Chapter-8

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Summary

Introduction to alopecia
Alopecia is a common hair loss problem affecting 8-10% population worldwide. Although scalp hair seems to serve the seemingly inconsequential purpose of ornamentation and sexual virility, it plays an important role in the psychology of humans. While hair disorders are not life threatening they are a form of major psychological distress for the affected individual. With increased affordable healthcare, better nutrition and sanitation, the average lifespan of man has increased considerably; this increasing longevity coupled with the ancient preoccupation with hair, the desire to extend youthfulness is inevitably fuelled. This ever-increasing fascination with hair-care is reflected in the incessant growth of the hair-care market, already a multi-billion dollar enterprise world-wide. The bizarre expansion of hair products industry has been ascribed to the flourishing markets, premature aging crisis, and the infomercial appreciation of organic benefits.

Possible causes of alopecia
Factors causing alopecia have been elucidated as hormonal imbalance, genetic predisposition, chemotherapy, stress, diabetes, vitiligo, trauma, autoimmune diseases, rheumatoid arthritis, major weight gain or loss, abnormal kidney and liver function, lupus erythematosus, heat and chemical damage, childbirth, and fungal infection.

Hair cycle
The hair follicle grows in a life-long complex, continuous and cyclic process with three phases viz, anagen; the growth period, catagen; the transitory period and telogen; the resting or quiescence period.
Although the exact mechanism that initiates follicular formation and regulates the cycle is yet to be understood, it is known that transition from one phase to another is based on the signaling and expression of various transcription factors, cytokines, neurotransmitters, growth factors, hormones and enzymes.

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Hair cycle in mice

The hair growth cycle in mice is precisely time synchronized with the physiological growth cycle, especially the first two cycles post birth. The hair cycle starts with catagen at two weeks post birth continuing with telogen in the third week and growing with anagen from the fourth week till the sixth week. The second hair cycle post morphogenesis starting with catagen in the sixth week post birth, progressing to telogen in the seventh week and later anagen in the twelfth week. Also, these changes are evidenced by changes in skin color from pink in telogen to grey in catagen and black in anagen.

Hence, from four to six weeks after birth and twelfth to fourteen weeks subsequently the hair cycle is in a stage of anagen or active growth whereas from three to four weeks and seven to twelve week, it is in a state of rest or telogen.

Since follicular melanogenesis and HF cycling are synchronized with each other in C57BL/6 mice, it is evident that anagen development is associated with distinctive changes in skin pigmentation. Also, the time synchronized HF cycling induces profound alterations in the histomorphology and thickness of the skin, which are most evident proofs of the various phases of the hair cycle.

Catagenic changes in HF histomorphology are initially observed on day 17 after depilation in the neck region, and are recognizable visually by a change in skin color from black to grey-pink. This catagenic wave reaches the tail region 2 days later on day 19-20.

Mechanism of progression of alopecia

Hair growth is a result of reproduction and division of matrix keratinocytes forming the hair shaft and inner root sheath. It is these specialized mesenchymal cells that are the key signalling centre in hair follicles. The removal of the dermal papilla and the lower dermal sheath has led to loss of hair growth. Nutrition is as necessary for the papilla as the overlying matrix cells and is provided via a capillary loop located within the dermal papilla of terminal hair follicles, it has been observed that dermal papillae of vellus hair follicle typically do not contain capillaries.

Alopecia is a progressive ailment subsequent to decreased density of terminal hair and a consequential rise in vellus hairs. Primarily, the anagen phase becomes progressively shorter over the course of several hair growth cycles indicating that the number of actively growing
scalp hairs is decreasing. This is followed by the next transformation which is the progressive miniaturization of the follicles leading to the gradual conversion of thick, long terminal hairs into shorter vellus hairs or vellus like follicles. The general effect is to shrink perceptible scalp density. However, the affected follicles show no other abnormalities and maintain their potential for cyclic growth until they reach a very advanced stage of baldness.

Current status of indigenous formulations for treatment of alopecia
The current volatile growth in the use of indigenous hair care products is partly due to the fact that modern medicine has run out of promising leads and blockbuster drugs are running off patent. Drug development in modern medicine is time consuming extremely expensive with relatively low periods of patents thereby restricting innovative companies to a limited period to enjoy the fruits of their labour. Promising leads which have incurred billions of dollars worth research may turn out to be non-marketable or have serious side effects so as to be pulled out of shelves, not to mention the danger of lawsuits that might follow for compensation.

Indigenous medicine come into the picture at this juncture as centuries old tried and tested formulations that need to be developed further into clinically safe and acceptable compounds. The major spade work has been already done for the companies and all that remains is preclinical and clinical trials with standardized and validated formulations. It is not only the industry that stands to gain from this but also the patients and holistic practitioners too. The patient gets a variety of treatment methodologies to choose from and is not restricted by the lack of information of indigenous therapies. It is a win-win situation for all with scope for industry, patient, doctors etc.

However, it is also extremely important to develop standard operating protocols, standardized and validated extraction techniques, stringent laws to regulate safety and efficacy and prevent adulteration of these therapies. Man can harness the tremendous powers of nature by using his intelligence and creativity. The greatest need of the hour, however, is to create safe bioactive products whose therapeutic and cosmetic claims are founded on a good scientific basis and which can be standardized as well as validated like its synthetic pure molecular cousin.
Problem statement and proposed research approach

In humans, the main function of the hair shaft is its role as an important facet of appearance. Across cultures for centuries, the decoration and styling of scalp hair has been a means of social communication and display of social identity or status. Hair is so important in our society that hair loss, as well as the overgrowth of terminal hair on the body or face, has deleterious effects on self-esteem. The clinical relevance of hair and hair growth impairment goes far beyond the diagnosis and treatment of hair disorders as evidenced by the billion dollar hair care industry.

The aim of this research was to provide a safe, effective and easily available form of hair growth promoter sans the adverse side effects that come packaged with modern medicine. It is the purpose of this research to bring objectivity to traditional systems of medicine by way of moving in parallel lines with modern science and technology.

Comparison of conventional treatments with novel indigenous formulation (NIF)

The progress of science through the last 50 years has yielded two drugs, topical Minoxidil and oral Finasteride as approved treatments for alopecia. Other drugs under research for treatment of alopecia include squaric acid dibutyl ester, diphenylcycloprenone, sulfasalazine, anthralin, dutasteride, spironolactone, bexarotene, capsacin, hydroxychloroquine, alefacept, roxithomycin, oral cyclosporine, oral, injectable and topical corticosteroids. The current research had cumulated into the development of two validated novel indigenous formulations, NIF1 and NIF2, which have provided relief for alopecia with added advantage of transcending barriers of age, gender, and disease. Also they are relatively safer than conventional therapies and provide permanent relief [Table 45]. It is evident from the [Table 45], that finasteride and Minoxidil are effective only in androgenetic alopecia. These medicines cannot be used by all populations and have serious side effects listed in [Table 45]. On the other hand, the newly developed NIF’s are effective in all types of alopecia and is suitable for all population types. On application of NIF1, leech extract, which is a known permeation enhancer increases the absorption and penetration of NIF’s into the dermis. The lipophilic nature of NIF’s further facilitates the passage through the Lipid-Protein-Lipid layer of skin. Normal regulatory T cell epitopes present in leech extract provide
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protection to the dermal papilla and act on the local immune system by suppressing dysfunctional T cells from attacking the dermal papilla and follicular keratinocytes. NIF stimulates the neuronal meshwork surrounding the dermal papilla fibroblasts, follicular keratinocytes, and melanocytes. Leech extract has neurite stimulating effect on the neural pathways responsible for growth and development of hair leading to enhanced rate of growth. When NIF reaches the capillary network, it dilates the vasculature thereby increasing vascular irrigation to the dermal papilla and other parts of hair root, thus nurturing the hair root.

Table 45. Comparative mechanistic effect of conventional therapies with NIF.

<table>
<thead>
<tr>
<th>Mechanism of action</th>
<th>Indications</th>
<th>Contraindications</th>
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<tr>
<td>Vasodilatation, smooth muscle relaxation, inhibition of opening of sarcolemmal $K_{\text{ATP}}$ channels.</td>
<td>Male/female pattern baldness, androgenetic alopecia</td>
<td>People with cardiovascular disease, cardiac arrhythmias, hypertension, pregnant &amp; nursing women, hypotension,</td>
</tr>
<tr>
<td>5-alpha reductase inhibition</td>
<td>Androgenetic alopecia</td>
<td>People with cardiovascular disease, cardiac arrhythmias, hypotension, pregnant &amp; nursing women,</td>
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<tr>
<td>1. Immuno suppression by Tregitopes.</td>
<td></td>
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<tr>
<td>2. Neurite stimulation</td>
<td>All types of alopecia including alopecia areata, androgenetic alopecia, pattern baldness etc.</td>
<td></td>
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<tr>
<td>3. Vasodilatation</td>
<td>No known contraindications.</td>
<td></td>
</tr>
<tr>
<td>4. Topical sensitization/Irritant effect</td>
<td></td>
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<td>5. Enhanced Nutrition</td>
<td></td>
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<td>6. Permeation enhancement</td>
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<table>
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<th>Side Effects</th>
<th>Remission</th>
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<tr>
<td>Burning or irritation of the eye, itching, redness, severe allergic reaction, hypertrichosis</td>
<td>Reversal of regrown hair on cessation of treatment</td>
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- **Prostate and male breast cancer, teratogenic, impotence, abnormal ejaculation etc.**
- **Reversal of regrown hair on cessation of treatment**
- **Regrown hair doesn’t fall on cessation of treatment**

**No known side effects.**
- **Proven to be safe in C57BL/6 and swiss albino mice**

**Experimental work envisaged**

1. To develop standardized methods of extraction of selected indigenous medicine.
2. To develop standardized and validated analytical methods for analysis of the selected hair growth promoters.
3. To investigate the efficacy of selected indigenous medicine for treatment of alopecia in in-vivo models e.g., C57BL/6 mice and swiss albino mice with the optimized formulations and compare it with the conventional and placebo formulations.
4. To formulate a semi solid dosage form of equable efficacy for topical application using experimental design.
5. To study acute dermal and systemic toxicity of the prepared formulations on mice skin.
6. To analyse accelerated stability and predict the shelf life of the optimized formulations.
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Achievements in research

Four indigenous drugs, juniper extract, egg oil, leech extract and a mixture of egg oil with leech extract were selected out of a database of 32 plants and medicine on the basis of novelty, uniqueness, efficacy, availability, and patentability. The drugs were accurately identified and the respective specimens were stored in Jamia Hamdard.

Physical characterization including appearance, odor, density, iodine value, acid value, solubility in water and organic solvents was carried out and results were recorded. NMR spectra scan of egg oil, NIF1 and NIF2 illustrate that the peaks are of many types of which include β-Sitosterol glucoside and Stigmasterol glucoside amongst others.

Phytochemical characterization of juniper berries indicated the presence of reducing sugars, anthracene derivatives, tannins, and flavonoids apart from diterpene acids, sesquiterpenes, sugars, resin, and vitamin C.

The biochemical characterization of the zootherapeutic drugs indicated that sterols were a major component in egg oil, leech extract and the mixture of egg oil with leech extract. Hence this group was selected as the biomarker group and consecutively analytical method development was done to standardize the extracts as well as the gel formulation. This was also validated by the HPTLC method that was developed that was relevant for both the extracts and gel formulation.

On the basis of various chemical tests it was concluded that the egg oil, leech extract/NIF1 and NIF2 contained carbohydrates and steroids. The extracts gave negative results for proteins and alkaloids.

Analytical Method Development

The developed HPTLC method was found to be reproducible, accurate, precise, rugged, robust and specific. The method was suitable and convenient for the quantification of biomarker in extracts and formulated dosage form.

The calibration curve was prepared by preparing the sample in known concentrations of the organic solvent and application of samples on TLC plates and scanning to obtain the densitometric spectra. The calibration curves were plotted between concentration and...
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average peak area. The statistical parameters were calculated and positive correlation was obtained between these two parameters. The method is highly specific for cholesterol since all the degradation products can be easily discerned from the biomarker compound. Since the developed method separates the degradant products of leech extract under different stress conditions; it may be used to study degradation kinetics in drug product.

In vivo studies on hair growth
Animal studies to elucidate the safety, efficacy, and toxicity of the selected drugs were carried out on C57BL/6 and Swiss Albino mice.
Briefly, 7 week old mice in the 2nd telogen phase post morphogenesis were grouped into separate polypropylene cages according to the drug coding. They were depilated on their dorsal backs using a standard hair removing cream, and drug was applied to the dorsal back each day through next 30 days.
Global photographs were taken each day to assess the onset of melanogenesis which is indicative of the anagen phase. The mice were examined each day for onset of anagen, percent anagen induction and percent telogen frequency. Hair pluck test and length of hair grown were done on mice to assess the normal hair shedding of control mice as compared to test groups. Length of hair grown is an indication of the duration of anagen phase. If the anagen is regular, it would last for 19 days in C57BL/6 and swiss albino mice; however prolonged anagen would lead to longer hair than those observed in control groups.
The mice were sacrificed at the end of 30 days and punch biopsies of resected skin was taken.

Measurement of dorsal hair lengths
For the measurement of dorsal hair lengths in telogen C57BL/6 mice skin after topical treatment with various drugs; hair was removed on days 0, 14, 21, 28, 35 and 40. The hair length for Novel indigenous formulation 2 (NIF2) was extremely significantly different from that of vehicle control and of standard control on analysis with non parametric repeated measure ANOVA, using the Friedman test and Dunns multiple comparisons test [Fig 75].
Fig 75. Comparative lengths of regrown hair in C57BL/6 mice after drug treatment.

Fig 76. Changes in hair lengths of regrown hair in C57BL/6 mice after topical application of FK506, NIF1, NIF2 or minoxidil.

The length of hair follicles in C57BL/6 mice dorsal skin was evaluated after treatment with different modalities. Novel indigenous formulation 2 (NIF2) showed the maximum length of hair follicles at 12.5mm at day 49 followed by FK506 at 9mm as reported by Jiang et al in 1995 from Keio University, Tokyo, Japan [211] (Fig 76).
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NIF1 showed hair length comparable to that of FK506 at 9mm on day 49. Whereas, Minoxidil showed least hair growth at 6mm on day 49 post depilation. Hence, it is concluded that NIF1 and NIF2 are superior to minoxidil in inducing faster hair growth as evidenced by length of hair grown in the above figure.

Average follicular count

The number of follicles observed in resected telogen skin in which anagen has been induced through extraneous treatment with various hair growth promoters is assessed here. The test is done to compare the effectiveness of hair growth promoters in comparison to conventional therapy.

![Graph showing follicular count](image)

**Fig 77. Comparative evaluation of number of hair follicles across treatment modalities in C57BL/6 and Swiss albino mice.**

NIF2 shows the maximum number of hair follicles in anagen phase in both C57BL/6 (47.17 follicles) and swiss albino mice (43.3 follicles) [Fig 77]. This is followed closely by the 3.2mg/15cm² methanolic extract of *Eclipta alba* (45 follicles)[230]. NIF 1, leech extract was observed to induce anagen in 36.17 follicles in C57BL/6 [Fig 78] mice and 30 follicles in swiss albino mice. Egg oil compared with 32.83 follicles in C57BL/6 mice and 29.67 follicles in swiss albino mice. The lower concentration of *Eclipta alba* was able to induce only 11 follicles to anagen phase.
Fig 78. Comparative effect of various treatment modalities on number of hair follicles in C57BL/6.

The number of hair follicles in C57BL/6 mice dorsal skin was evaluated after treatment with different modalities. Novel indigenous formulation 2 (NIF2) showed the maximum number of hair follicles in anagen phase followed by the methanolic extract of *Eclipta alba* (3.2mg/15cm²) as reported in literature [230].

**Anagen induction**

Anagen phase was induced in telogen skin of C57BL/6 mice by topical application of various drugs. The microscopic data obtained from the validation study shows that the topical
administration of NIF2 affects the normal cycle by inducing the resting follicles to anagen phase of hair growth in approximately 16.67% of the treated animals as opposed to 0% efficacy in all other treatment modalities by day 4 followed closely by NIF1 (16.67% at day 5). FK506 induced anagen in 16.67% animals only by day 6 [211] along with minoxidil. NIF2 induced anagen in 100% animals by day 11 whereas FK506 and minoxidil both induced 100% anagen in C57BL/6 mice only by day 16 [Fig 79].

![Fig 79. Percent anagen induction in telogen mice skin by topical application of various treatment modalities.](image)

In comparison, methanolic extract of *Eclipta alba* (3.2mg/15cm²) as reported by Datta et al from the Dabur Research Foundation, Ghaziabad, India in 2009 [230] induces anagen in 50% animals by day 7 and 87.5% animals by day 10. Thus NIF1 and NIF2 not only induce early anagen but also feature 100% anagen in all animals by day 11 as compared to 100% anagen induction by day 16 in both FK506 and Minoxidil. It is concluded that NIF1 and NIF2 are superior to tested formulations, FK506 and EA (3.2mg/15cm²) as reported in literature as well as the conventional drug minoxidil in inducing earlier anagen besides achieving complete anagen in resting hair follicles.
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Percent telogen frequency
On analysis of percent telogen frequency results data, it was found that results of the groups vary significantly. Thus, vehicle is least effective in controlling active hair shedding in both C57BL/6 and swiss albino mice, followed by juniper extract and minoxidil. The NIF2 shows maximum benefit in hair shedding/hair fall followed by followed by NIF1 and egg oil.

Fig 80. Percent telogen frequency in C57BL/6 and Swiss albino mice across various treatment modalities.

Hair pluck test
Hair pluck test was carried out for both C57BL/6 mice and swiss albino mice. The number of hair plucked in vehicle control group was maximum for both C57BL/6 and swiss albino mice, 8 and 10 respectively [Fig 81]. This was followed by minoxidil in both groups of mice with plucked hair being 7 and 8 respectively, NIF2 showed least hair lost due to plucking (2, 2), NIF1 had similar values (2, 3). Differences between the two species of mice were not significant testing with a student's t test leading to the conclusion that both species of mice showed consistent behavior across the groups with NIF 2 having least plucked hair and vehicle with maximum hair.
Quality of hair

The quality of hair in groups treated with egg oil, NIF1 and NIF2 is significantly different as opposed to that of the negative and positive control and juniper berry extract, i.e. in anagen phase as evidenced by the deeply melanised follicles present deep in the subcutis as opposed to superficially present in the dermis.

NIF1 and NIF2 induce early anagen, increase number of hair follicles, reduce shedding and prolong anagen. It is thus concluded that egg oil does act as a nutrient for the dermal papillae cells enhancing their growth and strength as observed by hair pull and pluck tests.

Leech extract is a promising new medicine that can be explored further. It acts as an irritant on the scalp, increasing the blood supply to the dermal papillae cells and promoting growth of hair. Also it elongates the anagen phase which is a new feature in this study. The hair color seen in the treated vs. vehicle group is significantly different.
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Histopathological studies
Histopathology of C57BL/6 mice treated with NIF2 presented several hair follicles in the anagen phase. The anagen follicles are larger, darkly pigmented and lie much deeper in the subcuticular fat. The dark pigmentation in follicles is due to heavy melanisation of the hair root sheath cells and is a characteristic feature of anagen transformation in this species. Animals in all other groups show hair follicles lying superficially in the dermis indicative of the telogen phase.

Formulation of a topical hair gel for hair growth
Formulation of a topical gel was done using Carbopol 940 (poly-acrylic polymer), Tween 20 (surfactant), propylene glycol 200 (solubilizer) and Triethanolamine (gelling and neutralising agent). Design of Experiments was carried out using the Box-Behnken design for the selected variables to identify the optimum levels of different process variables (drug concentration, solubilizer, surfactant) influencing the response i.e. topical permeation.

Characterisation of gel
Characterisation and evaluation of gel for physical appearance showed a homogenous smooth white gel. It was smooth and free from lumps and grittiness. No liquefaction or separation was observed on storage. Skin retention was found inversely proportional to Tween and PG200. Spreadability was found to be inversely proportional to viscosity whereas viscosity was directly influenced by concentration of Carbopol and Tween 20. The pH of the formulated gels was measured to be 6.5 for NIF1 gel and 6.2 for NIF2 gel. The mean viscosity of NIF1 gel composition was found to be about 6380.8cp and for NIF2 was found to be 6477.29cp.

Acute dermal toxicity
Acute dermal toxicity profile was carried out in mice. The animals showed little or no reaction to the patches in Modified Draize’s test. Erythema, edema and scaling/drying of skin were not observed at the site of application of the extract in any animal. Grade 0 of the Draize’s scale was assigned for erythema and dryness and wrinkling of skin. Grade 0 was assigned for edema. The formulations were thus proved to be safe for dermal use. All animals were healthy, active with bright red eyes and healthy fur. There were no significant
deviations from the normal control in haematological and biochemical parameters. Examination done by naked eye found no abnormality in the necrosed organs. Histopathological examination also found necrosed sections to be within average limits. Histological examination of heart, stomach, jejunum, colon, liver, pancreas, kidneys, spleen, adrenals, thyroid, testes and ovaries revealed no abnormalities.

After performing histopathological studies, indigenous formulations were found to be safe and non-irritating as no apparent signs of skin irritation (erythema and edema) were observed. Indigenous formulations were comprised of excipients belonging to the GRAS (Generally Regarded As Safe) category and hence appeared to be safe and biocompatible for topical delivery.

**Microbiological testing on optimised gel**

Destabilase lysozyme of leech extract has potent anti-microbial activity as reported in literature. Microbiological testing was performed as per USP 30/NF 25 for gel formulations. It was found that the concentration of test micro organisms was well under control limits for the formulations [Table 40].

**Stability studies on optimised gel formulation**

Stability studies of the optimized gel formulations of NIF 1 and NIF 2 were carried out as per ICH guidelines. The effect of temperature on the degradation was studied by plotting log K v/s 1/T. Degradation rate constant (K) was calculated from the slope of the curve at each temperature. The value of K at 25°C (K_{25}) was obtained by extrapolation of the plot and shelf life of the NIF 1 and NIF2 gels were found to be 1.379 and 1.361 years respectively.

**Mechanistic pathways of action**

It is only during embryonic development or morphogenesis of the organism that hair follicles are formed. It is rare for hair follicles to be formed after birth, although androgens can have drastic influence over individual hair follicles over time.
Fig 82. Illustration of tri pronged putative mechanistic considerations of anagen inducing, vasodilator, immunosuppressive and neurite stimulating effect of novel indigenous formulation on hair follicle cells of skin.

Hence it is clear that any growth, cessation or regrowth of hair is occurring through the active, dormant and re-activated hair follicle. Taking this analogy to hair growth treatments, when NIF1 and NIF2 are applied to the scalp, the lipophilic nature of these extracts helps them in passing through the stratum corneum into the dermis and hypodermis where they reach the dermal papilla cells.
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Leech extract is a known permeation enhancer and spreading agent which facilitates deeper penetration and better spreading of the extract in the dermis and subcutis. Once they have reached the dermis and subcutis, the biochemical moieties of the leech extract come into play. Leech extract and egg oil contain a cocktail of hormones, neurotransmitters, enzymes and growth factors which activate the dermal papilla cells leading to early transformation of telogen to anagen and elongation of anagen.

Normal regulatory T cell epitopes are known to suppress immune response, which is also a causative factor for hair loss/alopecia areata. Hence apart from stimulating the neuronal pathway leading to activation of dermal papilla differentiation and mitosis, leech extract also acts by suppressing immune response locally at the dermal papilla and hence reducing hair loss due to autoimmune malfunctioning.

Thus, the putative mechanistic considerations of the three pronged effect of novel indigenous formulation include the following [Fig 82]:

1. Immunosuppressive effect on skin epithelial fibroblast cells by regulatory T cell isotopes or Tregitopes leading to protection of epithelial keratinocytes including those in dermal papilla.
2. Neurite stimulating effect of the enzymes Destabilase and Bdellin leads to stimulation and subsequent growth of specialised mesenchymal cells of the dermis and follicular papilla.
3. Vasodilatation of the blood vessels surrounding the hair follicle thereby increasing volume and force of blood flow to hair dermal papilla cells leading to increased supply and exchange of nutrients and gases.

Thus, two indigenous formulations have been developed which are safe, extremely effective and with no side effects.

Despite science having made progress each day through centuries, we still need to go back to our ancient roots, go back and trace the ancient pathways which were overlooked for decades and find that safe and effective solutions which lie within the ancient wisdom of our forefathers.
Conclusion

The research was conducted to investigate the hair growth promoting effect of various indigenous formulations. Three indigenous drugs were selected out of a database of 35 drugs on the basis of novelty, availability, feasibility of formulation and patentability. These drugs were tested for toxicity, safety and efficacy in 2 strains of mice; pigmented C57BL/6 and swiss albino mice. Standardization and validation of the selected drugs was done according to ICH guidelines. Further the selected drugs were formulated into a hair gel. During this research, the prominent results that were concluded are given as follows:

1. The hair growth promoting effect as affirmed by average follicular count in telogen skin of mice shows that NIF2 has the maximum number (47.17) of hair follicles as compared to 3.2mg/15cm² methanolic extract of Eclipta alba (45 follicles)[230]. NIF1 shows 36.17 follicles whereas egg oil has 32.83 follicles. These values are definitely better than 19 follicles for minoxidil which is a well known standard drug widely used for alopecia. Vehicle control group shows a low of 11.33 follicles only. Hence, NIF2 has more than 4 fold average follicles as compared to vehicle control and almost 2.5 fold better than minoxidil.

2. Length of hair follicles in NIF2 group were longer by 15% as compared to those in NIF1 and FK506 [211] and 2.08 times more than that of minoxidil. Hence, it is concluded that NIF1 and NIF2 are superior to minoxidil in inducing faster hair growth.

3. NIF2 was almost 6 times or 83.3% more effective as compared to vehicle control in inducing anagen; which is the principal phase indicating hair growth. NIF1 and NIF2 not only induce early anagen but also feature 100% anagen in all animals by day 11 as compared to 100% anagen induction by day 16 in both FK506 and Minoxidil. It is concluded that NIF1 and NIF2 are superior to tested formulations, FK506 and EA (3.2mg/15cm²) as reported in literature as well as the conventional drug minoxidil in inducing earlier anagen besides achieving complete anagen in resting hair follicles.

4. NIF2 and NIF1 prolong anagen phase to 30 days as compared to 20 days shown in C57BL/6 mice as reported in literature and observed for synthetic standard drug
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minoxidil. In reference to these 2 formulations, our results are better than minoxidil which is a synthetic compound having many other properties.

5. Another indirect yet important parameter relates the decrease in active shedding of hair to maintain hair density as substantiated by percent telogen frequency test. This property has been complimented by NIF2 giving 4 fold decrease in active shedding of hair proving the synergistic effect of egg oil with leech extract.

6. The number of hair plucked in vehicle control group was maximum followed by minoxidil in both groups of mice. NIF2 showed least hair lost due to plucking (2, 2). NIF1 had similar values (2, 3) validating the fact that application of NIF1 and NIF2 strengthens the roots leading to decrease in loose anagen hair and telogen effluvium.

7. Histopathology data of C57BL/6 mice treated with NIF2 presented several hair follicles in the anagen phase. The anagen follicles are larger, darkly pigmented and lie much deeper in the subcuticular fat. Animals in all other groups show hair follicles lying superficially in the dermis indicative of the telogen phase.

8. The putative mechanistic considerations of the novel indigenous formulation has been elucidated at least partly through Tregitopes or normal regulatory T cell isotopes leading to immune suppression of skin follicular papilla cells. Enzymes destabilase and bdellin enhance neurite stimulating effect of leech extract on epithelial keratinocytes and dermal fibroblast cells. Lastly, vasodilatation of $K^+$ channels leads to better vascular irrigation and hence increased exchange of blood, oxygen etc leading to better growth.

9. NIF1 and NIF2 were formulated into patient compliant, long staying gels. The formulation was optimized using Box Behnken Design using 4 independent and 3 dependent factors. The optimized formulation was characterised on the basis of pH, spreadability, skin retention, and viscosity.

10. Accelerated stability analysis was carried out which showed that formulations were stable after 6 months. The shelf life of NIF1 and NIF2 gel formulations were found to be 1.379 and 1.361 years respectively.