

Original Research Article

Restoring natural hair pigmentation: clinical assessment of safety and efficacy of an ayurvedic leave-on treatment

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ABSTRACT

Background: This clinical study evaluated the safety and efficacy of an Ayurvedic leave-on treatment for premature hair greying in healthy Indian adults (male and female). It also investigated the product's mechanism of action through in vitro analyses.

Methods: Healthy male and female volunteers aged 20–42 years with early-onset hair greying and no underlying medical conditions were enrolled in the clinical study. Participants were screened based on predefined criteria, including a specific percentage of grey hair. The study included a washout period before treatment initiation, followed by a four-month application phase with regular follow-ups. An in vitro study using B16F10 melanocytes (ATCC) was also conducted to explore the Ayurvedic leave-on treatment's mechanism of action.

Results: The Ayurvedic leave-on treatment significantly increased natural black hair density and reduced grey hair count in both male and female subjects. In vitro analysis reveals that the Ayurvedic leave-on treatment enhances melanogenesis by upregulating melanin production in melanocytes.

Conclusions: The present study concludes that the Ayurvedic leave-on treatment promotes melanogenesis, reduces oxidative stress, provides cytoprotective benefits, and effectively enhances dark hair density while reducing grey hair count. Clinically, 100% of users showed improved dark hair density, 97% reported reduced grey hair. No adverse effects were observed, confirming its safety and efficacy for both men and women with greying concerns.

Keywords: Premature hair greying treatment, Jatamansi, Ayurvedic, Melanogenesis

INTRODUCTION

Dark and lustrous hair on scalp is considered sign of youth, health and pride across cultures. Dark colour of the hair is due to the presence of two pigments, eumelanin and pheomelanin in hair.¹⁻³ It is the ratio of these two pigments that determines the tone of the hair colour, ranging from black to brown and blond. Greying, (scientifically known as canities) is loss of pigment from the hair and is a visible sign of natural aging of hair, while also observed in younger people most often associated with genetic predisposition, nutritional

deficiencies, psycho-emotional stress, metabolic or redox-imbalance on scalp and in hair follicles.⁴

Chronological aging of hair is manifested as greyness of hair and prevalence studies indicate that 6%–23% of people have 50% grey hair by the age of 50 years, however premature greying develops in younger age, which is considered different in ethnic groups like in Asians before 25 years, Europeans before 20 years, before 30 years in African population.^{5,6} Human hair grow in cycles, the anagen, catagen and telogen, can have 10-30 cycles in one's life time.⁷ In healthy hair follicles, pigment production is usually linked to the growth phase,

the anagen during which the melanocytes in the hair bulbar region actively synthesis melanin and transfer to the hair keratinocytes imparting colour to hair.⁸ During catagen phase these melanocytes cease to produce melanin and undergo rapid apoptosis (cell death), followed by resting phase of the hair, the telogen.⁹

Oxidative stress has been reported in aging scalps and hair. In aging hair follicles, at least after 10 cycles of hair growth, either there is depletion of melanocyte stem cells in the follicle or loss of melanogenic activity with amelanotic cells,¹⁰ culminating into canities, the grey hair. In hair follicles undergoing premature greying, there is reduction of antioxidant enzymes and redox imbalance with no reported damage to melanogenic stem cells.¹¹ This distinction presents a unique opportunity to develop targeted premature greying treatment with antioxidants and melanogenic stimulators to reverse this condition, and along with cytoprotective agents, strategically to support survival of sensitive cells in chronologically & prematurely greying hair follicle. Anti-grey treatment is an unmet need in the field of cosmetics and therapeutics with no proven technology that can address above mentioned issues together, owing to the complex aetiology of canities/premature greying. To address this, FMCG and other cosmeceutical industries across the world developed multiple technologies (synthetic or herbal derived or herbal ingredients) to treat this condition like α -MSH agonists which is a synthetic peptide (palmitoyl tetrapeptide-20), derivatives of plant-based molecules or herbal blends, with some level of proven effectiveness. Ayurveda, Indian classical system of medicine, suggests use of natural ingredients like Jatamansi, Bringha, Hibiscus etc., in different formats to treat premature greying.¹² The present studies demonstrate the *in vitro* efficacy of the ayurvedic complex, and safety and clinical efficacy of leave-on treatment made with ayurvedic complex in improving density of dark hair and reducing grey hair in (male and female) participants (age group of 20-42 years) suffering from hair greying problems.

METHODS

Cell culture

B16F10 melanocytes procured from ATCC (passage 23) were sub cultured in Dulbecco's Modified Eagle's medium (DMEM from GIBCO™, USA) with high glucose supplemented with 10% heat-inactivated foetal bovine serum (FBS; MP Biomedicals, USA), 1X penicillin-streptomycin-amphotericin B (MP Biomedicals, USA). Cells were maintained at 37°C in a humidified atmosphere at 5% CO₂ in the PhCbi incubator and 23th and 25th passage cells were used for the experiments.

Determination of non-toxic concentrations

The MTT assay was carried out to identify the noncytotoxic concentrations of ayurvedic complex. This

assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by metabolically active cells, resulting in purple colored formazan. The amount of formazan is quantitated using a microplate reader. Briefly, B16F10 were seeded into 96-well plates (4×10^3 cells/well) and were exposed to different concentrations (0.001%, 0.01% and 0.1%) of ayurvedic concoction. After a 48-hour incubation period, cells were treated with 150 μ l MTT (0.5 mg/ml) at 37°C for 3 hours. Following, the resulting formazan was solubilized using 150 μ l of DMSO and the absorbance was read in Multiskan SkyHigh Microplate reader (Thermo scientific, USA) at 570 nm.

Melanin assay

B16F10 cells (passage 23) were trypsinized using 0.25% trypsin-EDTA and 1.5×10^6 cells/plate were seeded in 60 mm well plates with 5 ml of 10% DMEM media and incubated for 24 hours. The media was prepared for all tests in phenol-red-free DMEM media along with 10% FBS. The consumed media was removed, and prepared phenol red-free media along with different concentrations of ayurvedic complex incubated for 72 hours. Cells were lysed, in lysis buffer (RIPA buffer) and centrifuged at 15000 RPM and pellets were solubilized in of 1M NaOH with 10% DMSO, incubated at 80°C for 1 hour and absorbance was measured at 405 nm and quantified using synthetic melanin standard. Cell proteins were measured with Protein estimation kit (Genie™) and melanin content normalized with protein levels. α -MSH (200nM) was used as positive control.

Statistical analysis

The results obtained from 3 separate experiments are expressed as mean \pm standard error of the mean (SE). The results were analysed in the GraphPad Prism using unpaired t test where differences with $p < 0.05$ were considered significant.

Study design and methodology

This is a controlled, single arm, efficacy and safety clinical study. Study was executed at MS Clinical Research, Bangalore, India with Dermatologist as the principal investigator and Ayurvedic doctor as co-investigator. Study was conducted between September 2024 to March 2025.

Male and Female participants with 20-30% greying across of the scalp, in the age group of 20-42 years were recruited in the present study. Pregnant and lactating women were excluded. Volunteers with severe hair fall or any scalp related problems or allergies that can potentially interfere in the study results or on food supplements were excluded from the study. Volunteers undergone hair transplantation and have participated in similar studies in last 6 months were excluded.

Participants who are currently using henna/dye were also excluded from the study.

Study volunteers were restricted from using any hair care product or treatments except the products provided to use during the study period. Test products were used by volunteers at home as per the instructions provided, and applied twice a day on scalp. There was a wash-off phase of 3 days with neutral shampoo, followed by 4 months of treatment phase and assessments were done periodically at Baseline, 1 month, 2 months, 3 months and 4 months.

During the visit to the site the participants were acclimatized at a temperature of 22°C + 5°C and relative humidity of 50%-60% for 15 minutes prior to scalp imaging and assessments. Trichoscan based analysis was done to assess improvement in dark hair number and reduction in grey hair on a localized trimmed area using Trichoscan. Trimmed area containing both black and grey hairs was selected for assessment. As Trichoscan detects only black hair, the area was first assessed before the temporary dye application, and then assessed again after dyeing the hair. The difference in hair count between the pre- and post-dye scans was considered for the analysis to estimate the number of grey hairs. Manual counting of grey was also done based on visual counting with naked eye on the Trichoscan generated images. Greyness on the hair was assessed close the scalp region on the hair using imaging software (J image) that measures the greyness of the hair (with values ranges from 0-255, with 0 being absolute black and 255 being white. L* (Luminosity) on the hair was measured using the same imaging software, which determines the lightness of the hair. The lower the L* value the higher the darkness of the hair.

Ethics and informed consent

Present clinical study was carried out as per GLP and ICH guidelines in compliance to the local government regulations. The study was reviewed and approved by independent ethics committee in India and an informed consent was obtained from all study subjects. The study was registered on Clinical Trial Registry of India, under ayurvedic study with study number CTRI/2024/10/074760 [Registered on: 04 October 2024] prospectively.

Statistics

All statistical tests were performed at a two-sided 5% level of significance & 95% confidence interval was reported. The Statistical software, R- ver. 4.1.3 was used for the analysis of the data. For Efficacy checking based on the p value, either Wilcoxon test or parametric test i.e., t-test (paired) was performed to compare each visit with baseline.

For Trichoscan based analysis, 3 subjects' data was found to be outliers, and was not considered for the data analysis hence 35 subjects' data was considered. Due to

Trichoscan image quality concerns, the month 2 data for Trichoscan (black hair count and grey hair count) for the nine subjects were excluded. As a result, the remaining sample size at month 2 was deemed insufficient for meaningful analysis.

Demographics

53 participants were recruited in the study, with 38 subjects completing the study. Demographic characteristics of the participants are depicted in Table 1.

Table 1: Demographics and volunteers characteristics at baseline.

Demographics and volunteers characteristics	
Age range (years)	20-42
Gender category	Male and Female - 38 (1:2)
Hair greyness	20-30% grey hair across the scalp
Race/category	Asians with Indian Origin

RESULTS

Determination of viability of B16F10 cells (melanocytes)

To determine the effect of Ayurvedic complex on the viability of melanocytes, MTT assay was performed. No significant loss in cell viability was observed in the tested range of concentration (0.001%, 0.01% and 0.1%) as shown in Figure 1, and were used to assess the efficacy of the same on melanin modulation.

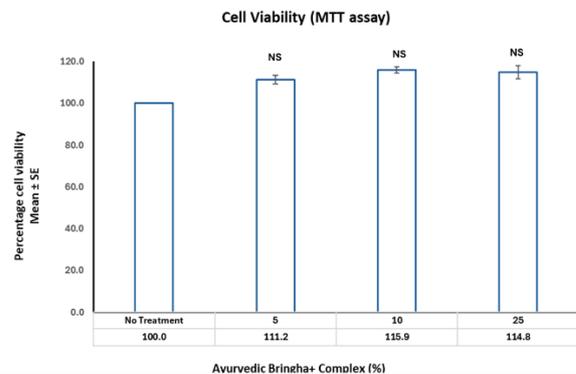


Figure 1: B16F10 cells treated with different concentration of Ayurvedic complex.

Data expressed as mean percentage viability ±SE; NS: No significance difference between the tested concentrations.

Quantification of melanin synthesis

One of the reasons in the etiopathology of greying hair is either loss/reduction of melanin production in the anagen phase. To determine the effect of Ayurvedic complex on synthesis of melanin by melanocyte and above-mentioned non-toxic concentrations were used to. There is a dose dependent increase in melanin production upon treatment

with Ayurvedic complex 0.001%, 0.01% and 0.1% by 20%, 37% and 108% respectively compared to no treatment control, as shown in Figure 2, which could be the mechanism of action of this Ayurvedic complex, that can potentially help to treat grey hair issues.

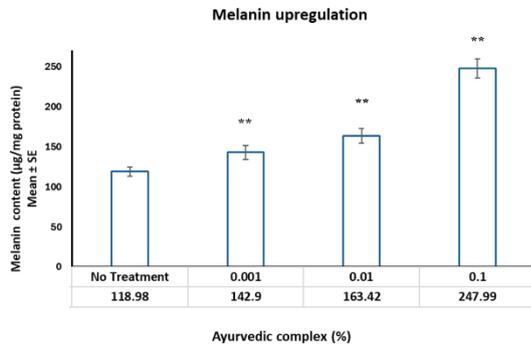


Figure 2: Melanin quantification.

Data expressed as Mean melanin content ± SE, **p value <0.05 vs No treatment control.

Improvement in black hair density (Trichoscan analysis)

With regular use of scalp leave-on treatment there is a significant improvement in black hair density from month 1 of treatment phase till 4 months with 13.7% improvement in black hair density from baseline (Figure 3). By the end of 4 months of treatment there was an increase of ~ 24 hair strands per cm² based on Trichoscan analyzed scalp area. Extrapolating this improvement in hair density to average scalp area (~650 cm²), it is estimated to have improved by ~15600 new black hair on the scalp by the end of 4 months.

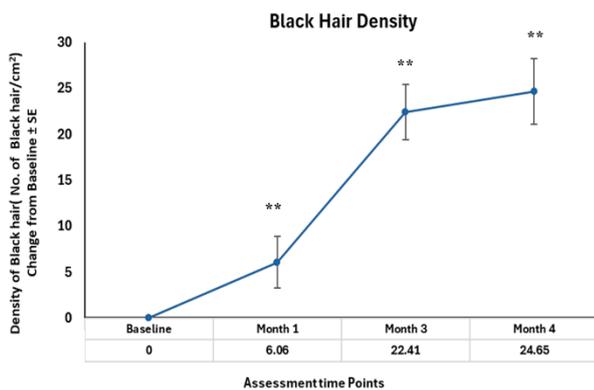


Figure 3: Black hair density (Trichogram assessment).

Data expressed as change form baseline ± SE; **p value <0.05 vs baseline.

Reduction in grey hair density (Trichoscan analysis)

With regular use of scalp leave-on treatment there is a significant reduction in grey hair density from month 1 of treatment phase till 4 months (Figure 4) with ~30%

reduction in hair density from baseline. By the end of 4 months of treatment there was reduction of ~5 hair strands per cm² based on Trichoscan analyzed scalp area. Extrapolating this improvement in hair density to average scalp area (~650 cm²), it is estimated to have reduced by 3,250 grey hair on the scalp by the end of 4 months.

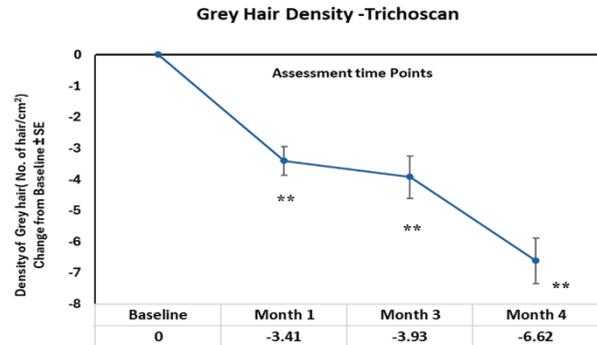


Figure 4: Grey hair density (Trichogram assessment).

Data expressed as change form baseline ± SE; **p value <0.05 vs baseline.

Reduction in grey hair density (visual counting)

With regular use of scalp leave-on treatment there is a significant reduction in grey hair density from month 1 of treatment phase till 4 months (Figure 5) with ~50% reduction in hair density from baseline. By the end of 4 months of treatment there was reduction of ~5 hair strands per cm² based on Trichoscan analyzed scalp area by visual/manual counting. Extrapolating this improvement in hair density to average scalp area (~650 cm²) it is estimated to have reduced by 3250 grey hair on the scalp at the end of 4 months.

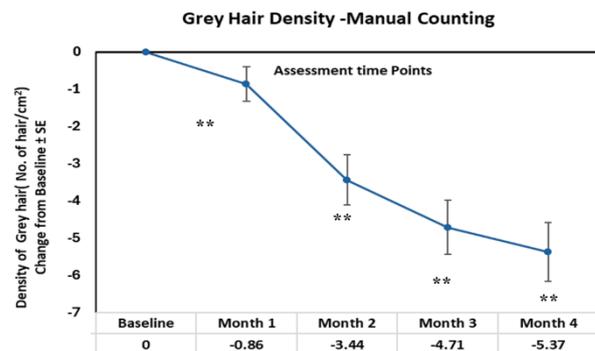


Figure 5: Grey hair density (manual count).

Data expressed as change form baseline ± SE; **p value <0.05 vs baseline.

Improvement in darkness of hair

This is an image-based assessment. The assessment was based on the grey-tone evaluation using pixel composition. A standard NIH published software Image J* is used for the analysis. The software maps pixel by pixel luminosity on a grey scale (0-255); where “0”

means absolute black and “255” means absolute white. The lower the value, the darker the hair. There is a change in 35 units from baseline to 4-month treatment (Figure 6), indicating the improvement in darkness of the hair, which is global measure of hair colour on the scalp.

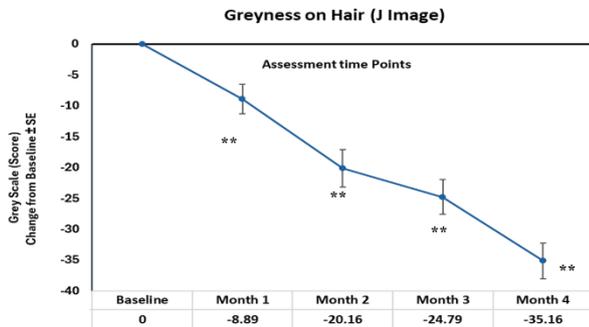


Figure 6: Greyness of hair (J-Image assessment).

Data expressed as change form baseline ± SE; **p value <0.05 vs baseline.

Reduction in greyness on hair

Measurement of L* on hair provides relative (arbitrary) values of intensity of lightness on hair vs baseline. With regular use of scalp leave-on treatment there is a significant reduction in L* value on hair from month 1 of treatment phase till 4 months (Figure 7) with reduction in value by 2 units from baseline indicating the reduction in greyness of hair.

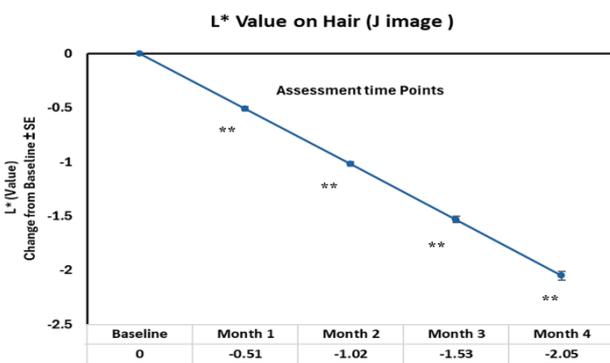


Figure 7: Lightness of hair (L*value).

Data expressed as change form baseline ± SE; **p value <0.05 vs baseline.

DISCUSSION

Greying of hair is a result of chronological aging. It has been reported that approximately 50% of people develop 50% of grey hair by the age of 50 years as per thumb rule of 50:50:50.^{13,14} However, can also happen in younger people, commonly referred as premature greying. Age for premature greying is considered different for different ethnic population, having said that development of grey hair before 30 years may be considered as premature greying in all of culture.¹⁵ Premature greying has a

considerable negative impact on the confidence, self-esteem and social acceptance in the sufferers. The cause of this premature greying is reported to be multifactorial like genetics, hormonal, pollution, nutritional deficiencies like Ca, Vitamin insufficiencies like D, B₁₂, smoking, emotional stress, pollution and using harsh grooming products with oxidants or generating oxidants etc.¹⁶⁻¹⁸ Prevalence of premature greying is reported across geographies known to have considerable psychological burden to the younger generation. In the present clinical study, ayurvedic complex was studied for its efficacy to enhance pigment production melanocytes and showed improvement in melanin synthesis at cellular level in a dose dependent manner and leave-on treatment made with the same reduced greyness and increased density of black hair by 13.7% in 4 month of treatment phase in participants suffering from greying hair.

Mechanism of hair greying was reviewed by several researchers supporting the role of oxidative stress as one the underlying mechanisms. Accumulation of millimolar levels of hydrogen peroxides in the greying follicles have been reported.¹⁹ Role of diminished antioxidant defence system with major reduction of catalase activity has been reviewed by Seiberg et al along with down regulated anti-apoptotic protein BCL-2, which is essential for cellular survival and cytoprotection.^{20,21} In hair, pigment production in linked to hair cycle in which melanocytes proliferate in early anagen to reach to maturation in mid anagen phase, while melanocytes undergo apoptosis during catagen with no pigment production in telogen phase.²² Considering the initiation of chronological greying age in the humans, it is obvious to note that first 10 cycles of hair is tightly linked to pigmentary unit. Imbalance of innate antioxidant network leading to damage to cellular DNA, dysregulated pro/anti-apoptotic mediators causing loss of pigment producing melanogenic precursors could play a potential role in both chronological and premature greying.²³ With increase in grooming awareness and aging population global anti-greying product market is poised to grow and cosmeceutical industries across the world are eyeing to capture the market by introducing new technologies to address this. Ayurveda, Indian traditional system of medicine proposes holistic approach with natural ingredients and lifestyle change to treat greying and premature greying problems.

Ancient ayurvedic scholars Charaka and Sushruta have referred premature greying of hair as Akaal Palitya.²⁴ Ayurvedic experts have classified this condition under kshudrarogadhikara and Kapala Roga. Vardhakya (chronological aging) is considered as main reason of palitya, however, the symptoms are observed early in young age due over work or excessive grief and anger where sharir ushma (heat) and pitta diverted to head leading to greying.²⁵ Ayurvedic experts suggested ingredients like Bringha, Jatamansi, Hibiscus etc to treat the same, processed as concoctions / oils or mask like Mansyadi lepa.²⁶ Kayyanyadi taila is referred in

ayurvedic texts in management of palitya and akalapalitya. This is a classical ayurvedic medicine referred to treat premature greying using ingredients like Amla and Bringha as per shastroyogam.²⁷ Improvement in hair colour was reported from a clinical study with above mentioned oil by performing siroabhyngana for 90 days.²⁸ Indravaruni beej siddha taila was recommended by Acharya Sarangadhara in ayurvedic texts to treat greying.²⁹ Clinical studies using Indravaruni beej (*Citrullus Colocynthis* seeds) siddha taila as shiroabhyanga (massaging scalp) was found to be effective in reducing grey hair count in subjects suffering from premature greying.³⁰ High polyphenolic content and antioxidants were reported in seeds of this plant which could be one of reasons for using this in treatment of premature greying in ayurveda. Bringhadi thilam and Nili bringhadi thailam are also commonly referred in ayurveda to treat greying/premature greying and hair loss.³¹⁻³³ The present ayurvedic leave-on treatment is thoughtfully made the ayurvedic ingredients like Jatamansi, Bringha, Amla etc in right proportions to treat greying scalps i.e., premature greying and greying.

Bringha (*Eclipta alba*), the Keshraj is widely referred in ayurvedic texts in various classical formulation to hair and scalp health. Ethanolic extracts of Bringha has been reported to increase melanogenesis in experimental *in vitro* models by upregulating the rate limiting enzymes of melanin synthesis.³⁴ Bringha extracts were proven to upregulate cyclic adenosine monophosphate (cAMP)—responsive element-binding protein (CREB), tyrosinase and tyrosinase related protein-1 which are essential for synthesis of melanin.³⁵ Proprietary Formulations with Bringha have been clinically proven to increase hair density and reduce hair fall in subjects suffering from hair thinning issues.³⁶ Optimised levels of Bringha extracts were used in the present leave-on treatment (which has shown significant increase in melanin production in melanocytes) and strategically designed to improve both hair growth and improve pigment production in hair from roots.

Role of psycho-emotional stress has been implicated in the development of early or premature greying. Hans Selye, proposed that intense psychic shock may also exert pronounced effects on the skin like greying and generalized hair loss.³⁷ As per Ayurveda anger and emotional stress also causes premature greying and suggested multiple ingredients and formulas to treat this condition.³⁸ Mansiadi lepam made with Jatamansi & other ingredients is an ayurvedic classical formulation recommended in ayurvedic texts to treat premature greying and chronological greying problems. The roots and rhizomes of Jatamansi were proven to have anti-depressive effects and have been used to treat hysteria & mental weakness, indicating its role in addressing emotional stress.³⁹ Jatamansi rhizome extracts were proven to exhibit inhibition of free radicals, scavenge superoxide anions and prevent lipid peroxidation.⁴⁰ Hair growth benefits of Jatamansi rhizome extract was

documented by Rao *et al* based on *in vivo* experimental models supporting its role in hair growth and cytoprotection benefits against oxidative stress in cell based *in vitro* studies.^{41,42} Use of this plant in ayurveda to treat premature greying could be a differentiated strategy in addressing one of the root causes of premature greying, and is backed by scientific support of cytoprotection and antioxidant effects of Jatamansi. The present leave-on treatment is strategically crafted with Jatamansi at functional levels to target mediators that may cause premature greying for anti-grey benefit and is convincingly supported by reduced number of grey hair by the end of 4 month of treatment phase in the present clinical study.

Nilini (*Indigofera tinctoria L.*) is commonly referred in ayurvedic classical formulations like Nilibringhadi taila to treat greying problems. Nilini with broad range of pharmacological activities like anti-inflammatory, antimicrobial and antioxidant benefits with proven inhibitory effects on hydroxyl and nitric oxide radical could serve as a prospective ingredient in the treatment of greying scalps.^{43,44} Adhirajan *et al* demonstrated that leaf extract of *Hibiscus rosa-sinensis Linn.* has hair growth-promoting effects in experimental models by *in vivo* and *in vitro* studies.⁴⁵ Oxidative stress not only have implication in premature greying but also cause hair loss and hair thinning. Hence incorporation of ingredients with hair growth promotion to support new hair growth helps in faster and visible results in treatment of premature greying. Nilini and Japa were incorporated at optimum levels as a part of this ayurvedic leave-on treatment, owing to the above benefits and the present clinical study demonstrates significant improvement in black density from month 1 till the end of treatment phase, 4 months.

Kutiki, (*Picrorrhiza kurroa*) extracts were proven to increase melanin synthesis both from basal levels and under oxidative stress and upregulates NRF2 gene that boosts cells natural antioxidant defence systems in melanocytes.⁴⁶ Premature greying hair follicles exhibit redox-imbalance and whereas aging hair follicles also need cryoprotection due to accumulation of high levels of oxidants with depleting melanogenic progenitor cells and melanin synthesis machinery.⁴⁷ Kutiki is proven to have cell damage prevention effects in experimental models.⁴⁸ Considering the cytoprotective and cellular antioxidant augmenting effects, it is prudent to use this ingredient as a part treatment strategy to treat greying problems in the present ayurvedic leave-on treatment.

Rosemary (*Rosmarinus officinalis*) is widely used in cosmetic formulations for its efficacy in improving hair growth.⁴⁹ Essential oils from Rosemary were proven to protect dermal papillary cells from oxidative stress along with increased levels of VEGF and IGF-1.⁵⁰ It is an established fact that pigmentation process in hair linked to anagen phase and cellular messengers like VEGF improves vascular supply to hair and hence

nutrient supply. Accelerated hair regrowth was reported in experimental depilated hair growth models, with upregulation of VEGF expression. Hence ingredients with aforementioned mechanism actions must be part of anti-grey treatment strategies to support faster and denser dark hair. The present leave-on treatment made with optimum levels of rosemary extract is proven to improve dark hair density & total hair density by the end of the 4 month treatment phase.

Kaidrya, (*Murraya koenigii*) commonly called as curry leaves used in Indian culinary as a flavouring agent, but also used for hair growth and delaying premature greying benefits Indian folklore, house hold remedies and traditional medicine.⁵¹ Having the convincing role of oxidative stress in greying and prematurely greying hair follicles, with proven damaging role to cellular macromolecules like DNA, lipids, enzymes due to oxidative stress, it is prudent to use antioxidants to protect and revive the same.⁵² Proteins isolated from curry leaves demonstrated the cellular membrane protective benefits in cell based oxidative stress experimental models and rich in tocopherol, β -carotene and lutein that are known to protect membrane lipid, proteins and DNA.⁵³⁻⁵⁶ Use of this potent ayurvedic ingredient in the present leave-on treatment is justifiable as a part treatment strategy to treat greying issues.

The limited sample size and non-comparative study design are the two drawbacks of the study. Additionally, a comparative study design with a greater sample size and a wider population range can increase the scientific value of our clinical investigation.

CONCLUSION

The present leave on treatment is strategically crafted using multiple prong approach with ayurvedic complex, the ingredients of which with unique mechanism of actions like melanogenesis, antioxidant effects, cytoprotection etc., to deliver improved dark hair density and reducing greyness. 100% of the study participants experienced improvement in dark hair density and 97% experienced reduction grey hair and 100% of the users liked the color, consistency and spread ability of the leave-on treatment. There were no adverse events reported throughout the study, indicating that the leave-on treatment developed is not only efficacious in boosting dark hair density and reducing grey hair but also safe to use (without side effects like irritation/redness/greasiness/oiliness in both men and women with greying problems).

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Conflict of interest: None declared

Ethical approval: The study was approved by the Institutional Ethics Committee

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