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RESEARCH ARTICLE

In Vivo Phytotherapy in BALB/c Athymic Nude Mice: Hair Growth Promotion using *Ficus religiosa* L. and *Morus alba* L.

Pramod S. Joshi[1*], Yogesh B. Patil[2], Bhagyashri Nagarkar[3], Tania S. Paul[3] and Kishori G. Apte[3]

[1]Symbiosis School of Biological Sciences, Symbiosis International (Deemed University), Pune, Maharashtra – 412115, India; pramodjoshi02@yahoo.com
[2]Symbiosis Centre for Research and Innovation, Symbiosis International (Deemed University), Pune, Maharashtra – 412115, India
[3]APT Research Foundation, Pune, Maharashtra – 411041, India

Abstract

Ficus religiosa L. (FR) and *Morus alba* L. (ML) belonging to the family Moraceae have been tested as novel herbal agents for hair growth promotion and Hair Follicles (HFs) regeneration in BALB/c athymic nude mouse model. Current study tested different mixtures of 5% aqueous fractions: Test 1 (ML2+ML3+ML4+FR4), Test 2 (FR1+FR2+FR4), or Test 3 (ML2+ML3+ML4+FR1+FR2+FR4) from leaves of both plants including standard of care 2% minoxidil. Control mice were untreated. Animals were treated for 33 days by topical application on the back skin and changes in hair growth patterns were evaluated. Histology was performed to assess the HFs morphology, and modulation of hair cycle phases. Gene expression analysis was performed to understand potential mechanisms of action. All treatment groups had significantly higher anagen phase HFs compared with untreated control group based on histology analysis. Also, expressions of CD34, CD200R and Oct4 genes were upregulated in all treatment groups compared with untreated control. Present study demonstrated that a combinatorial therapy using either fractions of FR or FR and ML promoted hair growth and HFs regeneration through induction of anagen phase in conjunction with stem cells associated genes upregulation in BALB/c athymic nude mouse model of hair loss.

Keywords: Anagen Phase, *Ficus religiosa* L., Hair Follicles, *Morus alba* L., Nude Mouse

1. Introduction

Skin is considered as the largest organ in mammals that protects against external insults while, hair have evolved to protect skin and as a mode of communication via signals and cues. Epidermis is the outermost layer of the skin, which is extended into numerous HFs[1]. HFs are dynamic structures that post-birth undergo cycles of anagen, catagen and telogen phases in a repetitive manner[2]. C3H/HeJ mice and Dundee Experimental Bald Rat (DEBR) models are considered as the basis of developing new treatments for alopecia areata[3–5] and put rodents in forefront of preclinical research for human hair loss indication. Mouse has short hair cycle (3 weeks) with well delineated anagen and catagen phases divided into six and eight sub phases respectively[6]. There appear to be no structural differences between mouse and human hair cycles except, catagen phase in mouse exhibits hair bulb alterations in absence of retraction of vibrissae follicles[1]. Rat model has ability to transform human stem cells into dermal papilla followed by new HFs generation[7].

Athymic nude mouse is devoid of fur and has been studied in biomedical research for more than five decades[8,9]. Phenotypically this mouse has hairless skin, however, its dermis has numerous active HFs that are anomalous with impaired keratinization[8,10]. The compromised differentiation of HFs in athymic nude mice is attributed to defects of the ectoderm that lead to structural abnormalities of cortex, hair cuticle and inner root sheath[11]. Since nude mice lack hair keratins, their hair shafts undergo twisting and coiling in the hair follicle infundibulum causing its dilation[12]. Studies on mutant mouse models of hair loss have implicated proteins such as bone morphogenic protein receptor, epidermal growth factor receptor, fibroblast growth receptor 2, insulin – like growth factor 1 receptor, and fibroblast growth factor 7

*Author for correspondence

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in hair growth pattern and overall hair cycle turnover[13]. Nude mouse model has been efficiently utilized to identify therapeutic targets such as Keratinocyte Growth Factor (KGF)[14], tellurium immunomodulator AS101[15], and cyclosporin A (CsA)[16]. *Eclipta alba* L. has been demonstrated to play a key role in facilitating nude mice follicular keratinocytes proliferation and delaying their terminal differentiation via TGF-β1 down regulation[17]. A study comparing C57BL/6 wild type (WT) and Foxn1-/- (B6.Cg-Foxn1nu/J) mice revealed that the very hairless phenotype in nude mice exists because of Foxn1−/− mutation influencing the downstream mechanisms of terminal keratinocyte differentiation and in addition, transcription factor Lhx2 (regulator of hair cycling and stem cells) appears to have a significant impact on mechanisms of stem cells regulation within the HFs bulge region[18]. Importantly, human and murine hair follicle stem cells share certain markers such as CD34[19,20] and CD200[19–21]. HFs in the frontal parts of the scalp are deficient in CD34 in androgenetic alopecia (AGA), while, its expression is conserved in HFs of the occipital region[22]. Expression of CD200 is minimal in matrix cells in patchy alopecia, which may be an indication of compromised immune tolerance[22,23] therefore, transmission of immunoregulatory signal from CD200 through CD200 receptor (CD200R) is vital to reduce inflammatory reactions and facilitate immune tolerance[24].

Ficus religiosa L. leaves containing carbohydrates, proteins, lipid, calcium, sodium, potassium, and phosphorus possess pharmacological activity against bacteria and fungi and they can also work as a wound healing agent[25]. A decade ago, an *in vitro* study was conducted using combination of extracts containing *Aloe vera* whole plant, *Eucalyptus globulus* leaves, *Ficus infectoria* bark, *Ficus religiosa* bark and *Piper betel* leaves to study antibacterial activity against resistant and sensitive strains isolated from infectious skin tissue (*Staphylococcus, Klebsiella, Enterobacter, Escherichia* and *Micrococcus* species). A mixture containing hot alcoholic extracts of *Ficus infectoria, Ficus religiosa* and *Piper betel* turned out to be the most efficacious against all bacteria including its bactericidal activity against resistant strains of *Pseudomonas*[26]. In more recent study, *Ficus religiosa* leaves extract was orally administered at doses up to 400 mg/kg for 55 days that significantly diminished the motor defects and provided brain protection from oxidative stress in rat models of Parkinson using 6 hydroxydopamine (6-OHDA) and catalepsy using haloperidol at test preparation doses of 200 and 400 mg/kg[27]. *Ficus religiosa* leaves extract also provided protection against isoniazid-rifampicin and paracetamol induced liver toxicity in rats at doses of 100, 200 and 300 mg/kg[28]. In guinea pig model of asthma, administration of *Ficus religiosa* leaves extract (150 and 300 mg/kg, ip) generated significant inexpression effect to develop histamine & acetylcholine induced pre-convulsive dyspnea[29]. *Morus alba* L. leaves predominantly containing flavonoids have activity as anti-microbial, anti-diabetic, anti-atherosclerotic, anti-obesity, and as a cardioprotective agent[30]. Abnormal overproduction of melanin in skin leads to dots, freckles, bruising keratosis, and hyperpigmentation. Compound 3 isolated from *Morus alba* leaves was demonstrated to impede melanin production via regulation of melanogenesis-related protein expression[31]. Till to date, there is little evidence generated that established efficacy of *Ficus religiosa* L. or *Morus alba* L. in skin or hair growth associated abnormalities. Considering scarcity of approved products for hair loss in humans and no evidence of hair growth promotion and HFs regenerative activities produced in rodent models using *Ficus religiosa* L. and *Morus alba* L. either as single agent or in combination, authors decided to test these two herbal agents in BALB/c athymic nude mouse model.

5% aqueous fractions from leaves of *Ficus religiosa* L. (FR1, FR2, FR3 and FR4) or *Morus alba* L. (ML1, ML2, ML3, and ML4) were tested for hair growth efficacy in pilot study using testosterone induced hair loss model in rats as described previously[32]. These fractions were topically applied on dorsal skin area for 28 days. Additional treatments included 5% aqueous FR leaves or ML leaves extract including a standard of care 2% minoxidil. In this study, 5% aqueous fractions ML2, ML3, ML4 of *Morus alba* L. leaves or 5% aqueous ML leaves extract yielded a biologically significant increase (based on % change) in hair weights compared to disease control (DC) and standard of care 2% minoxidil on day 21 of treatment in animals induced with alopecia, while *Ficus religiosa* L. leaves 5% aqueous fractions FR2, FR3, FR4 or 5% aqueous FR leaves extract generated overall lesser or comparable increase compared with 5% aqueous ML leaves fractions or 5% aqueous ML leaves extract including standard of care 2 % minoxidil (supplemental Figure 1). In pilot study using BALB/c athymic nude mice, 5% aqueous leaves extract of *Ficus religiosa* L. or *Morus alba* L. including standard of care 2% minoxidil were applied as topical preparations on dorsal skin of animals for 21 days. The genes expression analysis data of skin tissues collected at terminal sacrifice revealed that CD34, CD200R or Oct4 expressions were biologically upregulated in all treatments groups compared with vehicle control (supplemental Figure 2). These preliminary data indicated promising hair growth associated efficacious effects of fractions or extracts of both plants prompting authors to pursue current study, wherein same fractions as testosterone induced alopecia rat pilot study such as FR1, FR2, FR4 or ML2, ML3, ML4 in the form of different mixtures of 5% aqueous preparations were tested in BALB/c athymic nude mouse as a rodent model of choice to assess hair

growth promotion and HFs regenerative activities and associated modulations of previously tested stem cells associated genes (CD34 and CD200R) and totipotency specific stemness transcriptional factor Oct4 after 33 days of topical application on dorsal skin area. Overall, this research opened the door of validating more therapeutic options for human hair loss indication and increased our understanding of roles of certain genes involved in stem cell biology of nude mouse HFs.

In the context of current study, a combinatorial therapy approach certainly is a viable option to promote hair growth and HFs regeneration.

2. Materials and Methods

The experiments were conducted as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, India with approval from the Institutional Animal Ethics Committee (IAEC) of APT Testing Research Pvt. Ltd, Pune, Maharashtra, India. Study was assigned a project number RP50/1819.

2.1 Plants Samples Collection and Formulation

Dried leaves of *Ficus religiosa* L. and *Morus alba* L. were collected from the Pune region of the state of Maharashtra, India. The plants samples were identified by Botanical Survey of India (BSI), Western Regional Center, Pune, Maharashtra, India. 5% gel was prepared from aqueous fractions of *Ficus religiosa* L. and *Morus Alba* L. in combination according to modified protocol based on procedure described previously[33]. Briefly, the individual fractions were dissolved in 4 ml of distilled water followed by addition of 4% propylene glycol and 3% ethanol. In another beaker, 1% Carbopol was added to 4 ml of distilled water followed by addition of 0.2 % methyl paraben, 0.02 % propyl paraben and 0.03 % EDTA. The contents of both the beakers were mixed thoroughly using mortar and pestle, and a drop wise triethanolamine was added till gel formation was visible.

2.2 Animals and Treatment Protocol

BALB/c athymic nude mice at 7 weeks of age were purchased from Vivo Biotech, Hyderabad, India. Nude mice were maintained under aseptic conditions in a laminar-flow rack and 12 h light: dark periods at 24 ± 2 °C with relative humidity of 40–60 %. These conditions also included sterile food and bedding, reverse osmosis water, and sterilization of water bottles and cages. After at least 5 days of acclimatization to new environmental conditions, 30 mice were randomized into 5 groups of 6 mice (3 males and 3 females) each. Topical application of test preparations was performed when the synchronized telogen phase was observed in all the experimental groups. *Ficus religiosa* L. and *Morus alba* L. in the form of mixtures of 5% aqueous fractions from leaves such as Test1 (ML2+ML3+ML4+FR4), Test 2 (FR1+FR2+FR4), or Test 3 (ML2+ML3+ML4+FR1+FR2+FR4) including standard of care 2% minoxidil were topically applied daily at 0.1 ml/mouse on the back skin from suprascapular region to cover dorsal cervical region, dorsal thorax, and region caudal to the ribcage until completion of two hair growth generations (33 days) (Table 1). Control mice were untreated.

Table 1. Study design

Groups	Number of Animals
Untreated Control	M (3/3), F(3/3)
Standard of Care (2% Minoxidil)	M (3/3), F(3/3)
Test 1, 5 % aqueous mixture (ML2+ML3+ML4+FR4)	M (3/3), F(3/3)
Test 2, 5% aqueous mixture (FR1+FR2+FR4)	M (3/3), F(3/3)
Test 3, 5% aqueous mixture (ML2+ML3+ML4+FR1+FR2+FR4)	M (3/3), F(3/3)

2.3 Determination of Hair Length

Images of dorsal skin region from thoracic area to region caudal to ribcage area were taken using Nikon digital camera before (Day 0) and after Day 14 and 28 of initiating daily tests treatment. Care was taken to measure hair length consistently in same representative region in all groups i.e. dorsal thoracic area to region caudal to the ribcage. Microware 1000X 8 LED USB digital microscope was used to measure hair length.

2.4 Skin Sample Collection and Histology Analysis

The study animals were sacrificed by using CO_2 asphyxia and skin tissue samples including HFs of dorsal region from thoracic area to region caudal to the ribcage were collected at terminal sacrifice (Day 33) from different treatment groups including 2% minoxidil and untreated control groups in 10% NBF (neutral buffered formalin). After 72 hours, the fixed skin tissues were processed using

ascending series of graded ethanol, and xylene[34]. Tissues were embedded in paraffin blocks for micro-sectioning on automated tissue microtome (Leica, Germany) and tissue transverse sections of 5 µm were placed on clean grease free glass slides. Subsequently, tissue sections were stained with hematoxylin and eosin (H&E). These tissue sections were observed under binocular microscope with microphotography unit (Nikon, Japan). The morphology and the number of HFs in various phases (viz. Anagen, Catagen and Telogen) were evaluated microscopically in 5 fields per section at a magnification of 100×.

2.5 Gene Expression Analysis: CD34, CD200R, and Oct4

Total RNA was extracted from Day 33 skin samples using GeneJET RNA Purification Kit (Thermo Fisher Scientific). cDNA was then synthesized by reverse transcription using High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Thermo Fisher Scientific). Quantitative PCR (RT-PCR) was performed with Quant Studio™ 5 Real-Time PCR System (Thermo Fisher Scientific). Genes expressions were evaluated using the primers as described previously for CD34[35], CD200R[36], and Oct4[37]. Relative gene expression was determined using the $2-\Delta\Delta Ct$ method and presented as the mean.

2.6 Statistical Analysis

The experimental data are expressed as the means. One-way ANOVA was used for the assessment of significance between the different treatment groups. Statistical analysis was performed using Graphpad Prism Version 5. A value of $p < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1 Effects of *Ficus religiosa* L. and *Morus alba* L. on Hair Growth

The authors evaluated in-vivo hair growth promotion and HFs regenerative activities in BALB/c athymic nude mice after topically applying *Ficus religiosa* L. and *Morus alba* L. 5% aqueous fractions from leaves in the form of mixtures (Test 1, Test 2, or Test 3) or 2% minoxidil on dorsal surface of skin from suprascapular region to cover dorsal cervical region, dorsal thorax, and region caudal to the ribcage. Care was taken to measure hair length consistently in same representative region in all groups i.e. dorsal region from thoracic area to region caudal to the ribcage. The untreated control group of nude mice exhibited thin, transient, and variable hair growth during the entire treatment period of 33 days. Mean hair length did not differ among all groups (data not shown). Incidence of hair growth in mice on Day 0 (pre dose), Day 14, and Day 28 are shown in Table 2. Although, untreated control mice showed a variable background hair growth on Day 0, Day 14, and Day 28; Day 14 had greater hair growth and relatively higher incidence of animals showing hair growth in Test 1, Test 2, or Test 3 treated groups compared to 2% minoxidil and untreated control groups (Figure 1, Table 2). Lack of hair growth on Day 28 of treatment in some of the same mice can be attributed to wave like pattern of hair growth[38]. Also, around 2 weeks of time frame of anagen phase[6,39], and animal to animal variability noticed in mice may add to scarce hair growth.

3.2 Effects on Number and Development of HFs

Mixtures of 5% aqueous fractions from *Ficus religiosa* L. and *Morus alba* L. leaves stimulated HFs development that is evident from the photomicrographs obtained from the histology analysis (Figure 2). Follicular development was determined according to the guideline previously proposed[40]. The quantitative analysis of the representative transverse skin tissue sections (from thoracic area to region caudal to the ribcage, Table 3, Figure 3) indicated that Test 1, Test 2, Test 3 or 2% minoxidil groups showed statistically and biologically higher number of HFs due to induction of anagen phase in the dermis and hypodermis region compared with untreated control. Also, telogen phase HFs were statistically significantly increased in Test 1, Test 2, Test 3 or 2% minoxidil groups compared with untreated control group. Telogen phase modulation appears to be treatments associated however, regular wave pattern of hair growth cycle phases in nude mice may be an additional factor contributing to modulation (Table 3). Overall, qualitative analysis under high power (100X) microscope field of skin tissue sections from thoracic area to region caudal to the ribcage revealed presence of different phases of hair cycle including anagen, catagen or telogen with anagen phase HFs significantly increased in Test 1, Test 2, Test 3 or 2% minoxidil groups as compared with untreated control group suggestive of initiation of HFs growth in these groups (Figure 2). Although, Test 1, Test 2, or Test 3 showed biologically marginally higher anagen phase HFs than 2% minoxidil, it was Test 3 group that had relatively higher number of HFs in anagen phase suggestive of progressive HFs growth compared with Test 1 or Test 2 groups.

Figure 1. Hair growth on Day 0, Day 14 and Day 28 of treatments, representative dorsal skin area of groups **(A)** Untreated Control, **(B)** 2% Minoxidil, **(C)** Test 1, **(D)** Test 2, **(E)** Test 3 from same animals. Note: Test 1 = ML2+ML3+ML4+FR4, Test 2 = FR1+FR2+FR4, Test 3 =ML2+ML3+ML4+FR1+FR2+FR4.

Table 2. Incidence of hair growth in mice on Day 0 (pre dose), Day 14, and Day 28

Groups	Day 0	Day 14	Day 28
Untreated Control	M(0/3), F(2/3)	M (3/3), F(0/3)	M (0/3), F(2/3)
Standard of Care (2% Minoxidil)	M(0/3), F(0/3)	M (0/3), F(2/3)	M (1/3), F(1/3)
Test 1	M(0/3), F(0/3)	M(2/2)*, F(2/3)	M (0/2), F(2/3)
Test 2	M(0/3), F(0/3)	M (3/3), F(3/3)	M (1/3), F(2/3)
Test 3	M(0/3), F(0/3)	M(3/3), F(1/2)*	M(2/3), F(2/2)*

*One Test 1 male found dead on day 9 and one Test 3 female found dead on Day 7 of treatment, cause (s) of death undetermined. Note: Test 1 = ML2+ML3+ML4+FR4, Test 2 = FR1+FR2+FR4, Test 3 = ML2+ML3+ML4+FR1+FR2+FR4

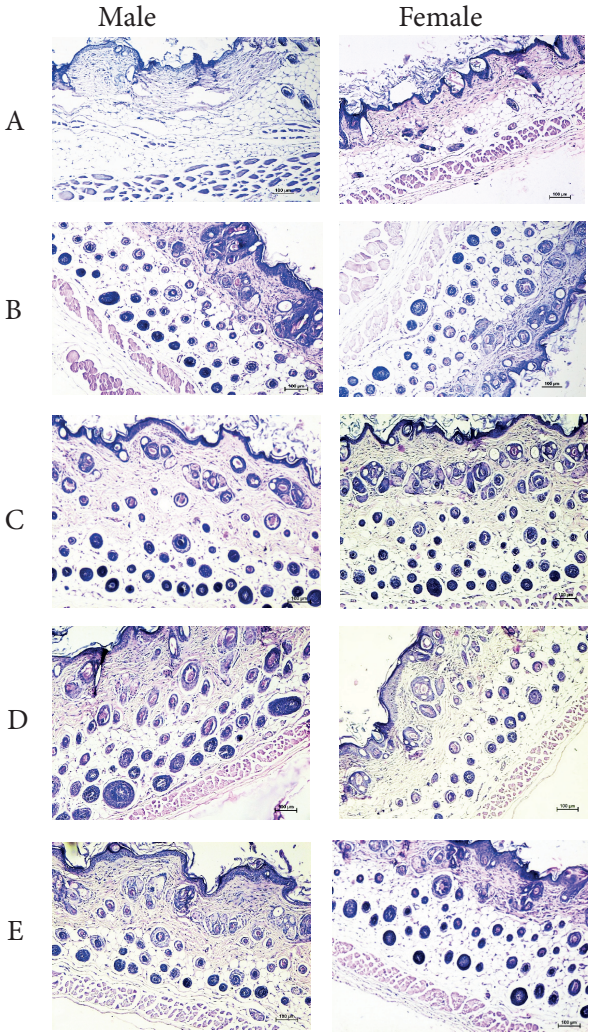

Figure 2. Representative transverse sections of dorsal skin specimens of groups **(A)** Untreated Control, **(B)** 2% Minoxidil, **(C)** Test 1, **(D)** Test 2, **(E)** Test 3 stained with Hematoxylin and Eosin (H&E). Scale bar 100 µm, same animals as Figure 1.Note: Test 1 = ML2+ML3+ML4+FR4), Test 2 = FR1+FR2+FR4, Test 3 =ML2+ML3+ML4+FR1+FR2+FR4.

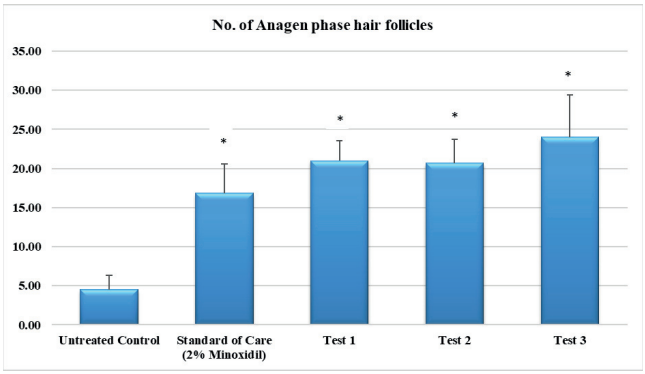

Figure 3. Mean # of anagen phase HFs after quantitative analysis performed on transverse sections of dorsal skin specimens stained with Hematoxylin and Eosin (H&E). *p < 0.05 when compared with untreated control.Note: Test 1 = ML2+ML3+ML4+FR4, Test 2 = FR1+FR2+FR4, Test 3 =ML2+ML3+ML4+FR1+FR2+FR4.

3.3 Expression of CD34, CD200R, and Oct4 during Follicular Morphogenesis

Current study compared the expressions of stem cells associated genes such as CD34, CD200R and a key transcriptional factor involved in induction of pluripotent stem cells (iPS); Oct4 using RT-PCR analysis of skin tissue collected at terminal sacrifice (Day 33, Figure 4). Mean relative gene expression (2−ΔΔCt) was statistically nonsignificant, however biologically higher in Test 1, Test 2 or Test 3 treated mice including 2% minoxidil compared with untreated control for CD34, CD200R or Oct4 with exception of CD200R that was statistically higher in Test 3 group compared with untreated control. Data suggest that expression of CD34 was 40-70 fold higher in Test 1, Test 2, Test 3 or 2% minoxidil treated mice over untreated control mice with Test 3 (69.52) showing higher fold increase than 2% minoxidil (58.58). CD200R expression was 5-47 fold higher in Test 1, Test 2, Test 3 or 2% minoxidil treated mice over untreated mice with Test 3 (46.68) showing biologically higher fold increase than 2% minoxidil (17.01) (Table 4). Overall, fold increases in Oct4 (4-12) were lower than CD34 or CD200R however, Test 3 (11.53) showed marginally higher fold increase compared with 2% minoxidil (7.63).

Table 3. The quantitative analysis of the representative transverse skin tissue sections stained with H & E

Groups	# Anagen phase HFs*	# Catagen phase HFs*	# Telogen phase HFs*
Untreated control	4.5	0.67	1.50
Standard of Care (2% Minoxidil)	16.83**	1.83	2.83**
Test 1	21.00**	0.80	3.60**
Test 2	20.67**	1.67	3.33**
Test 3	24.00**	1.00	3.40**

*Mean number of HFs (M +F combined) based on microscopic evaluation (5 fields per section at a magnification of 100×). **$p < 0.05$ as compared to untreated control.Note: Test 1 = ML2+ML3+ML4+FR4, Test 2 = FR1+FR2+FR4, Test 3 = ML2+ML3+ML4+FR1+FR2+FR4

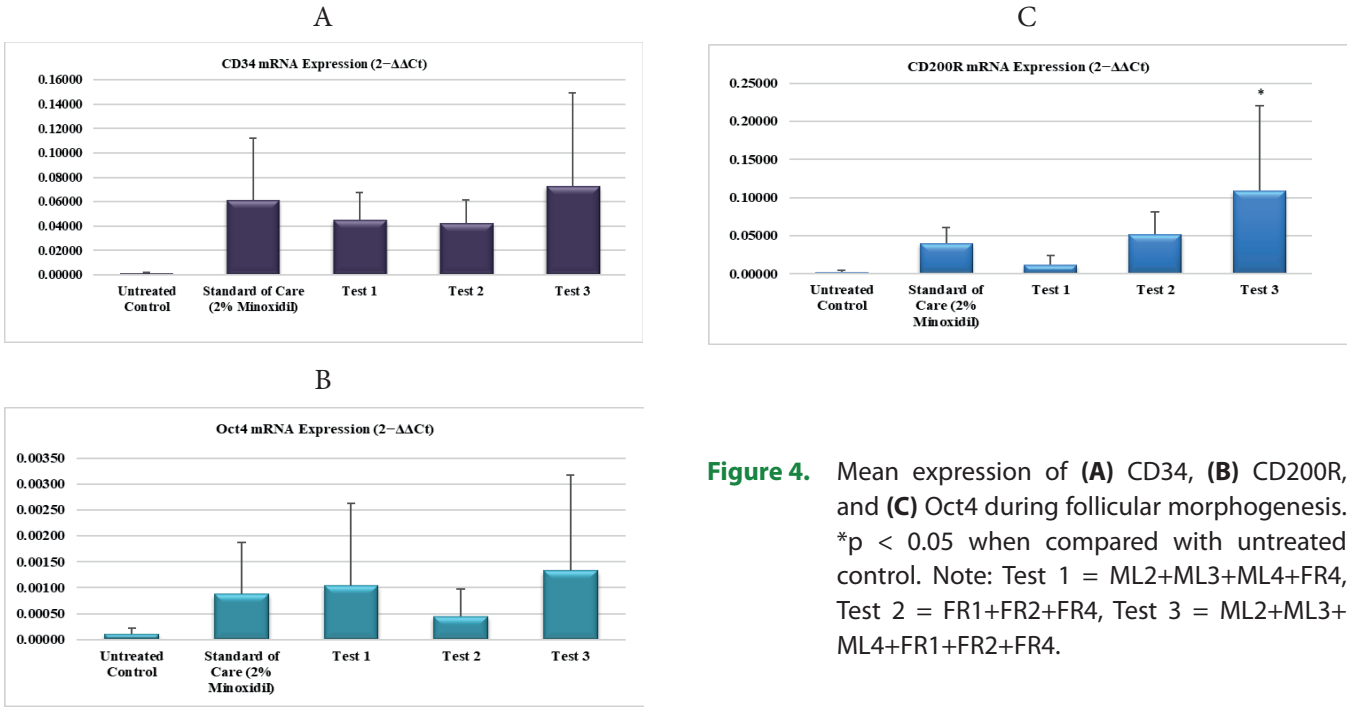

Figure 4. Mean expression of **(A)** CD34, **(B)** CD200R, and **(C)** Oct4 during follicular morphogenesis. *$p < 0.05$ when compared with untreated control. Note: Test 1 = ML2+ML3+ML4+FR4, Test 2 = FR1+FR2+FR4, Test 3 = ML2+ML3+ML4+FR1+FR2+FR4.

Table 4. Fold increase in mRNA expression (2−ΔΔCt) over untreated control

Gene	Standard of Care (2% Minoxidil)	Test 1	Test 2	Test 3
CD34	58.58	43.05	40.75	69.52
CD200R	17.01	5.08	22.09	46.68
Oct4	7.63	9.07	3.84	11.53

Note: Test 1 = ML2+ML3+ML4+FR4, Test 2 = FR1+FR2+FR4, Test 3 = ML2+ML3+ML4+FR1+FR2+FR4

4. Discussion

In this study, the researchers investigated the hair growth promotion and hair follicle regenerative activities of different mixtures of 5% aqueous fractions from leaves of *Ficus religiosa* L. and *Morus alba* L.

Treatments were performed by topical application of Test 1 (ML2+ML3+ML4+FR4), Test 2 (FR1+FR2+FR4), or Test3(ML2+ML3+ML4+FR1+FR2+FR4) including 2% minoxidil as standard of care on the back skin from suprascapular region to cover dorsal cervical region, dorsal thorax, and region caudal to the ribcage of BALB/c athymic nude mice. Untreated control mice showed a

variable background hair growth on Day 0, 14, and 28. Notably, Day 14 had higher incidence of animals showing hair growth in Test 1 (4/5), Test 2 (6/6), or Test 3 (4/5) treated groups compared with 2% minoxidil (2/6) and untreated control (3/6) groups. It is not uncommon to notice variability in hair growth pattern in mice which, during its regular cycles of anagen, catagen and telogen phases further gets modulated due to interventions such as in our study coupled with more asymmetrical and asynchronous pattern of hair wave[41]. Authors additionally investigated HFs associated morphological changes by histology and genes expression analysis. Histology analysis of Test 1, Test 2, Test 3, or 2% minoxidil groups provided evidence of an increased number of HFs in the deep subcutis and, fully established HFs in Test 1, Test 2 or Test 3 groups matched well with the anagen phase of the hair growth cycle in other studies[42,43]. Genes expression data ($2-\Delta\Delta Ct$) suggest that mean expression of CD34 was 40-70 fold higher in Test 1, Test 2, Test 3 or 2% minoxidil treated mice over untreated control mice. Mean CD200R expression was 5-47 fold higher in Test 1, Test 2, Test 3 or 2% minoxidil treated mice over untreated control mice with Test 3 associated increase statistically significant over untreated control. Oct4 fold increases (4–12) were relatively lower than CD34 or CD200R when compared between test preparations including 2% minoxidil groups vs untreated control. Noteworthy finding included a general trend for Test 3 treated mice to show biologically higher mean gene expression vs 2% minoxidil for CD34; 69.52 vs 58.58, for CD200R 46.68 vs 17.0, and for Oct4 11.53 vs 7.63. Multitude of evidence have been generated attributing different factors such as Fork head transcription factor, Hfh11 (also called whn) to hairless defect in nude mice[44]. Downregulation of TGF-β1 expression after treatment with *Eclipta alba* L. in nude mice was associated with stimulation of follicular keratinocytes proliferation and delay in keratinocytes terminal differentiation[17]. In nude mouse, a vital gene of epithelial cellness called LIM homeobox protein 2 (Lhx2) has been demonstrated to be constitutively upregulated along with significant changes in CD49f/CD34/CD200 cell subsets, that is indicative of changes in subpopulations of epithelial progeny[18]. The same research also indicated that Oct4+ stem cells population in dermal fractions of skin isolates from nude mice showed a greater expansion as compared with wild type counterpart. It has been demonstrated that defect in conversion of hair follicle stem cells to progenitor cell populations expressing CD200hiITGA6hi and CD34hi markers plays a pivotal role in the pathogenesis of AGA[45]. Scientists have also demonstrated that CD200 expression on mouse follicular epithelium in skin region plays a critical role in decreasing inflammatory responses and potentially facilitates maintenance of immune tolerance to HFs associated autoantigens after active engagement with CD200R[24].

Current study findings are well aligned with previous data on Foxn1−/− mouse phenotype with respect to hair growth promotion[17,46] and robust modulatory effects of CD34, or Oct4 genes upregulation are indicative of beneficial effects of Test 1, Test 2 or Test 3 in HFs regenerative activity. Further, the authors speculate that, this phytotherapy may have downstream effects of stimulating progenitor cells population and facilitation of immune tolerance via upregulation of CD200R message. In addition, Test 3 results indicated overall advantage over rest of the treatments including 2% minoxidil in terms of marginally higher upregulation of all 3 genes. Despite animal to animal variability, overall histology and genes expression analysis data demonstrated hair growth promotion from thoracic region through caudal region to rib cage in Test 1, Test 2, Test 3 or 2% minoxidil treated groups compared with untreated control group. Future dose response studies in mutant rodent models using either same combinations of fractions used in current study or using different combinations and / or using different treatment preparations may enable more insight into pharmacology of both plants in hair loss indication.

5. Conclusion

Present study demonstrated that a combinatorial therapy using either fractions of FR or FR and ML promoted hair growth and HFs regeneration through induction of anagen phase in conjunction with stem cells associated genes upregulation in BALB/c athymic nude mouse model of hair loss.

6. Conflict of Interest

The authors declare no conflict of interest.

7. Acknowledgments

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8. References

1. Porter RM. Mouse models for human hair loss disorders. J Anat. 2003; 202(1):125–31. https://doi.org/10.1046/j.1469-7580.2003.00140.x.PMid:12587927PMCid:PMC1571051

2. Schneider MR, Schmidt-Ullrich R, Paus R. The hair follicle as a dynamic miniorgan. Curr Biol. 2009; 19(3):R132–42. https://doi.org/10.1016/j.cub.2008.12.005. PMid:19211055

3. Sundberg JP, Cordy WR, King LE, Jr. Alopecia areata in aging C3H/HeJ mice. J Invest Dermatol. 1994; 102(6):847–56. https://doi.org/10.1111/1523-1747.ep12382416. PMid:8006447

4. McElwee KJ, Freyschmidt-Paul P, Sundberg JP, Hoffmann R. The pathogenesis of Alopecia areata in rodent models. J Investig Dermatol Symp Proc. 2003; 8(1):6–11. https://doi.org/10.1046/j.1523-1747.2003.12164.x. PMid:12894987

5. Sundberg JP, McElwee K, Brehm MA, Su L, King LE, Jr. Animal models for Alopecia Areata: What and where? J Investig Dermatol Symp Proc. 2015; 17(2):23–6. https://doi.org/10.1038/jidsymp.2015.35. PMid:26551940 PMCid:PMC4722955

6. Muller-Rover S, Handjiski B, van der Veen C, et al. A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages. J Invest Dermatol. 2001; 117(1):3–15. https://doi.org/10.1046/j.0022-202x.2001.01377.x. PMid:11442744

7. Sun J, Silva KA, McElwee KJ, King LE, Jr., Sundberg JP. The C3H/HeJ mouse and DEBR rat models for alopecia areata: Review of preclinical drug screening approaches and results. Exp Dermatol. 2008; 17(10):793–805. https://doi.org/10.1111/j.1600-0625.2008.00773.x. PMid:18798913 PMCid:PMC2778023

8. Flanagan SP. 'Nude', a new hairless gene with pleiotropic effects in the mouse. Genet Res. 1966; 8(3):295–309. https://doi.org/10.1017/S0016672300010168. PMid:5980117

9. Wortis HH, Nehlsen S, Owen JJ. Abnormal development of the thymus in "nude" mice. J Exp Med. 1971; 134(3 Pt 1):681–92. https://doi.org/10.1084/jem.134.3.681. PMid:15776569. PMCid:PMC2139084

10. Pantelouris EM. Athymic development in the mouse. Differentiation. 1973; 1(6):437–50. https://doi.org/10.1111/j.1432-0436.1973.tb00143.x. PMid:4547146

11. Kopf-Maier P, Mboneko VF, Merker HJ. Nude mice are not hairless. A morphological study. Acta Anat (Basel). 1990; 139(2):178–90. https://doi.org/10.1159/000146996. PMid:2288204

12. Mecklenburg L, Tychsen B, Paus R. Learning from nudity: lessons from the nude phenotype. Exp Dermatol.2005; 14(11):797–810. https://doi.org/10.1111/j.1600-0625.2005.00362.x. PMid:16232301

13. Nakamura M, Sundberg JP, Paus R. Mutant laboratory mice with abnormalities in hair follicle morphogenesis, cycling, and/or structure: annotated tables. Exp Dermatol. 2001; 10(6):369–90. https://doi.org/10.1034/j.1600-0625.2001.100601.x. PMid:11737257

14. Danilenko DM, Ring BD, Yanagihara D, et al. Keratinocyte growth factor is an important endogenous mediator of hair follicle growth, development, and differentiation. Normalization of the nu/nu follicular differentiation defect and amelioration of chemotherapy-induced alopecia. Am J Pathol. 1995; 147(1):145–54.

15. Sredni B, Gal R, Cohen IJ, et al. Hair growth induction by the Tellurium immunomodulator AS101: association with delayed terminal differentiation of follicular keratinocytes and ras-dependent up-regulation of KGF expression. FASEB J. 2004; 18(2):400–2. https://doi.org/10.1096/fj.03-0552fje. PMid:14656992

16. Gafter-Gvili A, Sredni B, Gal R, Gafter U, Kalechman Y. Cyclosporin A-induced hair growth in mice is associated with inhibition of calcineurin-dependent activation of NFAT in follicular keratinocytes. Am J Physiol Cell Physiol. 2003; 284(6):C1593–1603. https://doi.org/10.1152/ajpcell.00537.2002. PMid:12734112

17. Begum S, Lee MR, Gu LJ, Hossain J, Sung CK. Exogenous stimulation with Eclipta alba promotes hair matrix keratinocyte proliferation and downregulates TGF-beta1 expression in nude mice. Int J Mol Med. 2015; 35(2):496–502. https://doi.org/10.3892/ijmm.2014.2022. PMid:25484129

18. Bohr S, Patel SJ, Vasko R, et al. Highly upregulated Lhx2 in the Foxn1-/- nude mouse phenotype reflects a dysregulated and expanded epidermal stem cell niche. PLoS One. 2013; 8(5). https://doi.org/10.1371/journal.pone.0064223. PMid:23696871 PMCid:PMC3656088

19. Purba TS, Haslam IS, Poblet E, et al. Human epithelial hair follicle stem cells and their progeny: Current state of knowledge, the widening gap in translational research and future challenges. Bioessays. May 2014; 36(5):513–25. https://doi.org/10.1002/bies.201300166. PMid:24665045

20. Inoue K, Aoi N, Sato T, et al. Differential expression of stem-cell-associated markers in human hair follicle epithelial cells. Lab Invest. 2009; 89(8):844–56. https://doi.org/10.1038/labinvest.2009.48. PMid:19506554

21. Ohyama M, Terunuma A, Tock CL, et al. Characterization and isolation of stem cell-enriched human hair follicle bulge cells. J Clin Invest. 2006; 116(1):249–60. https://doi.org/10.1172/JCI26043. PMid:16395407 PMCid:PMC1323261

22. Elmaadawi IH, Mohamed BM, Ibrahim ZAS, et al. Stem cell therapy as a novel therapeutic intervention for resistant cases of alopecia areata and androgenetic alopecia. J Dermatolog Treat. 2018; 29(5):431–40. https://doi.org/10.1080/09546634.2016.1227419. PMid:27553744

23. Li J, Jiang TX, Chuong CM. Many paths to alopecia via compromised regeneration of hair follicle stem cells. J Invest Dermatol. 2013; 133(6):1450–2. https://doi.org/10.1038/jid.2012.511. PMid:23673497. PMCid:PMC4378644

24. Rosenblum MD, Olasz EB, Yancey KB, et al. Expression of CD200 on epithelial cells of the murine hair follicle: a role in tissue-specific immune tolerance? J Invest Dermatol. 2004 Nov; 123(5):880–7. https://doi.org/10.1111/j.0022-

202X.2004.23461.x. PMid:15482475

25. Chandrasekar SB, Bhanumathy M, Pawar AT, Soma sundaram T. Phytopharmacology of *Ficus religiosa*. Pharmacogn Rev. 2010; 4(8):195–9. https://doi.org/10.4103/0973-7847.70918.PMid:22228961. PMCid:PMC3249921
26. Pawar PL, Nabar BM. Effect of plant extracts formulated in different ointment bases on MDR Strains. Indian J Pharm Sci. 2010; 72(3):397–401. https://doi.org/10.4103/0250-474X.70494 PMid:21188057. PMCid:PMC3003181
27. Bhangale JO, Acharya SR. Anti-parkinson activity of petroleum ether extract of *Ficus religiosa* (L.) Leaves. Adv Pharmacol Sci. 2016; 2016. https://doi.org/10.1155/2016/9436106. PMid:26884755. PMCid:PMC4738991
28. Parameswari SA, Chetty CM, Chandrasekhar KB. Hepatoprotective activity of *Ficus religiosa* leaves against isoniazid+rifampicin and paracetamol induced hepatotoxicity. Pharmacognosy Res. Oct 2013; 5(4):271–6. https://doi.org/10.4103/0974-8490.118828. PMid:24174821. PMCid:PMC3807992
29. Kapoor M, Jasani N, Acharya N, Acharya S, Kumar V. Phytopharmacological evaluation and anti-asthmatic activity of *Ficus religiosa* leaves. Asian Pac J Trop Med. 2011; 4(8):642–4. https://doi.org/10.1016/S1995-7645(11)60163-6
30. Chan EW, Lye PY, Wong SK. Phytochemistry, pharmacology, and clinical trials of *Morus alba*. Chin J Nat Med. 2016; 14(1):17–30.
31. Li HX, Park JU, Su XD, et al. Identification of Anti-Melanogenesis Constituents from *Morus alba* L. Leaves. Molecules. 2018; 23(10). https://doi.org/10.3390/molecules23102559. PMid:30297610. PMCid:PMC6222840
32. Dhanotia R, Chauhan NS, Saraf DK, Dixit VK. Effect of Citrullus colocynthis Schrad fruits on testosterone-induced alopecia. Nat Prod Res. 2011; 25(15):1432–43. https://doi.org/10.1080/14786410802632820. PMid:19764005
33. Shahtalebi MA, Asghari GR, Rahmani F, Shafiee F, Jahanian-Najafabadi A. Formulation of herbal gel of antirrhinum majus extract and evaluation of its anti-propionibacterium acne effects. Adv Biomed Res. 2018; 7:53. https://doi.org/10.4103/abr.abr_99_17. PMid:29657938. PMCid:PMC5887696
34. Luna LG. Armed Forces Institute of Pathology (U.S.), Armed Forces Institute of Pathology (U.S.). Manual of histologic staining methods of the Armed Forces Institute of Pathology. 3d ed. New York,: Blakiston Division; 1968.
35. Lee J, Choi BI, Park SY, An SY, Han J, Kim JH. Fetal hematopoietic stem cells express MFG-E8 during mouse embryogenesis. Exp Mol Med. 2015; 47 pp.https://doi.org/10.1038/emm.2015.42.PMid:26206421. PMCid:PMC4525298
36. Cui W, Cuartas E, Ke J, et al. CD200 and its receptor, CD200R, modulate bone mass via the differentiation of osteoclasts. Proc Natl Acad Sci U S A. 2007; 104(36):14436–41. https://doi.org/10.1073/pnas.0702811104. PMid:17726108 PMCid:PMC1955461
37. Xiao L, Song Y, Huang W, et al. Expression of SOX2, NANOG and OCT4 in a mouse model of lipopolysaccharide-induced acute uterine injury and intrauterine adhesions. Reprod Biol Endocrinol. 2017; 15(1):14. https://doi.org/10.1186/s12958-017-0234-9. PMid:28253866. PMCid:PMC5335735
38. Militzer K. Hair growth pattern in nude mice. Cells Tissues Organs. 2001; 168(4):285–94. https://doi.org/10.1159/000047845. PMid:11275695
39. Toyoshima KE, Asakawa K, Ishibashi N, et al. Fully functional hair follicle regeneration through the rearrangement of stem cells and their niches. Nat Commun. 2012 Apr 17; 3:784. https://doi.org/10.1038/ncomms1784. PMid:22510689. PMCid:PMC3337983
40. Paus R, Muller-Rover S, Van Der Veen C, et al. A comprehensive guide for the recognition and classification of distinct stages of hair follicle morphogenesis. J Invest Dermatol. 1999; 113(4):523–32. https://doi.org/10.1046/j.1523-1747.1999.00740.x. PMid:10504436
41. Plikus MV, Chuong CM. Complex hair cycle domain patterns and regenerative hair waves in living rodents. J Invest Dermatol. May 2008; 128(5):1071–80. https://doi.org/10.1038/sj.jid.5701180. PMid:18094733 PMCid:PMC2705329
42. Begum S, Gu LJ, Lee MR, et al. In vivo hair growth-stimulating effect of medicinal plant extract on BALB/c nude mice. Pharm Biol. 2015; 53(8):1098–103. https://doi.org/10.3109/13880209.2014.959614. PMid:25612775
43. Zhang NN, Park DK, Park HJ. Hair growth-promoting activity of hot water extract of Thuja orientalis. BMC Complement Altern Med. 2013; 13:9. https://doi.org/10.1186/1472-6882-13-9.PMid:23305186.PMCid:PMC3637267
44. Kurooka H, Segre JA, Hirano Y, et al. Rescue of the hairless phenotype in nude mice by transgenic insertion of the wild-type Hfh11 genomic locus. Int Immunol. 1996 Jun; 8(6):961–6. https://doi.org/10.1093/intimm/8.6.961. PMid:8671685
45. Garza LA, Yang CC, Zhao T, et al. Bald scalp in men with androgenetic alopecia retains hair follicle stem cells but lacks CD200-rich and CD34-positive hair follicle progenitor cells. J Clin Invest. 2011 Feb 2011; 121(2):613–22. https://doi.org/10.1172/JCI44478. PMid:21206086. PMCid:PMC3026732
46. Begum S, Lee MR, Gu LJ, Hossain MJ, Kim HK, Sung CK. Comparative hair restorer efficacy of medicinal herb on nude (Foxn1nu) mice. Biomed Res Int. 2014; 2014. https://doi.org/10.1155/2014/319795. PMid:25478567. PMCid:PMC4247959

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