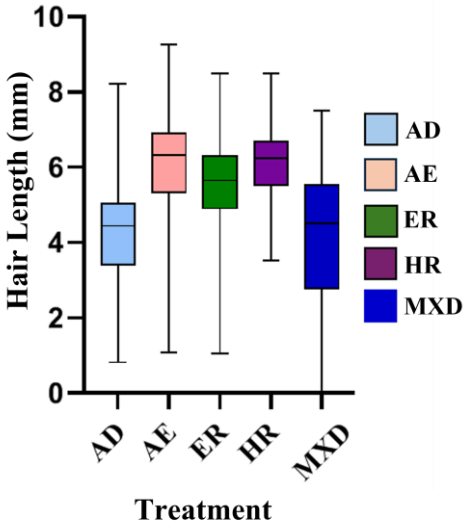


**Figure 9:** Comparison of hair growth (measured in mm) in the form of boxes, for the AD, AE, ER, HR and MXD groups between weeks 2 and 5.

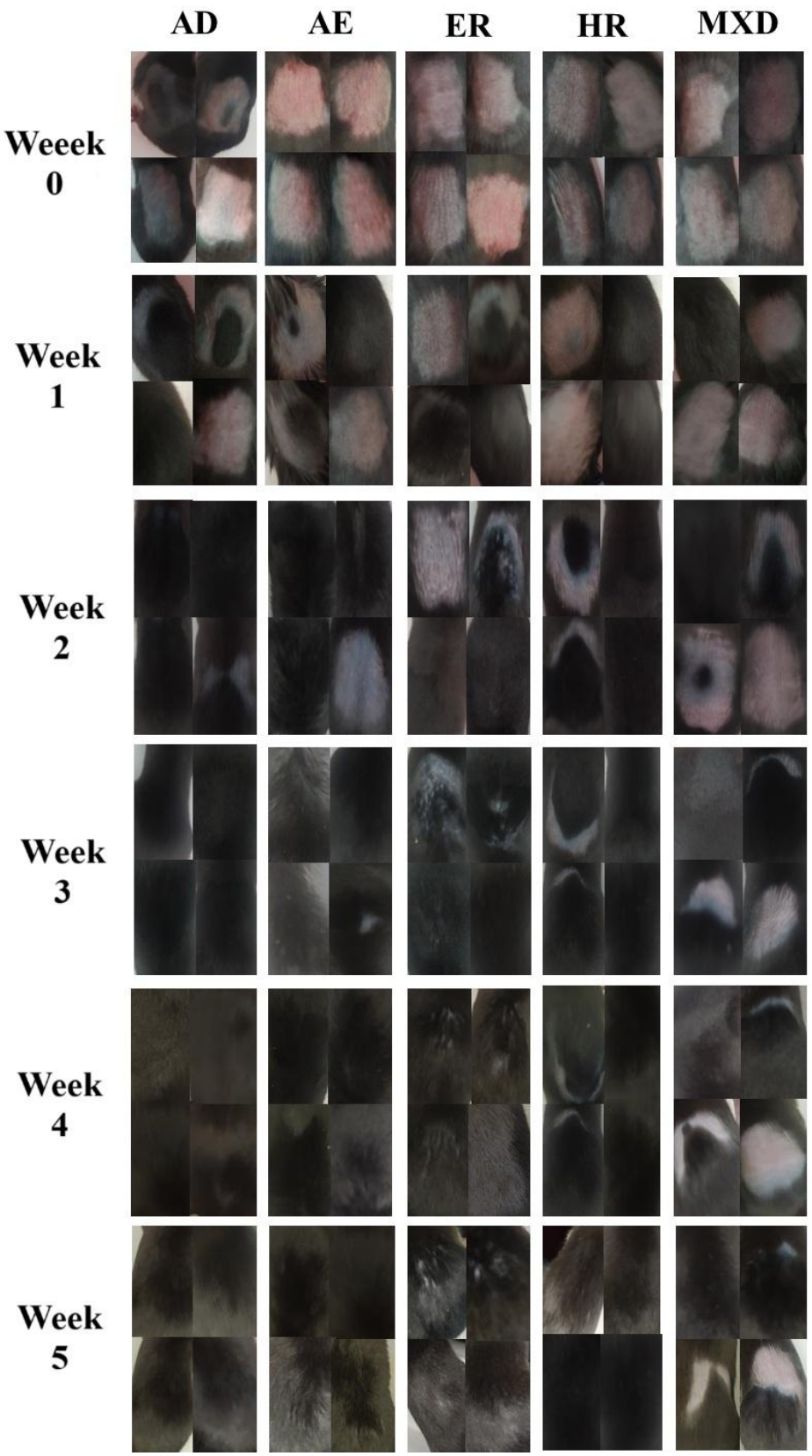


**Figure 10.** Macroscopic observation of the dorsal region of C57BL6//BIOU mice. AD (distilled water), AE (rosemary essential oil), ER (rosemary extract), HR (rosemary hydrolate) and MXD (5% minoxidil).

The results of the p-value for the Shapiro-Wilk test (Table 3) demonstrate that the hair length values throughout the study fit the normality model in all study groups.

**Table 3:** Shapiro-Wilk normality test for data from the AD, AE and MXD groups between weeks 2 and 5.

| | AD | AE | ER | HR | MXD |
|---|---|---|---|---|---|
| | Shapiro-Wilk Test | | | | |
| **W** | 0,9893 | 0,8245 | 0,9304 | 0,8423 | 0,9561 |
| **p-value** | 0,9538 | 0,1539 | 0,5970 | 0,2022 | 0,7545 |
| **Normality** | Yes | Yes | Yes | Yes | Yes |

**Note:** Data normality is checked if p-value>0.05.

For its part, the results of the ANOVA test carried out considering all the study groups showed that there are statistically significant differences ($p<0.05$) in the hair growth of these (Table 4 and Figure 11).

**Table 4:** Results of the one-way ANOVA test, comparing the hair length of all study groups.

| | SS | DF | MS | Value of F | Value of P |
|---|---|---|---|---|---|
| **Between groups** | 642,3 | 4 | 160,5 | 71,7 | 0,006 |
| **Within the groups** | 1778,1 | 795 | 2,23 | | |
| **Total** | 2420,4 | 779 | | | |

**SS:** sum of squares. **DF:** degrees of freedom. **MS:** mean squares. **Note:** There are statistically significant differences between group means if p<0.05.

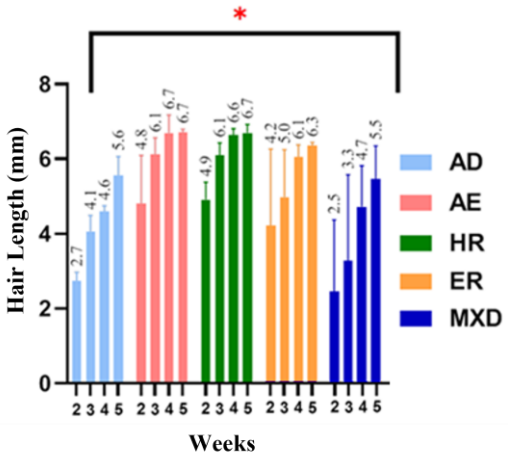


**Figure 11:** Results of the ANOVA test on hair length of the AD, AE, ER, HR and MXD groups, at weeks 2 and 5. The test result is indicated with (**), which expresses that p 0.05.

With this in mind, the means of each treatment group (AE, ER and HR) were compared with those of the AD and MXD groups using the Tukey test. This test determined that the hair length of the treatment groups was statistically different (longer) than that of the AD and MXD groups (Table 5 and Figure 12).

**Table 5:** ANOVA test with post-Hoc analysis under Tukey's test of the AE, ER and HR groups, assuming the AD and MXD groups as HDS.

| Stimulus evaluated | p Value | |
|---|---|---|
| | Distilled water (AD) | 5% Minoxidil (MXD) |
| **AE** | 0.0039* | 0,0079* |
| **ER** | 0,0075* | 0,0057* |
| **HR** | 0,0044* | 0,0086* |

**Note:** The symbol (*) indicates that p < 0.05, and H1 is approved.

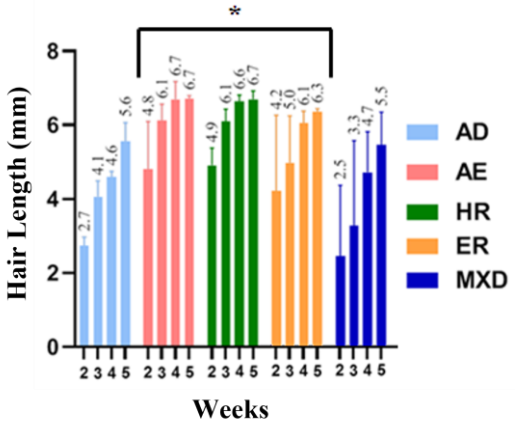


**Figure 12:** ANOVA with post-hoc analysis using Tukey's test for the AE, ER, HR, TC, and CH groups, assuming the AD and MXD groups as HDS. The symbol (*) indicates that the group mean presents statistically significant differences with the AD and MXD groups (p<0.05).

## 4 Conclusion

A hair growth study was conducted comparing the effect of rosemary-derived compounds, individually (oil, extract, and hydrolate) and in combination (hair tonic and shampoo). The results demonstrated that the hair scale method (method 1) has serious limitations when evaluating hair growth in mice with melanocytic nevus. On the other hand, method 2 demonstrated that the groups treated with rosemary-derived products (AE, HR, ER, CH, and TC) showed statistically superior hair growth (p-value 0.05) compared to the negative control group (distilled water) and the positive control group (5% minoxidil).

The results of this research demonstrate that the treatments used stimulate hair growth to a greater extent than the

commercial drug minoxidil, and that they could be a possible therapeutic alternative.

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