Original Article

Evaluation of Herbal Hair Lotion loaded with Rosemary for Possible Hair Growth in C57BL/6 Mice

Asia Begum[1], Sandhya S[2], Anoop Kumar N[3,4], Syed S. Ali[1]

[1]Department of Pharmacognosy, Nalanda College of Pharmacy, Cherlapally, Nalgonda, Telangana, India, [2]Department of Pharmacology, P. S. M. College of Dental Science and Research, Thrissur, Kerala, India, [3]School of Family Health Studies, Kerala University of Health Sciences, Thrissur, Kerala, India, [4]Department of Oral Pathology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, Tamil Nadu, India

Abstract

Background: *Rosmarinus officinalis* (rosemary) is a common household plant with needle-like leaves and white flowers that belongs to the family Lamiaceae and has various medicinal properties including ailments of hair and scalp, cardiovascular, nervous disorders, etc., In the current work, we have focused on formulation and evaluation of 1% hair lotion incorporated with methanolic extract of *R. officinalis*.

Materials and Methods: The aerial parts of the plant were extracted with methanol and then the nature of phytochemicals were identified by chemical tests. It showed the presence of proteins, amino acids, fats and oils, steroids, glycosides, phenolic compounds, flavonoids, volatile oil, and vitamins. The extract was formulated to a suitable hair lotion and then evaluated for its various quality control parameters. Finally, the lotion was evaluated for hair growth promoting activity on C57BL/6 mice, using water as control and 2% minoxidil hair lotion as standard.

Results: It was observed that the formulated 1% herbal hair lotion passed all the evaluation parameters and showed a significant hair growth promoting activity than the standard drug-treated animals.

Conclusion: Although several researches have been carried out on the rosemary, an investigation on formulation of hair lotion adding the extract of the aerial part of the plant is for the first time. Since our formulation exhibited an excellent activity, it can be well thought out to be an alternative to the commercially available hair growth promoters with a lot of unwanted effects.

Keywords: C57BL6 mice, flavonoids, hair follicles, herbal formulation, minoxidil, *Rosmarinus officinalis*, skin pigmentation

Address for correspondence: Dr. Sandhya S, Department of Pharmacology, P. S. M. College of Dental Science and Research, Thrissur, Kerala, India.
E-mail: drsanpharm@gmail.com
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Introduction

The hair is one of the fundamental parts of the body which is considered to be a complex organ derived from the ectoderm of the skin. It is made up of dead keratinocytes, the matrix cells, and dermal papillae situated in the innermost end of the follicle. They employ protection, sensory functions, controls temperature, and provide sexual attractiveness. They are also considered to be an additional structure of the integument along with sebaceous glands, sweat glands, and nails.[1,2] A healthy hair shaft has cuticle, medulla, and cortex where the cuticle is the outermost layer that protects the medulla and cortex. It is transparent in nature and gives a shiny appearance. The center layer cortex is made up of keratin and coloring pigments.[2] The size and shape of the hair shaft is determined by the cortex and large size cells make the innermost layer called the medulla. The hair growth and loss are completely random and are not seasonal or cyclic. The main problems associated with hair are loss of pigmentation, dandruff, and hair fall.[3,4] Nutrition plays a major role on the quality and quantity of hair. Lack of nutrition may result in dull, dry, brittle, or thin hair coat.[4,5]

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Minoxidil is a synthetic drug generally preferred for the treatment of hair loss and is found that with several years of use, enhanced hair growth was observed to reach the peak in 1 year followed by decline over the following years.[6] Plants rich in flavonoids is reported to exhibit potential hair growth property since it possess antiandrogenic activity (testosterone, 5-alpha-reductase inhibition) and antioxidant activity.[7]

Rosmarinus officinalis Albus (Lamiaceae) also known as Garden Rosemary, Compass-Weed is a common dense, evergreen, aromatic shrub with fragrant, needle-like leaves, and white colored flowers. It is cultivated in the Mediterranean basin and India. Chemical properties of the plant include essential oil with main constituents including camphene, camphor, cineol, and borneol. It is reported to contain a lot of flavonoids, bitter principles, tannins, and terpenoids.[8,9] Rosemary is used for several health conditions as a digestant, diuretic, emmenagogue, laxative, etc., It is also used as a flavoring agent in various cosmetic preparations.[8,9] Hair loss is a dermatological disorder that is considered to be a problem from time immemorial and is reported to affect approximately 0.2–2% of the world population. Hair growth problems often affect the physical and mental health of individuals and are considered to play a significant role during the aging.[6,10,11] In an effort to determine such problems, an attempt was done to find plants having hair growth promoting activity and thus found that the volatile oil of *R. officinalis* is widely used for the above-mentioned purpose. Hence, this formed the basis for the selection of the plant and formulating into a hair cream.

Materials and Methods

Collection and authentication

The fresh plant of *R. officinalis* Albus was collected from Central Institute of Medicinal and Aromatic Plants in the region of Field Station, Allalasandra, Bangalore, India, in the month of December to January 2012. The plant material was identified and authenticated by Mr. Siddulu, Lecturer of Botany, Nagarjuna Government Degree College in Nalgonda, Telangana. The herbarium specimen was prepared and deposited in the Department of Pharmacognosy, under the voucher no: NCOP/Ph'cog/2011-2012/042, for future references.

Procurement of experimental animals and their maintenance

Healthy C57BL/6 male mice (7 weeks old, six mice per group) were obtained from Mahaveera Enterprises (Hyderabad). Mice were cared for in a controlled barrier facility within Nalanda College of Pharmacy, where the temperature (23 ± 2°C), humidity (35–60%), and photoperiod (12 hours light, 12 hours darkness cycle) were kept constant. The animal study was performed in accordance with the institutional guidelines. Ethical clearance for the use of animals was obtained from the Institutional Animal Ethical Committee under reference no: NCOP/IAEC/Approval/53/2012, Date: March 24, 2012. Sacrificed animals were disposed as per Committee for the purpose of Control and Supervision of Experiments on Animals guidelines.

Extraction

About 600 gm of the whole aerial part of the plant was taken and extracted with methanol in a Soxhlet apparatus and the extraction was completed in 32 hours when the syphoning was colorless. The extract was made solvent-free with the help of rotary vacuum evaporator and percentage yield was calculated. Further, the preliminary chemical tests were performed to identify the chemical nature of the extract.[12-14]

Formulation and evaluation of 1% herbal hair lotion

To prepare the oil phase stearic acid, tween 60, propylparaben, and ethylparaben in the concentration of 3.5%, 2%, 0.126%, and 0.126%, respectively, were taken. The aqueous phase consisted of water, triethanolamine, and plant extract in the concentration of 8%, 1%, and 1%, respectively. The required ingredients for oil phase and aqueous phase were taken and heated at 50°C separately. Then to the aqueous phase the required amount of plant extract was added and, mixed thoroughly at 50°C. Further, the oil phase was mixed with aqueous phase and stirred well until the desired consistency was obtained and it was stored in a well closed air tight container.[15,16]

Evaluation parameters for 1% herbal lotion

Organoleptic characters were studied by observing the color, odor, and physical appearance. Presence of foreign particles/grittiness was studied by spreading a small amount of lotion on a glass slide free from grease and observing against a diffused light.[17,18] The pH was noted using a pH meter at 27°C.

Irritancy: The hair from the neck and dorsal skin of C57BL6 mice were shaved and was cleaned with surgical spirit. Then, the formulated 1% herbal lotion was applied and was observed for erythema and edema for a time period of 24 hours, 48 hours, and 72 hours.[17,18]

Globule size determination: The globule size of the formulation was carried out using a compound microscope and the average of diameters of 20 particles were determined.[17,18]

Viscosity: The viscosity of formulated lotion was measured by Brookfield Viscometer (Model-RVTP) by using spindle CP-18 over the range of speed setting at 5–10 rpm with 60 s between two successive speeds as equilibration with the shear rate ranging from 0.20 s–1.0 s to 1.0 s–1.0 s at 25°C ± 2°C.[17,18]

Stability studies by freeze thaw method: The formulated product was stored at chilled temperature of 4°C, and then it was maintained at room temperature until its temperature normalized to 28°C. Likewise, three cycles were performed and viscosity was measured by Brookfield Viscometer (Model-RVTP).[17,18]

Fourier transform infrared spectroscopy (FTIR): FTIR was executed using potassium bromide pellets, to evaluate the drug excipient interactions.[17,18]

Sterility test: The sterility testing was performed on nutrient agar where the sterile discs were loaded with 1% hair lotion and then incubated for 24 hours at 32°C.[17,18]

Spreadability: The formulation was sandwiched between the two glass slides of 20 cm × 5 cm dimension by placing a weight of 100 g uniformly on the slides. The excess lotion was scrapped off after removing the weight and the slides were fixed to a stand at a 45° angle allowing the upper slide to slip when a weight of 20 g was tied to the upper slide. The time taken for the upper slide to separate from the lower glass plate was noted as per International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines 10. The experimental procedure was done in triplicates and the spreadability was calculated using the formula: S = W × L/T, where, S = spreadability, L = length of the glass plate, W = weight tied to the upper plate, and T = time taken (s).[17,18]

Hair growth promoting activity: C57BL/6 male mice weighing 25–30 g were retained below room temperature and provided with food and water *ad libitum*. The hair on the dorsal part was removed using hair removing cream in an area of 3 cm^2. The cream was applied gently on the area and was observed for possible side effects. We could not see any undesirable effects like rashes, itchiness, or inflammation. The animals were divided into three groups with six animals in each group. Group I was applied with water as control. Group II was applied with 1% *R. officinalis* Albus hair lotion as a test. Group III was applied with 2% minoxidil as standard. The control, test, and standard were applied two times a day for 30 days in the depilated area. At the end of each week, one animal per group was sacrificed by means of cervical dislocation and the animals were observed for hair growth and change in protein content.[19-21]

The parameters observed were:

1. Qualitative observation of hair growth.
2. Effect of 1% herbal hair lotion on hair growth.
3. Determination of hair length.
4. Change in concentration of total protein content.
5. Determination of hair weight.
6. Microscopical studies:
    i. Histological studies (observation of induction of anagen phase in telogenic C57BL/6 mice).
    ii. Hair follicle count (quantitative observation of hair growth).

The number of hair follicles and length of hair were determined and compared with the control and standard.[22,23]

Statistical analysis

Dunnett multiple comparison test by one-way analysis of variance was performed by comparing with control using GraphPad InStat #3. Averages ± S.E.M. of the means were calculated and the values of $^{**}P < 0.01$ were considered significant.

Results

The percentage yield obtained for the methanolic extract of aerial parts of the plant was determined to be 20%w/w. Preliminary phytochemical investigation was performed to reconfirm the presence of primary and secondary metabolites in the extracts. However, our extract exhibited the presence of several primary and secondary metabolites like amino acids, proteins, fats and oils, flavonoids, volatile oil, steroids, glycosides, phenolic compounds, and vitamins.

Evaluation of 1% herbal hair lotion

The color of the lotion was found to be light green and the odor was pleasant. It had a smooth texture and was free from grittiness and foreign particles. The formulated 1% herbal lotion showed a pH of 6.82 and the animals did not show any kind of primary skin irritation, sensitivity, erythema, or edema after 72 hours of application. The average globule size of the lotion was found to be 72.8 µ. Viscosity value was found to be 10.8 cPs at 10 rpm. The freeze thaw method was performed for the stability determination of lotion by using spindle no. 18 and finally, after the performance of three cycles it was found that there was not much variation in the globule size by microscopic observation and hence it was confirmed that the lotion was stable. First cycle showed a viscosity of 10.8 cPs, second cycle showed 10.2 cPs, and the third cycle showed 10.6 cPs. FTIR established that the peaks of the formulated lotion with extract, lotion without extract, physical mixture, and pure drugs were observed [Table 1]. The major vibrations like CH_3, C = O, C-C are seen in the same frequency in hair lotion, extract, physical mixture, and lotion excluding extract which shows that not much interactions are present. Vibration of CH_3 was seen in the frequency 2918.40 cm^{-1} and 2956.97 cm^{-1} in the infrared (IR) spectra of the lotion extract, respectively, same was found in the frequency of 3377.47 cm^{-1} and 3356.25 cm^{-1} in the physical mixture and lotion without the extract which depicts drug excipient interactions are not present. C = O was found in the same frequency in all the three spectra except in that of extract. Similarly, C-C was found almost in the same frequency in all the three spectra except in the physical

Table 1: Fourier transform infrared spectroscopy

Types of samples	Frequency cm^{-1}	Types of vibrations
Hair lotion	2918.40	CH3
	2850.88	
	1743.71	C=O
	1635	C-C
Extract	2956.97	CH_3
	2920.32	
	1633	C-C
Physical mixture	3377.47	
	2965.04	CH_3
	2922.25	
	1743.71	C=O
Lotion without extract	3356.25	
	2956.97	CH_3
	2922.25	
	2852.61	
	1743.64	C=O
	1643.41	C-C
	1635.69	

mixture. The result concluded that there was no drug-excipient interaction [Figure 1]. The results of sterility testing depicted that the prepared 1% herbal hair lotion was free of microbial contamination. The spreadability value obtained was within the range and was determined to be 30.02 ± 0.012 g.cm/s. Hence, the 1% herbal hair lotion passed the spreadability test.

Evaluation of herbal hair lotion for hair growth

Qualitative observation of hair growth: It was observed that the number of days taken for hair growth initiation and completion by the animals treated with 1% herbal hair lotion incorporated with *R. officinalis* was better than the standard 2% minoxidil treated group of animals.

Effect of herbal hair lotion on C57BL/6 mice: On the 0th day, the depilated dorsal skin of C57BL/6 mice appeared in pink colour which indicated that it was in the telogenic phase. In the first week of treatment, the dorsal skin of C57BL/6 mice showed black pigmentation which indicated conversion of telogenic phase to anagen phase [Figure 2]. The black pigmentation was observed to be more in test and standard treated group of animals than the control. The second week of treatment showed increased pigmentation and hair growth in the test and standard group of animals. The third week of

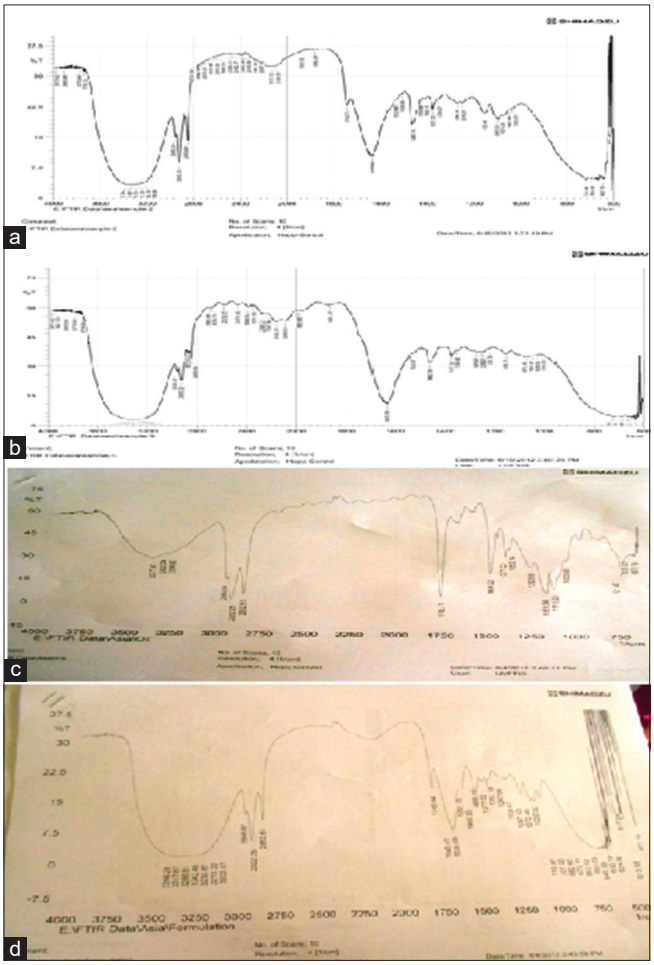

Figure 1: FTIR studies showing no drug excipient interactions. (a) Spectra of 1% herbal hair lotion incorporated with the methanolic extract of *R. officinalis*; (b) spectra of methanolic extract of *R. officinalis*; (c) spectra of physical mixture, and (d) spectra of excipients alone

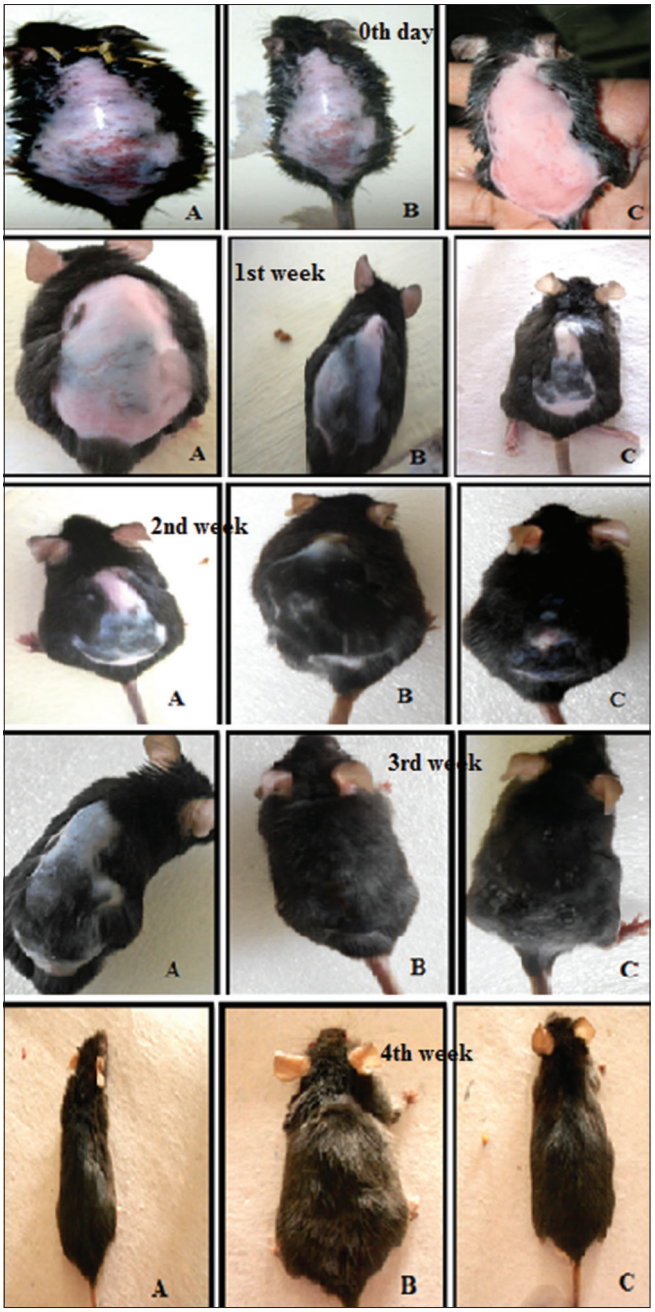

Figure 2: Animals on the 0th day, 1st week, 2nd week, 3rd week, and 4th week of treatment of C57BL/6 mice. (A) Shows animals belonging to Group I control (water) treated animals, (B) shows Group II is 1% herbal hair lotion incorporated with *R. officinalis* extract, and (C) shows group III is standard 2% minoxidil treated group of animals. On 0th day, the shaved dorsal skin of C57BL/6 mice appeared pink in color which indicated that it was in telogenic phase. By first week, mice showed black pigmentation which indicated conversion of telogenic phase to anagen phase. Second week of treatment showed increase in pigmentation and hair growth confined to the proximal parts of C57BL/6 male mice treated with the 1% herbal hair lotion. Fourth week of treatment showed complete growth of hair on the dorsal skin of C57BL/6 mice which showed the conversion of telogen phase to anagen phase

treatment showed that in the control-treated group the hair growth was not limited to the proximal parts of C57BL/6 mice whereas in the 1% hair lotion and the standard-treated group showed an increase in the hair growth which was limited to the proximal parts of C57BL/6 mice. The fourth week of treatment showed complete growth of hair on the dorsal skin of C57BL/6 mice which confirmed the conversion of telogen phase to anagen phase [Table 2, Figure 2].

Determination of hair length: The length of hairs of animals treated with 1% herbal hair lotion treated group was considerably longer than that of control. By the end of the fourth week, the test treated animals showed hair length of 76.3 ± 0.3457** cm, whereas 2% minoxidil treated animals showed a hair length of only 45.86 ± 0** cm which was almost similar to that of the control for which the hair length was observed to be 44.32 ± 0 cm [Table 3].

Determination of total protein content in blood: A remarkable increase in total protein content in blood from the 10th day to 30th day of treatment was seen in the group of animals treated with 1% herbal hair lotion, whereas the animals treated with control and standard showed a negligible increase in protein content [Table 4].

Determination of hair weight: The weight of newly grown hairs in test group treated animals were measured and compared with that of the control and standard group treated animals. The results obtained showed that the weight of hair was highest for mice with 1% herbal hair lotion. The hair weight was determined to be 52, 55, and 54 mg/cm^2 area of dorsal skin for the mice applied with the formulation, respectively, while it was found to be 46.741 mg/cm^2 area of dorsal skin for the control group. The standard-treated group of animals had shown a similar response as that of the control group of animals [Table 5].

Histological studies

Observations of induction of anagen phase in telogenic C57BL/6 male mice: In the case of the animals treated with 1% herbal hair lotion the longitudinal section and transverse section 0th day stained with hematoxylin and eosin showed less number of hair follicles which indicated that it was in telogenic phase. In the first week of treatment, the skin section was stained with hematoxylin and eosin which showed an increase in the number of hair follicles which indicated that the telogenic phase started converting to anagen phase. By second week, the histological studies revealed an increase in the number of hair follicles and hair shaft elongation in the epidermal layer had commenced. In the third week, the skin histology revealed an increase in the number of hair follicles and started hair shaft elongation extended to the subcutis layer. The histology sections taken on animals of fourth week exhibited an increase in the number of hair follicles and showed an increase in hair shaft elongation. The skin histology of the group of animals treated with control and standard drug showed induction of anagen phase but was slow when compared to the test treated group of animals [Figure 3].

Hair follicle count: The qualitative observation of hair growth revealed an escalation in the number and size of the hair follicle which was detected during anagen phase initiation in the animals treated with 1% herbal hair lotion. The upsurge in the size and number of hair follicles that lie deep inside the subcutis is related to the anagen phase in the subcutis as compared to that of the telogen phase where the hair follicles lie in dermis. The number of hair follicles of the relative area (0.09 mm^2) in *R. officinalis* Albus 1% hair lotion treated group was found to be more than the control and the standard group treated animals [Table 6].

Discussion

Hair growth promoters are one of the leading cosmetic preparations on demand that has led to a multibillion-dollar industry. In Japan, around 60% of the male population is distressed due to hair problems. Hair loss problems, though not life-threatening, can cause extreme emotional distress that makes afflicted patients vulnerable. Minoxidil is an established hair growth promoter, but can cause several adverse side effects like pruritus, dryness, scaling, local irritation, and dermatitis.[22] The mechanism by which minoxidil promotes hair growth in patients with androgenic alopecia is by inducing hair follicles where the transition from the early to late anagen phase occurs.[23] Although minoxidil was widely used for the treatment of hair fall, its use has been reduced due to disagreeable side effects and poor efficacy in improving the hair fall. C57BL/6 mice is a model often preferred to study hair growth since their truncal pigmentation is reliant on the follicular melanocytes which is usually produced only for the period of their anagen phase.[24,25] Conversion of telogen phase to anagen phase can be determined or identified by the conversion of pink coloured skin to black pigmentation.[26] C57BL6/N mice of 7-week-old which were in the stable telogen phase were selected for this study.

Table 2: Qualitative observation of hair growth of 1% herbal hair lotion

Treatment group	Dose	Number of days taken to initiate hair growth (avg. of group)	Number of days taken to complete hair growth (avg. of group)
Group I	Water	7	27
Group II	1%	3	15
Group III	2%	2	16

Group I is control (water) treated animals, group II is 1% herbal hair lotion incorporated with *R. officinalis* extract, and group III is standard 2% minoxidil treated group of animals.

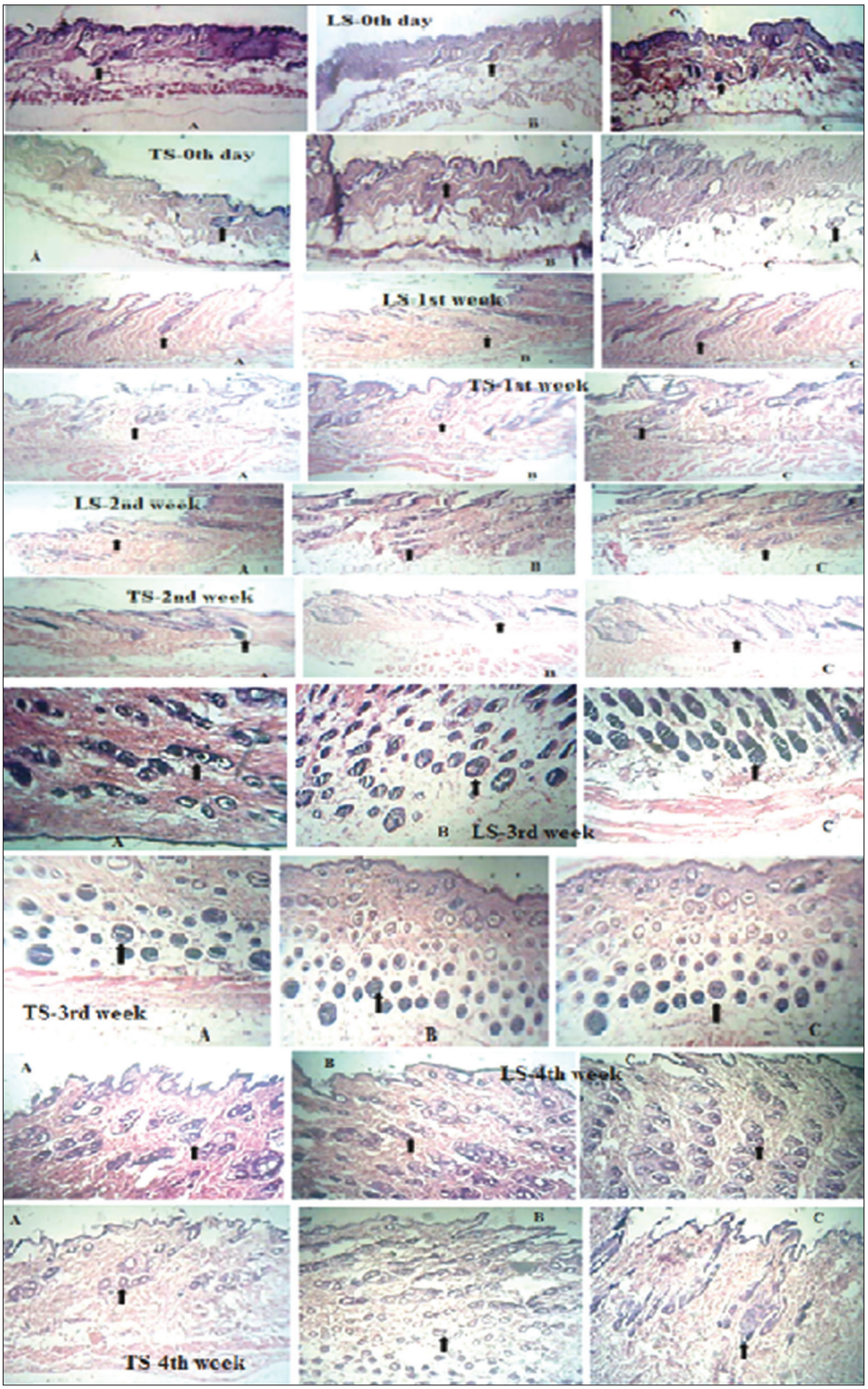

Figure 3: Longitudinal section (LS) and transverse section (TS) of the skin of C57BL/6 mice stained with hematoxylin and eosin stain on the 0th day, 1st week, 2nd week, 3rd week, and 4th week of treatment. (A) Shows skin section of animals belonging to Group I control (water) treated animals, (B) shows Group II is 1% herbal hair lotion incorporated with *R. officinalis* extract, and (C) shows group III is standard 2% minoxidil treated group of animals. ↑ Indicates the hair follicles and induction of anagen phase in telogenic C57BL/6 male mice treated by the 1% herbal hair lotion

The 1% herbal hair lotion incorporated with *R. officinalis* extract was formulated and evaluated. Organoleptic evaluations are performed to identify if the formulation is flawless or not. The 1% hair lotion possesses a pleasant colour and odour that can be easily acceptable. The organoleptic characters were found to be compatible and preparation had no gritty particles

Table 3: Determination of hair length

No. of weeks	Group 1 (cm)	Group II (cm)	Group III (cm)
2nd week	22.23±0	30.372±0.34**	24.45±0**
3rd week	30.65±0	57.70±1.033**	32.56±0**
4th week	44.32±0	76.3±0.3457**	45.86±0**

Group I is control (water) treated animals, group II is 1% herbal hair lotion incorporated with *R. officinalis* extract, and group III is standard 2% minoxidil treated group of animals. Experimental data were expressed as mean±SD, where n=10. The difference between experimental group was compared by one-way analysis of variance (ANOVA) followed by Dunnett multiple comparison test (control vs all) and the values are considered statistically significant when **P<0.01

Table 4: Determination of total protein content in blood for formulated animals

Treated groups	Concentration	Total protein content in blood at 660 nm (g/dl)		
		10th day	20th day	30th day
Group I	Water	0.96	1.21	1.74
Group II	1%	1.724	2.68	4
Group III	2%	0.98	1.21	1.73

Group I is control (water) treated animals, group II is 1% herbal hair lotion incorporated with *R. officinalis* extract, and group III is standard 2% minoxidil treated group of animals.

Table 5: Determination of hair weight

No. of weeks	Group I	Group II	Group III
1st week	41	46	42
2nd week	44	53	46
3rd week	47	55	49
4th week	50	57	52

Group I is control (water) treated animals, group II is 1% herbal hair lotion incorporated with *R. officinalis* extract, and group III is standard 2% minoxidil treated group of animals.

Table 6: Quantitative observation of hair follicle count

No. of weeks	Group I	Group II	Group III
1st week	1.33±0.5774	3±1.000ns	3.666±0.5774ns
2nd week	3.666±0.5774	10±1.000**	11.666±1.528**
3rd week	5±1.000	11±1.000**	13.666±1.528**
4th week	10±1.000	16±1.000**	18.333±1.528**

Group I is control (water) treated animals, group II is 1% herbal hair lotion incorporated with *R. officinalis* extract, and group III is standard 2% minoxidil treated group of animals. Experimental data were expressed as mean±SD, where n=10. The difference between experimental group was compared by one-way analysis of variance (ANOVA) followed by Dunnett multiple comparison test (control vs all) and the values are considered statistically significant when **P<0.01.

and had a smooth texture. The pH of hair products can affect the hair condition and cause damage by breakage. The pH of 1% hair lotion plays a key role in augmenting the quality of hair, reducing irritation, and stabilizing the ecological balance of the scalp. Mild acidic pH prevents inflammation and endorses tightening of the scales, thereby bringing shine.[7,18] The result of the 1% hair lotion had a pH of 6.82 which is near to the skin pH. It was observed that the hair of the animals treated with the 1% herbal hair lotion showed a shiny and healthy texture when compared to the hair of the control and standard treated animals. Skin irritancy was performed on C57BL/6 male mice by patch test. The results exhibited that the hair lotion was completely free of any primary skin irritation, sensation, erythema, or edema even after 72 hours of use. Increase in the globule size of a prepared emulsion is an indication of its physical instability.[15,27] The 1% hair lotion formulation showed a stable globule size in the first and third week of the study which indicates that the lotion was stable. Viscosity is a measure of a fluid's internal friction when one layer of fluid is forced to move in over another layer. Viscosity of a product is directly linked to patient compliance as it affects the cleansing efficacy and consumer's opinion.[18,27,28] The viscosity of 1% hair lotion was checked and it was observed that it was sticky in nature, so it sticks to the hair and shows the activity. Viscosity was found to be in the normal range hence it does not cause any irritation to hair. FTIR study showed that the CH3 and C-C peaks were found in the similar wavelength for the 1% herbal hair lotion, crude extract, physical mixture, and excipients alone which conclude that there was no drug-excipient interaction. The results obtained for spreadability indicate that the lotion quickly spreads by modicum of shear. The formulation displayed a potent hair growth promoting activity than the standard 2% minoxidil hair lotion. The animals treated with the 1% herbal hair lotion presented a faster onset of action and completion of hair growth. The quality of the hair, its appearance, length, and weight also were far better than the animals treated with the control and standard drugs. It was observed that an increase in total protein content was seen in the animals treated with the test lotion. Hence, it formed the basis for a healthy, long shiny hair with good texture. From the phytochemical screening it was found that the extract consists of volatile oils, flavonoids, alkaloids, saponins, and phenolic compounds. So, this potent hair promoting activity may be credited to the presence of the above-mentioned chemical constituents.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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