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Differences in Growth Response of Human Hair Follicle Mesenchymal Stem Cells to Herbal Extracts and a Growth Factor

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Abstract

Background: One of the key questions in biochemistry is why cell becomes aged and what are the involved factors? Why cell growth is stopped after some divisions and cells become senescent? This occurs in a greater frame in the whole body and cells dye after a while. Androgenetic alopecia (AGA) is characterized by a loss or decrease in hair follicle size, which could be related to the loss of hair follicle stem cells. Therefore, it is of great importance to develop novel therapies to increase hair follicle stem cells viability and proliferation.

Objective: In this study, we examined the effects of bFGF and aqueous Rosemary leaf and Marshmallow root extracts on human hair follicle mesenchymal stem cells (hHF-MSCs) proliferation in order to identify their potential for hair growth.

Methods: hHF-MSCs were isolated from hair follicle tissues and their mesenchymal nature confirmed by detecting cell surface antigens via flow cytometry. Bromodeoxyuridine (Brdu) incorporation assay was used to study the cell proliferation effect of herbal extracts in hHF-MSCs.

Results: Human hair follicle-derived mesenchymal stem cells (hHF -MSCs) were obtained by organ culture. They exhibited surface markers of mesenchymal stem cells as shown by positive staining for CD44, CD90 and CD105. Herbal extracts and bFGF were found to induce significant proliferation of human hHF-MSCs at concentrations ranging from 10 to 20 µl/ml and 15 to 25µl/ml.

Conclusion: These results suggest that herbal extract may produce positive effects on the hair growth promotion of hHF-MSCs and suggesting that herbal extracts may be a good candidate for helping hair growth promotion.

Keywords: Hair follicle, Herbal extracts, Proliferation, Stem cell

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Introduction

Hair follicles are renewable organs; they undergo repeated cycles of growth (anagen), regression (catagen), and rest (telogen) throughout the life of mammals [1]. It has been reported that there is an essential role for the follicular dermal papilla and its related dermal sheath cells in hair follicle development and regeneration [2]. At present, the majority of skin biology researches are focused on epidermal stem cells. However, the characteristics of MSCs also can be observed in hair follicle dermal cells [3, 4]. Pluripotent stem cells (PSCs) including human hair follicle stem cells have been demonstrated to be one of the best candidates for the generation of other cell lines [2].

The hair follicle stem cells (HFSCs) locating at the bulge region of hair follicle can be easily isolated; they possess the distinct characteristics of the adult stem cells, such as self-renewing, high proliferative potential [5, 6]. Therefore, hair follicle stem cells appear to be highly appropriate seed cells for tissue engineering and clinical application. Self-renewal and multilineage differentiation are other characteristics of stem cells. Since numerous undifferentiated stem cells are required for clinical applications, several laboratories have supplemented their expansion medium with growth factors to accelerate stem cell proliferation [7].

In Androgenetic alopecia (AGA), large terminal follicles diminish in size with time, and the resulting miniaturized follicle produces eventually microscopic velus hair [8]. The number of men and women who suffer from AGA is increasing, despite the development of several medical treatments [9]. Therefore, it is of great importance to develop novel therapies to prevent hair loss and to enhance hair growth. Multipotent adult stem cells have many potential therapeutic applications. One of these therapies is applying herbal extracts which are mentioned in Iranian traditional medicine such as *Rosemarinus officinalis* that improves microcirculation around hair follicle also has antioxidant properties, *Altheae officinalis* has antioxidant and anti-inflammatory properties so can relieve the micro-inflammation around hair follicle which are the result of environmental stress and exogenous and endogenous free radicals. Recent studies suggest that hair follicles are a source of easily accessible multipotent stem cells. Hair follicle stem cells could have important therapeutic applications [7].

Based on these lines, this study was planned to investigate the effects of some herbal extracts that have been used traditionally in oriental medicine for treating hair loss on hair follicle stem cells proliferation as well as its molecular mechanism.

Material and methods

Isolation of hair follicles stem cell and cell culture establishment

Hair follicles were isolated from the occipital scalp skin of three male individuals (age range 38–54 years, median 43 years) by plucking after full consent and ethical approval according to the methods of Yimei Wang [10]. We conformed to the Declaration of Helsinki Principle guidelines. Hair follicle root tissues

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were obtained by sterilized ophthalmic scissors and washed 3 times in PBS containing penicillin and streptomycin 1%, then placed into 96-well plates, cultured with hHF-MSCs medium: Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium (DMEM/F12), 10 % fetal bovine serum, 2 ng/ml basic fibroblast growth factor (bFGF) for 7 days. The wells populated with cells originating from the dermal sheath or papilla and which had the morphological appearance of mesenchymal cells were selected, pooled and expanded. Expanded cells were cultured in DMEM/F-12 containing 10% FBS and 1% antibiotic/anti-mycotic and supplemented with 2 ng/ml bFGF. Prior to the proliferation assay, the cells were cultured in stromal medium (DMEM/F-12, 10% FBS) for 7 days.

Immunophenotyping of human hair follicle stem cell by flow cytometry

Immunophenotyping of hHF-MSCs was carried out as previously described [11]. hHF-MSCs were detached from the tissue culture plate with trypsin–EDTA and counted. The cells (2×10^5) were divided into aliquots and centrifuged at 104 g for 5 min at RT. The pellet was resuspended in human serum and kept on ice for 30 min. After centrifugation at 104 g for 5 min, the pellet was suspended in 3% human serum albumin (HSA)/PBS and incubated with appropriate antibodies including Fluorescent Isothiocyanate (FITC)-conjugated anti-CD44, anti-CD90, anti-CD105, anti-HLA-DR (Biolegend, USA), anti-CD45, anti-CD31, anti-CD73 (BD, USA) and anti-CD15 (Abcam, MA) for 1 h in ice, washed twice in PBS, and centrifuged for 5 min. The cells were resuspended in 100 µL of PBS and studied by a Coulter Epics-XL flow cytometer (Beckman Coulter, Fullerton, CA, USA). An isotype control with FITC or PE labeling was included in each experiment, and specific staining was measured from the cross point of the isotype with the specific antibody graph. Histograms were created with Win MDI 2.8 software (Scripps Institute, La Jolla, CA, USA).

Plant Material

The root of *Althaea officinalis* and leaves of *Rosmarinus officinalis* were procured from local market and identified by comparing with standard herbarium specimens available in Islamic Azad University, Pharmaceutical Sciences Branch, Tehran, Iran. Voucher numbers: *Rosemarinus officinalis* L. is 250-PMP/A and *Altheae officinalis* is 312-PMP/A.

These parts of plant are crushed in mixer and passed through the sieve number 80. The various powder drugs were subjected to pharmacognostic studies for confirmation.

The herbs used in the present study for making herbal extracts were pre-washed, dried, crushed, grinded and passed through 100 mesh stainless steel sieves after 72 hours of maceration in 70°C (Indirect heat) to obtain a homogenous sample and water was used as base. The extracts were prepared utilizing this methodology; the extracts were filtered through muslin cloth then through filter paper and milipore under vacuum. The extracts were used immediately.

To standardize the herbal extracts thin layer chromatography and paper chromatography were used various mixtures of solvent systems

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were performed to verify the exact identity of the components discovered in the qualitative chemical tests.

Herbal Extracts' Standardization

To standardize the herbal extracts thin layer chromatography was used and performed to verify the exact identity of the components discovered in the qualitative chemical tests. For this aim the chromatography layers were prepared at first. The absorbent powder with volatile solvent such as acetone was deposited on a thin layer of a glass plate. After its dryness, a small drop of herbal extract sample was poured at the lowest area of column and allowed it to separate the various layers. Considering the active ingredients of *Rosemarinus officinalis* are rosemarinic and caffeic acid and the active ingredient of *Altheae officinalis* is flavonoids thus the samples were compared with these standardized pure materials.

Graphs Preparation

Graphs are prepared based on average of living cells in different concentrations of three groups: aqueous extract of leaf of *rosemarinus officinalis*, aqueous extract of root of *altheae officinalis* and the mix of two herbs as the third group after 48 hours. Considering that the Kolmogorov-Smirnov test confirmed the data of different concentrations of three groups are normal ($P>0.05$), to compare the different concentrations of various groups, One-way ANOVA and Tukey's post hoc tests are used.

Determination of Herbal Extracts Lethal Concentration 50

The herbs used in the present study for making herbal extracts were dried, crushed and passed through 80 mesh stainless steel sieves and water was used as base. The extracts was prepared described previously [9]. Treatments were designed in 6 groups 3 times 5, 10, 20, 30, 50 and 100µl/ml for *Rosmarinus officinalis* leaf extract and 5, 15, 25, 50 and 100 µl/ml for *Althaea officinalis* root extract.

Preparation of Combined Drug Herbal Extracts Formulation

After selection of method for preparation, multi ingredients of effective concentrations based on the preliminary physical and biological screening was prepared. The method selected was direct binge method and six different formulations having concentrations 5, 15, 25, 50, 100 and 200 µl/ml of extracts were prepared for maximum activity (Table 1).

Table 1- Selection of concentration of herbal extracts

Herbal extracts	LC50 (µl/ml)	Optimum dosages* (µl/ml)	
Rosemarinus officinalis leaf extract	30	20	10
Althaea officinalis root extract	50	25	15
Rosemarinus officinalis leaf extract+ *Althaea officinalis* root extract	25	15	5

* The best minimum and maximum dosages which induce maximum viability for MSCs

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Brdu cell proliferation assay

Cell proliferation was estimated in hHF-MSCs culture at different concentrations (10 and 20 µl/ml of *Rosmarinus officinalis* leaf extract, 15 and 25 µl/ml of *Althaea officinalis,* 5 and 15 µl/ml of combination) of herbal extracts using a colorimetric bromodeoxyuridine (Brdu) kit (Roche Diagnostics, East Sussex, UK) according to the previous study [12]. Briefly, cells labeled with 10 µM Brdu for 2 hrs at RT were fixed and made permeable with the FixDenat solution for 30 mins, then incubated with monoclonal anti-Brdu peroxidase-conjugated antibody for 90 min. The cells were then washed three times with wash buffer, and then peroxidase activity was measured using tetramethyl-benzidine as substrate. The reaction was terminated by adding 25 µl of 1M H2SO4, and absorbance at 450 nm was recorded in an ELISA plate reader. To study the rate of cell proliferation in the Brdu assay, the number of cells was adjusted to 20,000 to obtain an acceptable optical density (OD) in treatment groups.

Results

Isolation and Characterization of hHF-MSCs

The hHFM-SCs, resembling typical fibroblast morphology, migrated out from the hair follicle root tissue and adhered to the surface of the culture plate (Figure 1). The fibroblast-like cells at passage 3 were shown in Figure 1.

Immunophenotyping of hHF-MSCs:

Flow cytometry analyses showed that the majority of hHFMSCs expressed MSC markers CD44, CD90 and CD105 (25%, 30%, 29% resp.) (Figure 2).

Herbal extracts induced the proliferation of human hair follicle mesenchymal stem cells

The effect of herbal extracts on the hHFMSCs proliferation was examined. hHFMSCs were treated by various concentrations of herbal extracts and bFGF for 14 days and cell proliferation was measured by BrdU methods at days 0, 2, 4 and 14. BrdU assays showed a concentration dependent increased in cell growth activity in the hHFMSCs following exposure to *Altheae officinalis* extract but not *Rosmarinus officinalis* extract. As it is observed in Figure 3, proliferation promoting effects of *Rosmarinus officinalis* leaf extract on hHFMSCs start at 10 µl/ml and increased by

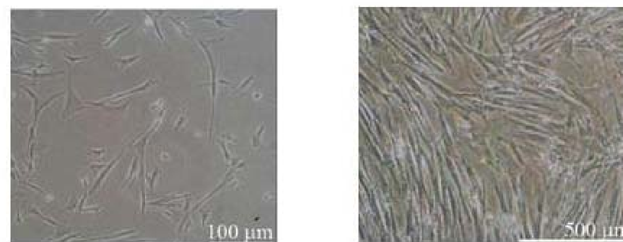

Figure 1- Isolation and characterization of human hair follicle mesenchymal stem cells (hHF-MSCs). The hHF-MSCs, resembling typical fibroblast-like cells

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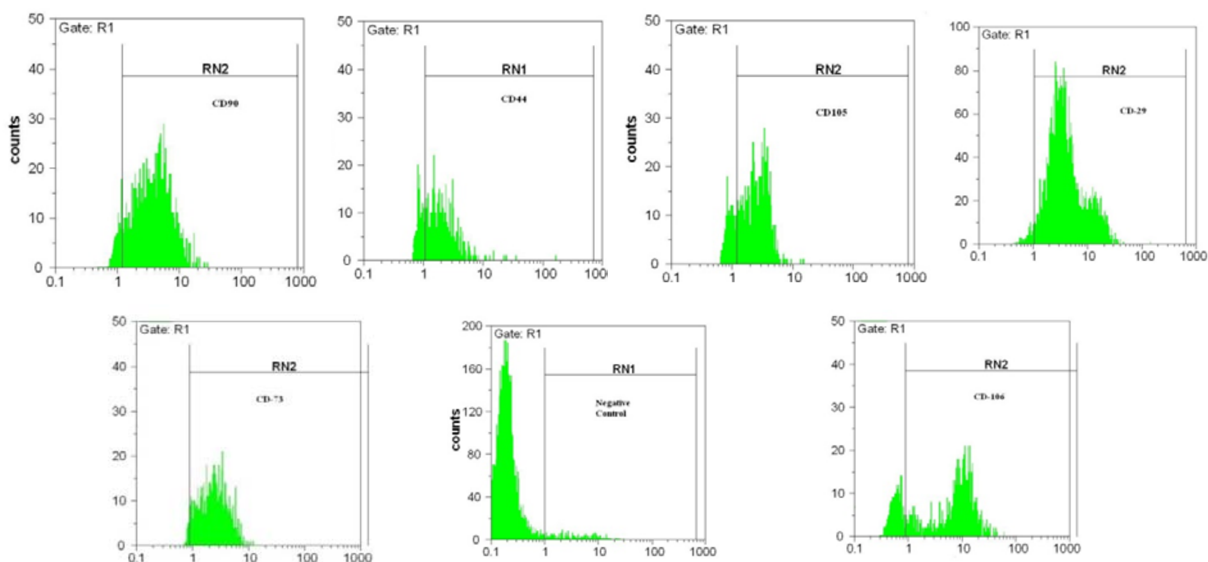

Figure 2- **Flow cytometric analysis of cell surface markers on hHF-MSCs cells. 2×10^5 cells were incubated with primary antibodies against CD29, CD73, CD105, CD90, CD106 and CD44 respectively, followed by incubation with a secondary FITC-labeled antibody. Controls were incubated with secondary antibody only. Percentages indicate the fraction of cells that stained positive.**

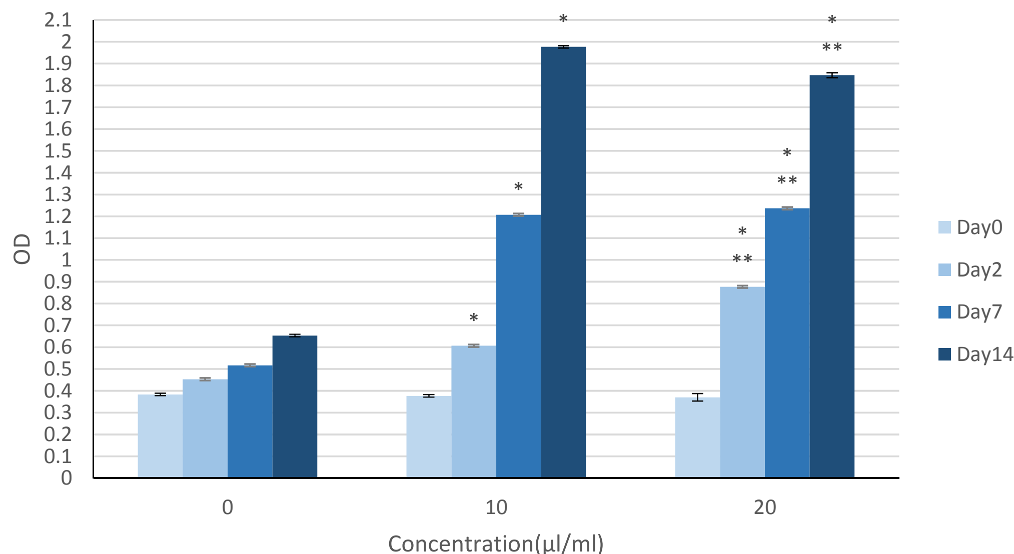

Figure 3- **Measurement the rate of human hair follicle mesenchymal stem cells proliferation in minimum and maximum dosages of *Rosemarinus officinalis* aqueous extract at days 0, 2, 7 and 14 of treatment in DMEM F12/FBS 10% and penicillin/streptomycin 1% by Brdu assay**

***: P <0.01 vs. Control at the same day**

****: P <0.01 vs. Minimum dosage at the same day**

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20 µl/ml but then decreased at day 14 in 20 µl/ml (from 1.64, 1.68 and 1.47% in 10µl/ml to 2.08, 2.02 and 1.69% in 20 µl/ml at day 2, 7 and 14 vs. control respectively; P < 0.01, one way ANOVA); also as observed in Figure 4, proliferation promoting effects of *Althaea officinalis* root extract on hHFMSCs start at 15 and increased by 25 µl/ml (from 1.3%, 1.4%, 1.28% in 15 µl/ml to 1.8%, 1.76%, 1.6% in 25 µl/ml at day 2, 7 and 14 vs. control respectively; P < 0.01, one way ANOVA);eventually as observed in Figure 5,

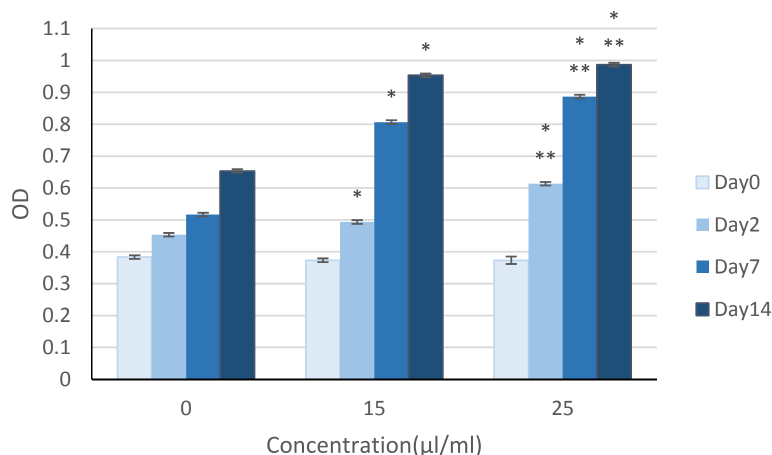

Figure 4- Measurement the rate of human hair follicle mesenchymal stem cells proliferation in minimum and maximum dosages of *Altheae officinalis* aqueous extract at days 0, 2, 7 and 14 of treatment in DMEM F12/FBS 10% and penicillin/streptomycin 1% by Brdu assay. *: P<0.01 vs. Control at the same day
****: P<0.01 vs. Minimum dosage at the same day**

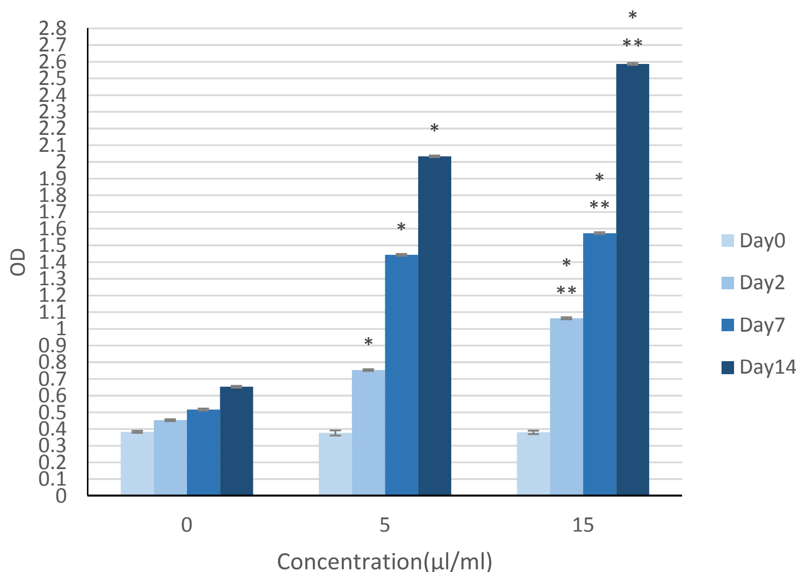

Figure 5- Measurement the rate of human hair follicle mesenchymal stem cells proliferation in minimum and maximum dosages of blend of *Rosemarinus officinalis* and *Altheae officinalis* aqueous extract at days 0, 2, 7 and 14 of treatment in DMEM F12/FBS 10% and penicillin/streptomycin 1% by Brdu assay
***: P<0.01 vs. Control at the same day, **: P<0.01 vs. Minimum dosage at the same day**

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proliferation promoting effects of combination of both herbal extracts on hHFMSCs start at 5 and increased by 15 µl/ml (from 1.6%, 1.19%, 1.55% at 5 µl/ml to 2.02%, 2.05%, 1.68% in 15µl/ml at day 2, 7 and 14 vs. control respectively; $P < 0.01$, one way ANOVA).

Discussion

During the growth of a hair, the follicle under-goes changes from an actively growing phase (anagen), to a remodeling phase (catagen), and finally to a quiescent phase (telogen), only to start growing again. One most key element that control hair follicle cycling is the follicular stem cells [13]. Recent studies demonstrated that alopecia is associated with destruction of hair follicle stem cells and hair loss [14]. The identification and characterization of stem cells is currently one of the most investigated areas of biological and biomedical research.

Multipotent MSCs were originally found in the bone marrow, and were considered to be the inherent stem cells at this location [15]. Later, these MSCs or MSC-like cells have been separated from, and identified in various tissues, including white adipose tissue, pancreas, skin, and the umbilical cord blood [16–19]. In recent years, studies have shown the presence of MSCs in the dermal sheath and dermal papillae of the hair follicle: In 2006, a comparative study investigated the properties of follicular dermal stem cells from whisker hairs of Wistar rats and bone marrow MSCs were isolated from femora of the same animals [20]. The results from that study showed that dermal stem cells from hair follicles have a similar morphology and population doubling time, and express the same cell-surface markers as the MSCs.

To our knowledge, the proliferation effects of herbal extracts in the hair follicle stem cell have not been previously elucidated. We hypothesized that the hair loss seen in AGA may result from loss of hair follicle stem cells. Based on these lines, this study investigated the effects of herbal extracts for treating hair loss in the hair follicle stem cells. In this study, we established a hair follicle stem cell culture model in vitro to clarify the effects of herbal extracts on the hair follicle growth. Our results are consistent with several previous studies that have demonstrated the multipotency of rat or human skin dermal fibroblasts [21-25]. It has been established that the bulge area of the hair follicle is an enriched source of epidermal stem cells [26-29]. The present study further supports these data by showing that HF-MSCs express CD44, CD73, CD90 and CD105 which are the surface markers that characterize MSCs.

In this study, herbal extracts were found to induce significant proliferation of human hair follicle stem cell at concentrations ranging from 5 to 25µl/ml.

In a study Roh SS *et.al* demonstrated that *Asiasari radix* extract increased the proliferation of both HaCaT and human DP cells in vitro [31]. Hay IC *et.al* have been investigated the efficacy of herbal extracts in the treatment of patients with alopecia and their results demonstrated that Nineteen (44%) of 43 patients in the active group showed improvement compared with 6 (15%) of 41 patients in the control group [32]. In another study, Bureau JP *et al.* demonstrated that the blend of *Pimenta racemosa*, *Myrtus*

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communis, *Cedrus atlantica*, *Laurus nobilis*, *Pogostemon patchouli*, *Rosmarinus officinalis*, *Salvia officinalis*, *S. sclarea*, *Thymus satureioides*, *Cananga odorata* increased hair density and the ratio of anagen hair per total hair in the double-blind randomized study vs. placebo in healthy male and female volunteers [33]. Although minoxidil, finasteride, and dutasteride including other synthetic therapeutic agents are mostly used for alopecia treatment, their adverse effects encourage sorting of alternative efficient treatment agents with a limited side effect particularly herbs. Thus, alternative herb medicine has attracted interest for hair loss treatment. Natural-derived compounds are able to prevent many diseases such as alopecia. Roh SS *et.al* was shown that *Sophora flavescens* is oriental medicine for hair loss treatment, which proven on hair growth promoting [30].

The effect of herbal extract on cancerous cell proliferation is studied for years and the results on DMBA induced cancerous cells showed that flavonoids of *Rosemarinus officinalis* and *Altheae officinalis* are able to activate PPARɣ which leads to inhibition of COX-2 over expression (that prevents apoptosis and induce cell proliferation); rosemarinic acid that exist in *Rosemarinus officinais* extract is able to do it as well by decreasing of COX-2 inhibition [34] but in another study, a combination of ethanolic extract of some herbs such as *Rosemarinic acid* and *Altheae officinalis* an increase in dermal pailla cell proliferation of human hair follicle was observed. While in another study showed that ethanolic extract of *Rosemarinus officinalis* and *Altheae officinalis* have positive effect on viability and proliferation of dermal papillae cells of human hair follicle; based on this study these herbal extracts have significant effects on expression of CyclinD1, Cdk4, Erk, Akt, Bcl-2 and Bax proteins in cel culture of human dermal papillae; It is suggested that activation of Akt and Erk pathways lead to this or maybe it is due to increase in expression of Cdk4 and CyclinD1 [35] which is the checkpoint of G1/S as the regulator of cell cycle [36]; the role of signaling pathway of Akt on mitogenesis and cell growth is previously showed [37] but maybe the antioxidant content of such herbal extracts helps to save the cellular energy therefor cell can focus on its vital activity such as proliferation and mitosis, gene expression and transcription instead of fighting to free radicals while it is previously showed that Akt has a key role on cell viability signals [38, 39].

Conclusion

Stem cell was isolated from human hair follicle tissues by noninvasive means. These results suggest that herbal extract may produce positive effects on the hair growth promotion Overall; we have demonstrated the potential hair growth promoting effect of herbal extracts, suggesting that herbal extracts may be a good candidate for hair follicle stem cells proliferation and helping hair growth promotion.

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