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Original Article

SOLID LIPID NANOPARTICLES OF FENUGREEK SEED EXTRACT IN A DERMATOLOGICAL BASE FOR ALOPECIA: AN *IN VIVO* STUDY

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ABSTRACT

Objective: The objective of the study was to investigate the potential of Solid Lipid Nanoparticles (SLN) of Fenugreek Seed Extract (FSE) in a dermatological base for its hair growth activity in alopecia

Methods: The optimized SLN formulation of FSE was loaded into neutralized Carbopol 934 gel, and its hair growth efficacy was studied in Wistar rats in terms of hair density and length. Alopecia was induced in the rats by administering cyclophosphamide at a dose of 40 mg/kg for three days. The formulations were applied to the skin for twenty-one days following the induction of the disease. The hair growth in FSE-SLN gel-treated groups were compared with disease control and other treatment groups using qualitative and quantitative assessments.

Results: FSE-SLN gel reduced the hair growth completion time comparable to that of the standard ($p<0.01$). The increase in hair length was significantly ($p<0.01$) greater in FSE SLN groups compared to groups treated with conventional gels, oils, and marketed formulations, demonstrating the superior hair growth efficacy of the developed FSE SLN.

Conclusion: SLNs can enhance the penetration of extract into the skin (stratum corneum) compared to oil and gels, thereby increasing treatment efficiency, targeting the epidermis, and reducing systemic absorption and related side effects. Consequently, the developed nanoformulation can be a substitute for *in vivo* hair growth activity.

Keywords: Fenugreek seed, Solid lipid nanoparticles, Alopecia, Cyclophosphamide, Wistar rats

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INTRODUCTION

Alopecia is considered a chronic dermatological disorder that affects hair follicles and is mainly characterized by loss of hair on the head as well as body regions, including eyebrows and beard [1]. The exact cause of alopecia is not fully understood, although it is considered an autoimmune disorder triggered by various genetic and environmental factors [2]. Even though the condition is not life-threatening, the mere prospect of being bald leads to emotional distress, anxiety, and depression in the individual [3].

The current treatment regimen includes various prescription drugs such as topical corticosteroids, minoxidil, and finasteride in topical formulations such as creams, lotions and foams. However, all these drugs and formulations have to be taken several times daily and regular use of these drugs leads to various localized and systemic side effects such as irritation to the scalp, tachycardia, and hypertrichosis [4]. Considering the disadvantages associated with these synthetic drugs, suitable alternatives have to be explored.

Traditional treatments have focused on extracting different species of herbs to obtain various phytochemicals with anti-alopecia properties [5]. *Trigonella-foenum-graecum*, commonly known as fenugreek, can potentially treat hair loss. It is an aromatic herb that belongs to the Leguminosae family. The primary components are flavonoids, quercetin, saponins, tigogenin, trigonelline, proteins, lipids, carbohydrates, and galactomannan [6]. Fenugreek seeds are well known to stimulate hair growth by interfering with the synthesis of dihydrotestosterone, which is bound to hair follicles and is categorized as one of the primary reasons for hair loss [7]. Various fenugreek formulations, such as hair tonics, pastes, and topical formulations, have been used to stimulate hair growth, albeit with little success [8-10]. These conventional formulations have various limitations, such as poor dermal retention and follicular penetration, which require frequent application. An alternative approach to overcome these limitations is using lipid nanocarriers such as solid lipid nanocarriers that can achieve deeper follicular targeting and improve uptake of the active constituents owing to their lipophilicity [11, 12].

In our previous work, we prepared solid lipid nanoparticles containing fenugreek seed extract and incorporated them into a carbopol gel base. The current study was an attempt to validate the therapeutic activity of the developed Solid Lipid Nanoparticles (SLN) *in vivo* by testing the efficacy of the formulation in a cyclophosphamide-induced alopecia rat model. The efficacy of the SLN gel containing fenugreek seed extract was compared with that of fenugreek seed extract alone, minoxidil topical solution (Standard), conventional gel formulation containing Fenugreek Seed Extract (FSE), marketed fenugreek oil, marketed fenugreek shampoo and control groups.

MATERIALS AND METHODS

Materials

Trigonella foenum-graecum (Fenugreek) seeds were purchased from a local market. Dr. Krishna Kumar G, Professor Department of Applied Botany, Mangalore University, Karnataka, identified and validated the fenugreek seeds. Pulverised seeds were sieved through 40 mesh to obtain coarse powder. Cyclophosphamide (CYP) was purchased from Himedia Laboratories (Mumbai, India), Rogaine (Minoxidil topical solution 2% w/v), was purchased from Johnson and Johnson (Mumbai), Khadi herbal fenugreek hair oil purchased from Khadi India, Bengaluru and Meera Fenugreek and Onion Shampoo, purchased from CavinKare Pvt Ltd, Chennai

Extraction of fenugreek seed and preparation of SLN gel containing FSE

Fenugreek seeds were subjected to Soxhlet extraction using ethanol as the solvent, and extraction was carried out according to the procedure outlined in our previously published article. The extract was then incorporated into an SLN and prepared using a combination of emulsification and hot homogenization, as per the method described in our previous work. The prepared FSE-SLN was

also evaluated for various *in vitro* parameters including the particle size, zeta potential, and entrapment efficiency [13]. The SLN was then introduced into a carbopol 934 gel base to obtain an aesthetically pleasing and clear topical formulation that can be readily applied and easily removed from the skin with water. Further *in vivo* investigation was carried out as follows.

***In vivo* studies**

Test samples

Cyclophosphamide (CYP) solution (125 mg/kg body weight) was prepared in distilled water.

Animals

Twenty-four male Swiss albino rats (3-4 mo of age, 120-140 g) were used in the experiments. Animals were housed in standard cages with free access to food and water. The Institutional Ethical Committee (IEC) of Nitte University (Reg. No. NGSMIPS/IAEC/JUNE-2020/212) approved the protocol for all animal experiments. The guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) were strictly followed.

Grouping of animals

The animals were randomly divided into 8 groups of 6 male Swiss albino rats and were treated as follows:

Group I: Normal control.

Group II: Alopecia-induced but not treated or disease control.

Group III: Standard [Rogaine (Minoxidil topical solution 2% w/v)

Group IV: Aqueous solution of extract

Group V: Fenugreek gel* (2.5% w/w)

Group VI: Fenugreek SLN Gel

Group VII: Marketed fenugreek hair oil-(2.5% w/v)

Group VIII: Marketed fenugreek shampoo (2% w/v, CavinKare Pvt Ltd, Chennai)

Induction of alopecia

The cyclophosphamide-induced alopecia model in rats was used to mimic alopecia in humans [14, 15]. The back of the skins of the rats of all groups was depilated for anagen induction, which occurred on the 9th-day post-depilation. Once the anagen phase was induced, cyclophosphamide injection of 120 mg/kg was administered in divided doses of 40 mg/kg for 3 days, and animals were observed for 45 d [16, 17]. Subsequently, one animal from each group was euthanized and further examined for histopathology. A dose of 120 mg/kg (40 mg for 3 d) induces epidermal hyperplasia, destroying hair follicles and reducing hair follicles [18].

Qualitative hair growth

Hair growth completion time and hair quality

Qualitative hair growth in Wistar rats was assessed visually based on two parameters: initiation time (minimum time to commence hair growth on denuded skin) and completion time (minimum time necessary for complete coverage of denuded skin with new hair growth) [19]. The denuded area of the skin was keenly observed, tiny hair growth was considered as the initiation time and total growth was observed as the completion time.

Quantitative hair growth assessment

Quantitative assessments were performed based on the length of hair growth and histopathological findings.

Hair length

To assess the length of the grown hair, the hair was plucked from the shaved dorsal area of the mice using sterile forceps after the 10th, 20th, and 30th d of treatment. For the estimation, an average length of 'n' hairs (n=25) was assessed and recorded [20, 21].

Hair density

Hair density was determined after total hair growth. Hairs from previously marked sites on the dorsal area of shaved skin were collected and counted manually. It was reported as total hairs for sq/cm area [22].

Histopathological Investigation for hair follicle count

After 30 d, one rat from each group was sacrificed. Skin biopsies were obtained and stored in 10% formalin. Tissue sections were taken and implanted in paraffin wax with a thickness of 10 µm. The hair follicles from the tissues were then stained with hematoxylin and eosin and examined using an eyepiece micrometer. The number of follicles in a 1 square mm area was determined [23, 24].

Statistical analysis

GraphPad Prism version 6.01 software (GraphPad Software, San Diego, CA, trial version) was used for analysis. Analysis of variance (ANOVA) was used to establish the degree of statistical significance, followed by Tukey's test for multiple comparisons, with $p<0.05$ regarded as statistically significant.

RESULTS

Evaluation of the prepared FSE SLN gel

Our previous published work has mentioned the results obtained from the assessment of the developed SLN [13]. In brief, the particle size of the SLN was found to be 223.36±2.14 nm with a narrow Polydispersity Index (PDI) of 0.313, indicating the developed formulation was homogenous. The Zeta potential of the formulation was found to be-16.34±1.15 mV and the entrapment efficiency was 74.56±1.22%. *In vitro* release studies revealed that FSE was released from the SLN in a sustained manner and achieved more than 70% skin deposition in the *ex vivo* studies.

***In vivo* studies**

Hair growth completion time and hair quality

The commencement of growth in the denuded region was noticed in the control group animals at 12.2 d. In the standard group, hair growth initiation was observed within 6 d. The FSE SLN gel formulation exhibited hair growth initiation on 7.5th d, whereas the gel formulation and extract solution showed hair growth on the 10th and 10.3th d, respectively. The normal control group achieved total hair growth after 23.7 d. The average time for complete hair growth in the standard group was observed to be 19 d, while the extract solution and gel groups required 22.3 and 22.1 d, respectively. Marketed fenugreek hair oil and shampoo showed complete hair growth on 21.5 and 21.7 d, respectively. In the FSE SLN gel group, the entire denuded area showed complete hair growth on the 20th day. Based on this observation, it could be inferred that the SLN gel caused s a faster hair growth completion time than all other groups except the standard. Initiation and completion times were compared between groups in fig. 1.

Quantitative hair growth assessment

Hair length

The animals treated with the SLN gel containing fenugreek seed extract had hair length of 3.2 mm at the end of 30th day when compared to 1.4 mm in the Fenugreek extract solution group, 1.58 mm in the gel group, 1.12 mm in the control group, 1.6 and 1.7 mm in marketed fenugreek hair oil and shampoo respectively. In addition, the hair length determined for the SLN gel group was similar to that of the standard group. This finding indicates that FSE-loaded SLN gel containing fenugreek had significantly better *in vivo* hair growth activity ($p<0.01$) than conventional gels, oils, and marketed formulations. A comparison of the hair length in different treatment groups is displayed in fig. 2.

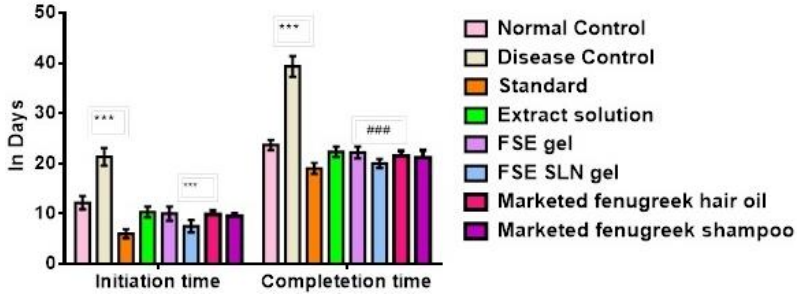

Fig. 1: Hair growth initiation and completion of rats at different times after beginning the treatment of standard, extract solution, FSE gel, FSE SLN gel, Marketed fenugreek hair oil and marketed fenugreek shampoo. Results were showed as mean standard deviation (SD). (* P<0.001, ### P<0.001), compared with normal control (n=6)**

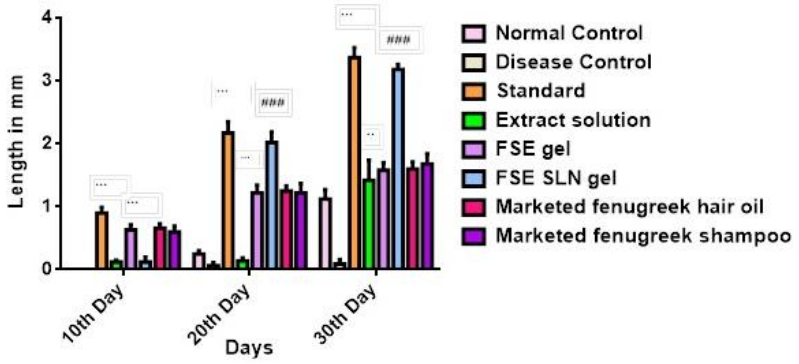

Fig. 2: Hair length of rats at different times after beginning the treatment of standard extract solution, FSE gel, FSE SLN gel, marketed fenugreek hair oil and marketed fenugreek shampoo. Results were showed as mean standard deviation (SD). (* P<0.001, ### P<0.001, **P<0.01), compared with normal control (n=6)**

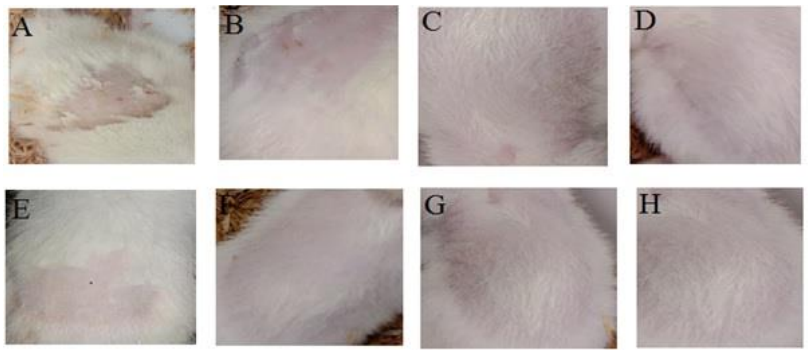

Fig. 3: Effects of topical formulation on hair density in albino rats. A-Normal control, B-Untreated, C-Standard (2 % w/v Minoxidil), D-Fenugreek seed extract solution, E-Fenugreek gel, F-FSE-SLN Gel, G-Marketed fenugreek hair oil (2.5% w/v) and H-Marketed fenugreek shampoo (2% w/v). Photographs were taken on 10th day after applying treatments on dorsal skin

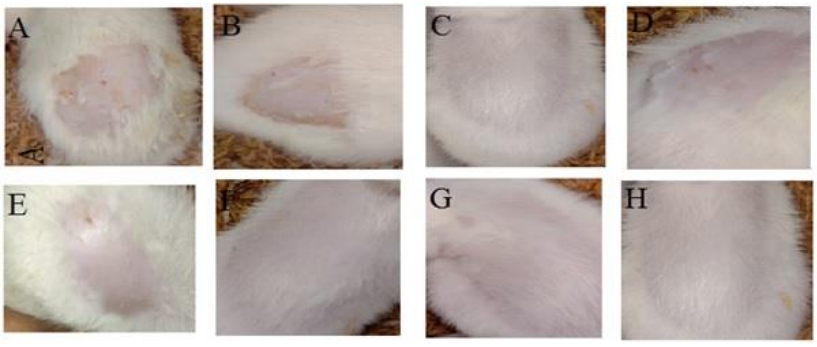

Fig. 4: Effects of topical formulation on hair density in albino rats. The back skin were topically applied with A-Normal control, B-Untreated, C-Standard (2 % w/v Minoxidil), D-Fenugreek seed extract solution, E-Fenugreek gel, F-FSE-SLN Gel, G-Marketed fenugreek hair oil (2.5% w/v) and H-Marketed fenugreek shampoo (2% w/v). Photographs were taken on 20th day after applying treatments on dorsal skin.

Hair density

The Standard minoxidil treatment and FSE-SLN gel groups had hair densities of 2588 per cm^2 and 2383 per cm^2, respectively, which were significantly higher than those of other treatment groups, demonstrating their superior hair growth activity. Fig. 3, 4, and 5 show images of hair density on 10th, 20th and 30th d of the treatment. The graphical representation of hair density (1 cm^2) of all groups is shown in fig. 6.

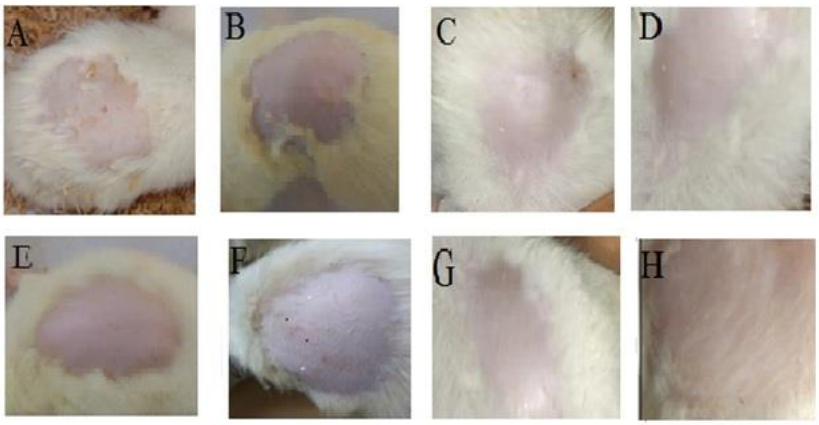

Fig. 5: Effects of topical formulation on hair density in albino rats. The back skin were topically applied with A-Normal control, B-Untreated, C-Standard (2 % w/v Minoxidil), D-Fenugreek seed extract solution, E-Fenugreek gel, F-FSE-SLN Gel, G-Marketed fenugreek hair oil (2.5% w/v) and H-Marketed fenugreek shampoo (2% w/v). Photographs were taken on 30th day after applying treatments on dorsal skin

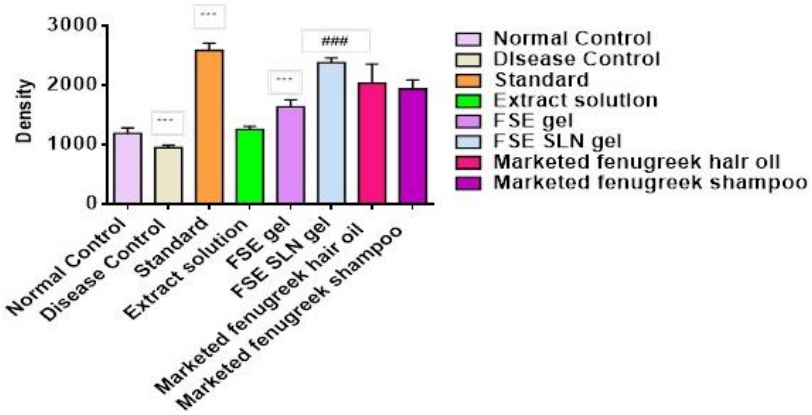

Fig. 6: Comparison of hair density of rats after beginning the treatment of standard, extract solution, FSE gel, FSE SLN gel, marketed fenugreek hair oil and marketed fenugreek shampoo. Results were shown as mean standard deviation (SD). (* P<0.001, ### P<0.001), compared with normal control (n=6)**

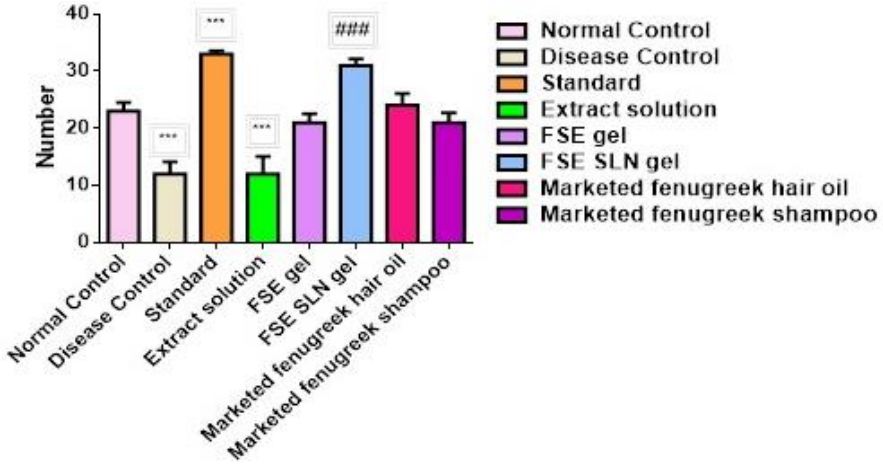

Fig. 7: Hair follicle count of rats after beginning the treatment of standard extract solution, FSE gel, FSE SLN gel, marketed fenugreek hair oil and marketed fenugreek shampoo. Results were showed as mean standard deviation (SD). (* P<0.001, ### P<0.001), compared with normal control (n=6)**

Histopathological investigations were performed to evaluate the efficacy of the developed formulation further. The primary focus of the histopathological studies was to examine the therapeutic activity of the formulation based on hair follicle count. In the untreated group, a dose of 120 mg/kg (40 mg for 3d) induced epidermal hyperplasia, destroying hair follicles and reducing hair follicles (fig. 7 and 8). The normal control group showed normal tissue architecture and was characterized by a regular thin stratified squamous keratinized epithelium consisting of two to four cell layers and dermis; there were no inflammatory cells in the normal control group. Hair follicles were observed in all the dermal layers. The normal control group had 27 hair follicles in the deep subcutis region. The groups treated with the extract solution and fenugreek gel formulations had 12 and 21 hair follicles in the deep cutis, respectively, higher than in the untreated group. Both the standard minoxidil group SLN gel had a significant increase in the size and number of hair follicles, with the standard minoxidil group having 33 hair follicles compared to 31 hair follicles in the SLN group indicating that hair growth activity of the developed FSE SLN gel was in line with the standard treatment.

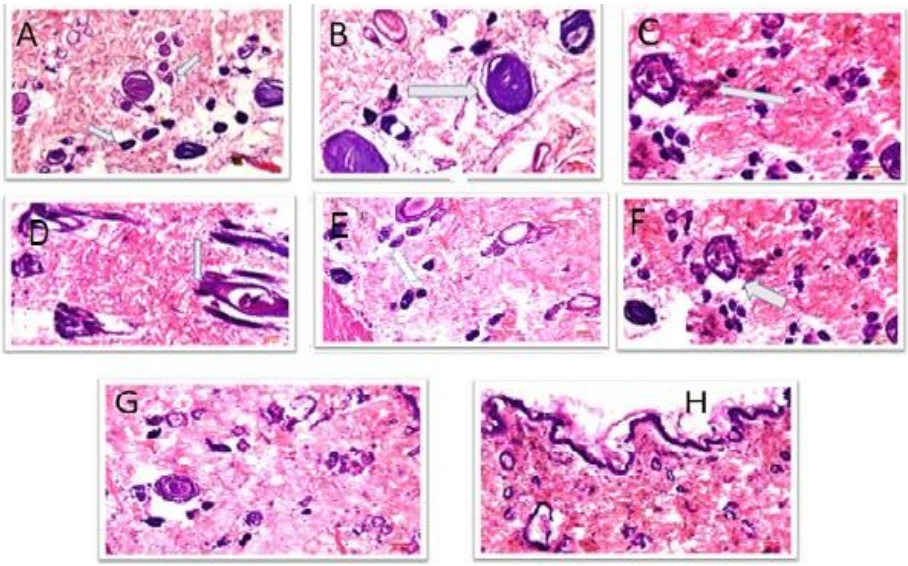

Fig. 8: Effects of topical formulation on hair follicle count in albino rats. A-Normal control, B-Untreated, C-Standard (2 % w/v Minoxidil), D-Fenugreek seed extract solution, E-Fenugreek gel, F-FSE-SLN Gel, G-Marketed fenugreek hair oil (2.5% w/v) and H-Marketed fenugreek shampoo (2% w/v). Haematoxylin and Eosin-stained slides depicting different treatment groups in 100x magnification (area 4 microns)

DISCUSSION

In our earlier study, we attempted to make SLN-containing fenugreek seed extract using the hot homogenization method. The nano range of the developed SLN was found to be ideal for topical delivery with the formulation achieving more than 70% Skin deposition. The high zeta potential values indicate a minimal chance of particle aggregation and ensure formulation stability. Moreover, the high entrapment efficiency of the formulation resulted in minimal loss of the extract and ensured sustained delivery as evident in the results obtained in the previous study [13].

In the current investigation, the hair growth activity of the prepared FSE SLN gel was evaluated in wistar rats using a cyclophosphamide-induced alopecia rat model. Results from qualitative hair growth indicated that when compared to the control, hair growth in the shaved area was promoted by the group treated with FSE SLN gel, which exhibited a notable depletion in time for complete hair growth, which was nearly the same as that in the minoxidil-treated group. It was also observed that the SLN gel formulation significantly shortened the time needed to initiate and complete hair growth compared to the other treatment groups. The time taken for complete hair growth was similar to that of the group treated with the standard. These findings were in line with several other studies suggesting that formulations containing nanoparticles could penetrate deeper into the hair follicles and increase their growth potential [26, 27].

Further, quantitative hair growth studies (histopathological investigations) reaffirmed the findings of qualitative studies. Compared to the untreated group, all treatment groups containing fenugreek (group 4,5,7 and 8) showed notable improvement in hair growth and follicle count at the end of the 30th day. This can be attributed to FSE, which promotes hair development by strengthening the capillary walls of blood vessels that direct blood to follicles and enhancing the blood flow to nourish hair follicles, increasing hair growth through dilating blood vessels in the scalp. It also provokes animal epidermal cells to start their growth again, especially hair cells [28].

However, compared to conventional gels, oils, and marketed formulations, FSE-loaded SLN was found to have significantly better *in vivo* hair growth activity ($p<0.01$). This can be because a high concentration of FSE-SLN could also have leached through the gel matrix, favoring an optimum contact time between the scalp and extract-loaded SLN. This phenomenon may facilitate the completion of hair growth.

The quantitative studies for determining hair length showed that topical FSE SLN gel therapy dramatically lengthened hair compared to the control and other treatment groups. In addition, an increased rate of cell proliferation was observed in the FSE SLN gel group, transforming short and barely noticeable hairs into long terminal hairs and increasing the follicle size. This may be due to vasodilation of blood vessels in the scalp and epithelial cell proliferation alongside the hair follicle base, which has been reported for herbal formulations [29]. These findings indicate the significant hair growth potential of these formulations. Sustained release of FSE and higher skin penetration can be held accountable for the enhanced therapeutic efficacy of FSE-SLN gel compared to conventional marketed formulations. Moreover, SLN gels have better occlusion of the nanoparticles compared to traditional creams and gels [30, 31].

According to the findings of the animal experiments, in the disease control group, H and E staining revealed enlarged layers of cells consisting of two to eight layers (epidermal hyperplasia) and a decrease in hair follicles compared to the normal control group. There was more keratin in the stratum corneum in the disease group than in the control group. In the extract-treated group, no such increase in hair follicle number was observed. There was an increase

in the hair follicle number in the FSE gel group, whereas SLN gel treatment had an increase in hair follicles but no epidermal hyperplasia. Moreover, no signs of inflammatory infiltrates were observed, and there were two to three layers of thin stratified keratinized squamous epithelium. This was due to better penetration and regulated release of the extract from the SLN. The minoxidil-treated groups showed similar results to the SLN-treated groups, but some areas had slightly more keratin in the stratum corneum than the normal control.

These observations indicated that most hair follicles in the control vehicle-treated groups were in the telogen phase (resting phase), and no hair follicles were observed in the anagen phase. However, the majority of hair follicles in the group treated with FSE SLN gel were in the anagen phase, indicating that the FSE SLN gel-treated group's hair follicles underwent telogen to anagen transition more frequently than those in other groups [32, 33]. Moreover, the group treated with FSE SLN gel formulation had soft and silky hairs, which was similar to the minoxidil-treated group. Therefore, except for the SLN gel group and minoxidil-treated groups, all the other groups showed sparse hair growth.

The above findings were reiterated in hair density studies, which suggest that incorporating the FSE in SLN has immense benefits, such as improving the permeation efficiency of the FSE to reach deeper targets and sustaining the release of trigonelline from the FSE-SLN gel. Thus, the developed SLN was as effective as the standard minoxidil formulation for all aspects of hair growth. Furthermore, minoxidil is discouraged due to its severe side effects, making the developed FSE SLN gel a suitable alternative for hair growth promotion.

CONCLUSION

The current study was an attempt to show that fenugreek seed extract nanoparticles may be used to promote hair growth and development. Investigations carried out in this aspect showed that topical application of a gel containing SLN of FSE has the potential to treat hair loss, as evidenced by results obtained from qualitative measurements, such as hair growth, length, and density, as well as histological analysis of hair follicles in Wistar rats. The hair growth-promoting ability of the FSE extracts was significantly enhanced by using the FSE SLN gel, which was as effective as minoxidil (2%) for promoting hair growth. The authors concluded that the developed SLN gel is efficacious, safe, and cost-efficient for the promotion of hair growth and tackling hair fall.

ACKNOWLEDGEMENT

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FUNDING

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ETHICAL APPROVAL

The study was conducted following the CPSCEA Guidelines. The research study obtained ethical approval (No NGSMIPS/IAEC/JUNE-2020/212, Approval Date: 25 June 2020) from the Research Ethics Committee, Nitte University, Mangalore.

AUTHORS CONTRIBUTIONS

AP carried out the research and data collection. MK supervised the study and approved the manuscript for submission. SSM organized, analysed the data, and reviewed the manuscript. DJ critically evaluated the data. All authors have critically examined and approved the final draft, and they are accountable for the content of the manuscript and similarity index.

CONFLICT OF INTERESTS

Declared none

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