Research Article

SesZen-Bio™: A Novel Modulator of Wnt/β-Catenin Signalling for Promoting Hair Growth and Follicular Regeneration

Ratna Upadhyay*, Mihir Gadani, Sneha Badak
Department of Phytochemistry, Zenherbs Lab Pvt Ltd, Mumbai, India.
***Corresponding author's E-mail:** ratna.upadhyay@fitcircle.in

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ABSTRACT

SesZen-Bio™, a proprietary extract, is obtained from *Sesbania grandiflora* leaves and is implicated in having a beneficial effect on hair growth but its mechanism of action is unknown. The present study aims to evaluate the SesZen-Bio™ effects on the growth and proliferation of Outer Root Sheath Cells and Dermal Papilla cells.SesZen-Bio™ effect on *in vitro* hair cell proliferation was evaluated by MTT assay and metabolic activity of DPCs by cell viability ATP assay. To characterize and further understand the mechanism of action of ORSCs and DPCs, gene expression analysis was performed by Real-time polymerase chain reaction . Hair growth analysis was performed using *ex-vivo* hair culture (Philpott test). The isolated ORSCs were shown to express *CK5, CK10,* and *CK15* and DPCs expressed *BMP4, Vcan,* and *Lef1.* The treatment with 10 µg/ml SesZen-Bio™ resulted in an increase in ORSCs proliferation, an increase in *Wnt, ß-catenin, Sox9,* and *CK15* expression respectively, and ATP production in DPCs, compared to the respective control group. The *ex vivo* organ culture of a complete hair strand showed that ten days of 10 µg/ml SesZen-Bio™ treatment significantly increased hair shaft length when compared to the control group. SesZen-Bio™ increases hair cell growth *in vitro* and *ex vivo* possibly by activation of the Wnt/β-catenin signalling and stem cell differentiation pathways. These favorable results indicate that the formulation can be used as a potential candidate for hair growth formulation.

Keywords: Hair growth, Dermal Papilla, Alopecia, SesZen-Bio™, Outer Root Sheath Cells.

INTRODUCTION

Alopecia or hair loss can happen because of many factors, such as hormonal dysregulation, dietary habits, environmental and genetic [1]. Androgenetic alopecia (AGA) is one of the most common alopecia known as male/female pattern baldness[2]. Many people are suffering from it and it's becoming more and more serious with ages. Although it is not deathly but causes emotional and physiological distress in patients. In AGA, over time the size of hair is shortened because of a decrease in the anagen phase and an increase in the telogen phase, and hair becomes thinner, eventually leading to permanent loss of hair [3,4]. For alopecia treatment, minoxidil is widely used as an FDA-approved drug but on the contrary, because of their side effects such as skin irritations with the typical symptoms of itching and scaling, increase in hair growth in untreated areas like the face, arms, and legs, sodium and fluid retention, and cardiovascular effect does not justify the use of it [5]. Therefore, more efficient and safe treatments are needed. SesZen-Bio™, a proprietary extract from Zenherb Labs Pvt. Ltd. in Mumbai, India, is obtained from *Sesbania grandiflora* leaves, commonly known as Agati. The HPLC study showed that SesZen-Bio™ contains 0.5 % biotin, antioxidants, and other cofactors. Clinical studies have shown that SesZen-Bio™ is well-tolerated and effective in enhancing hair health, and keratin production, improving hair density, and thickness [6,7].

After many decades of research, it was found that the Dermal Papilla cells (DPCs) in the bulb region and epithelial stem cells in the bulge region help in maintaining the hair cell cycle and growth [8]. The DPCs in *in vitro* culture are widely used for hair growth studies. DPCs are located in the bulb region at the bottom of the hair follicles, they are the specialized mesenchymal cells, that play an important role in hair growth by interacting with their microenvironment including Outer Root Sheath Cells (ORSCs) [9]. The ORSCs of hair follicles are made up of several multipotent stem cell populations and shown to produce fibroblast growth factor-5, which can partially regulate the duration of the anagen phase of the hair growth cycle and can also migrate out of the follicle to help regenerate the epidermis [1011,12].

The hair development and growth are a complex interaction of physical factors and signaling pathways. Importantly, the Wnt/β-catenin signaling pathway has been implicated to have a critical role and influence in the development of hair follicles and hair growth [13–15]. Downstream to this pathway, Lymphatic enhancer factor 1 (Lef1), a transcription factor, plays an important role in cell proliferation, apoptosis, bulge stem cell differentiation, and regulating the interaction between epithelial and mesenchymal cells [16,17]. The bulge region of the hair follicle is enriched with multipotent stem cells and the destruction of these cells is linked to irreversible hair loss [18]. The *Sox 9* expression is also seen to be associated with stem cell differentiation [19].

Although, Computational studies were conducted to predict the molecular interactions of SesZen-Bio™ components, particularly biotin, in promoting hair health [20]. The present study evaluated the effect of SesZen-Bio™ with regard to functional assessments on ORSCs and DPCs and its overall impacts on hair growth in the *in vitro* and *ex vivo* settings.

MATERIALS AND METHODS

ORSCs and DPCs isolation and culture

Post consent, healthy human hairs were plucked from the occipital area of the scalp and immediately processed for ORSCs and DPCs isolation. Intact hair follicles (HFs) in the anagen phase were selected under a dissection microscope.

The ORSCs were isolated using the enzymatic digestion method, as previously described, with some modifications[21]. The plucked hair from the head occipital region was washed twice with Hank's balanced salt solution with antibiotics and antifungal. The hair shaft and lower bulb were removed and the middle ORSCs region was treated with 3 ml of trypsin (0.25%) for 15 mins at 37 °C with intermediated shaking. After incubation, trypsin was neutralized with 10 ml of 10 % Dulbecco's Modified Eagle Medium (HiMedia, India). The suspension was centrifuged at 300 g for 5 mins after vigorous pipetting. The obtained pellet was resuspended in serum-free DMEM: F12 (3:1) supplemented with 10 µg/ml insulin, 10 ng/ml EGF, 10 ng/ml bFGF, and 100 ng/ml hydrocortisone and incubated in 5 % Co_2 incubator at 37 °C and humified air.

The DPCs were also isolated by enzymatic digestion with some modifications [22]. Hair bulbs dissected from the plucked hair were treated with trypsin (0.25%) for 15 min at 37°C. After incubation, the cell suspension was pipette up and down to lose the DP cells and further, treated with trypsin for 15 min. After that dissociated DP cells were pelleted down by centrifugation at 180 g for 3 min and maintained in serum-free DMEM: F12 (3:1) supplemented with 10 µg/ml insulin, 10 ng/ml EGF, 10 ng/ml bFGF, and 100 ng/ml hydrocortisone and incubated in 5 % Co_2 incubator at 37 °C and humified air.

Thiazolylblue (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) (MTT) assay

The cell viability of ORSc was assessed by MTT assay, as previously described by Carolin P [23]. 4 x 10^3 cells/well were seeded in a 96-well plate, and incubated for 16 h. After that cells were treated either with 10 or 50µg/ml of SesZenBio™ and 15ng/ml of EGF was used as a standard/positive control for 24 h. Post 24 h-drug treatment, 5 mg/ml MTT reagent was added for 4 hr at 37°C. The MTT solution was removed and 100 µl of isopropanol with 4mM HCL, was added to each well to dissolve the formazan crystals by gentle shaking for 20 minutes and measured OD at 570 nm by cytation5 plate reader. Cell viability (%) was determined by using the following formula- Cell Viability % = [OD (Treated) / OD (control)] * 100.

Quantitative real-time polymerase chain reaction (RT-PCR)

The ORSCs and DPCs were seeded in 24 well plates at a seeding density of 50 x 10^3 cells/well and after 16 h of incubation, ORSCs were treated with 10 and 50 µg/ml of SesZenBio™ for 24 h. The total mRNA was isolated from SesZenBio™ treated ORSCs and non-treated ORSCs and DPCs by GET Total RNA isolation kit (G-Biosciences, USA) according to manufacturer protocol. RNA quantified using NanoDrop 1000. The cDNA was synthesized for all treated groups according to the manufacturer (G-Biosciences, USA) protocol. To quantify the altered mRNA expressions due to SesZenBio™ treatment, RT-PCR was performed (Agilent, USA) using SYBR® Green based qPCR 2X master mix (G-Biosciences, USA) according to the manufacturer's instructions and using cycle conditions of 95° C for 2 min, followed by 40 cycles of 95° C for 10 s, 60° C for 20 s 72°C for 15 sec, and 25°C for 5 mins. qPCR-based relative gene expression data were evaluated by $2^{-\Delta\Delta CT}$ method and for characterization, $2^{-\Delta CT}$ method was used. For normalization, Beta-actin was used. The primer sequences are given in Table 1.

Table 1: Primer list of genes used in the study.

Gene Name	Forward Prime (5'- - -3')	Reverse Primer (5'- - -3')
β-actin	ATGATATCGCCGCGCTCG	CGCTCGGTGAGGATCTTCA
CK10	GGTGGTGGATTTGGAGGAGA	TCTTCCAGAGCCCGAACTTT
CK15	AGTGGATGGACAGGTGGTTT	CTGATGAGAGTGGGGAGTGG
CK5	GCTGACACGAGAACCCAAAG	ATTGGGGTGGGGATTCTGTT
SOX9	AGACAGCCCCCTATCGACTT	TAGGAGGGGCTGTAGTGTGG
Wnt10b	CATCCAGGCACGAATGCGA	CGGTTGTGGGTATCAATGAAGA
β-catenin	GCTGGTGACAGGGAAGACAT	CCATAGTGAAGGCGAACTGC
Lef1	CTTCCTTGGTGAACGAGTCTG	TCTGGATGCTTTCCGTCAT
BMP4	GCCCGCAGCCTAGCAA	CGGTAAAGATCCCGCATGTAG
Vcan	GGCAATCTATTTTACCAGGACCTGAT	TGGCACACAGGTGCATACGT
Bcl2	TCCGCATCAGGAAGGCTAGA	AGGACCAGGCCTCCAAGCT

Cell Titer-Glo luminescent cell viability assay

ATP Assay was performed to assess the metabolic activity of DPCs. Assay were performed from the Cell Titer-Glo® 2.0 assay kit according to manufacture protocol (Promega, USA). DPCs were seeded in 96 well plates in 2 x 10^3 density and were treated with bFGF (10 ng/ml) (standard/positive control), 10 and 50 ug/ml conc. of SesZenBio™ and incubated for 48 h. After incubation, 100µl of Cell Titer-Glo® 2.0 Reagent was added to 100µl of medium-containing cells and mixed for 2 min on a shaker to induce cell lysis. Further plate was incubated for 10 min to stabilize the luminescent signal. Luminescence was recorded in the luminescence channel on the Cytation5 multi-plate reader (Agilent, USA). Relative % Luminescence was calculated by normalizing untreated control as 100%.

Ex vivo culture of human hair follicle

The overall experiments were performed as described by Philpott *et al* with some modifications [24]. Post consent, intact plucked hairs from male donors suffering from alopecia were maintained in an individual wells of 24-well plates containing DMEM: F-12 media (3:1) with 10 µg/ml insulin, 10 ng/ml hydrocortisone, 10 ng/ml EGF, at 37 °C and 5% CO_2. Respective wells were treated with the 0.8 % SesZen-Bio, and 1% Minoxidil alongside control groups. Individual group-specific hair shaft length was measured across the groups at different time points (Day 0 and Day 10). The apical hair length differences were observed at day 10 (compared to day 0) among various treatment groups and were presented in terms of % hair growth change (in µm) format.

Statistical analysis

GraphPad Prism (version 8, GraphPad, USA) was used to analyze the data and to calculate the statistical significance. Student's t-test and one-way ANOVA were used for statistical significance and all quantitative data are presented as the mean ± SEM All data were from at least three independent experiments. The * $p \leq 0.03$, ** $p \leq 0.002$ and ***$p \leq 0.001$ were considered significant.

RESULTS

Isolated ORSCs and DPCs express respective signature genes

To evaluate the effect of SesZenBio™ on ORSCs and DPCs in hair growth, first, the isolated HF cells from plucked hairs were characterized by gene expression analysis of ORSCs and DPCs by RT-PCR. The ORSCs were characterized by signature markers, expressed in the middle ORScs region in the hair follicle, such as *CK 15, CK 5*, and *CK 10* [10,25,26]. and DPCs were characterized by signal transduction components (*BMP4*), transcription factors (*Lef1*), and extracellular adhesion gene (*Vcan*) [27]. As evident from Fig.1, all the signature marker expressions were prominent in the ORSCs and DPCs indicating the enrichment of isolated cells as ORSCs and DPCs respectively.

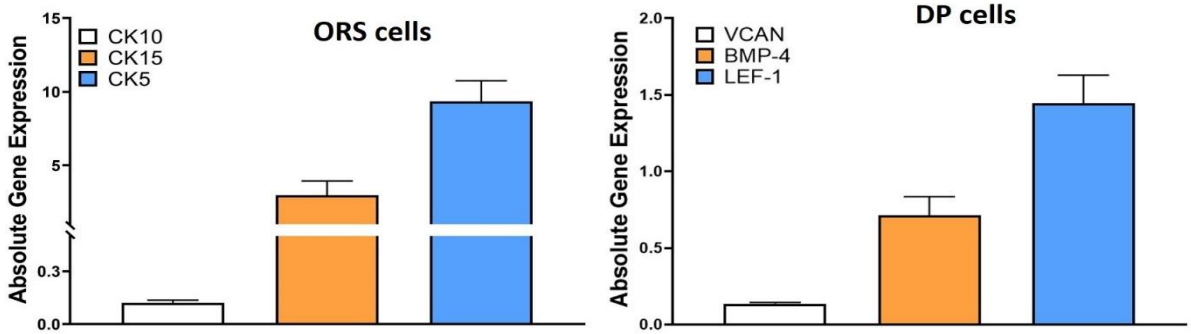

Figure 1: Characterization of ORSCs and DPCs isolated from Plucked hair. RT-PCR analysis of (A) ORSCs and (B) DPCs signature gene expression. B- actin was used as an internal control. Data are the mean ± SEM from three independent experiments.

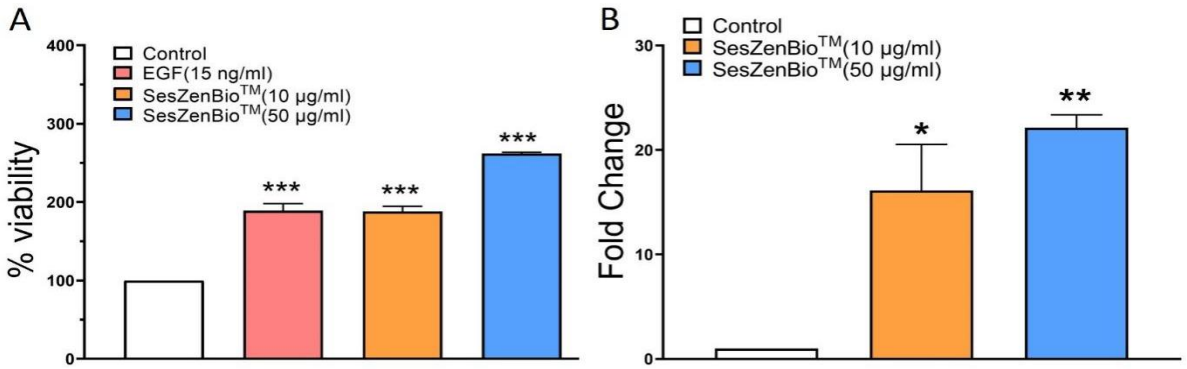

Figure 2: Effect of SesZenBio™ on ORSCs proliferation and apoptosis. 4 x 10^3 cells were seeded and treated with SesZenBio™ for 24 h and (A) viability was measured by MTT assay and (B) apoptosis by RT-PCR**.** The results are represented as the average of three independent experiments ± SEM performed in triplicates. Statistical significance (* $p \leq 0.03$, ** $p \leq 0.002$ ***, $p \leq 0.001$) was calculated with respect to the control group.

SesZenBio™ stimulated ORSCs proliferation and inhibited Apoptosis

To evaluate the effect of SesZenBio™ in the proliferation of ORSCs, respective cells were treated with 10 and 50 µg/ml concentrations and viability was assessed by MTT assay. As evident from Fig.2, treatment with both the concentration of SesZenBio™ increases the % viability of ORSCs. Both 10 and 50 µg/ml SesZenBio™ significantly ($p < 0.002$) increase the % viability to 187.8 ± 6.7 and 261.8 ± 1.6, respectively. It further demonstrated a dose-dependent increase in cell proliferation. Interestingly, both 10 and 50 µg/ml SesZenBio™ were also found to maintain the ORSCs proliferation at least or more to the level of the epidermal growth factor (EGF) treatment, a positive inducer for proliferation. The SesZenBio™ did not exhibit any cytotoxic effects on ORSCs and as a result increased the viability of ORSCs, which can help in hair growth. Further, we evaluated the impact of SesZenBio™ treatment on ORSCs apoptosis. The RT PCR result, Fig.2B, showed that both 10 and 50 µg/ml conc. significantly ($p \leq 0.03$) increase the expression of the *Bcl2* gene, encoding for anti-apoptotic functions, to 23.4 ± 7.9 and 18.9 ± 3.3-fold, respectively. This indicates that SesZenBio™ not only increases the proliferation it also helps in anti-apoptotic functions in ORSCs, which may positively influence hair growth.

SesZenBio™ promotes metabolic activity in DPCs

To evaluate the metabolic impacts of SesZenBio™ on DPCs, 10 and 50 µg/ml SesZenBio™ was treated on DPCs for 24 h and ATP production was measured, representing the cell metabolic activity. First, at 10 µg/ml treatment increased ATP production to 177 ± 3.5 %, whereas bFGF, as positive control, also increased ATP production by 114.3 ± 4.3 % as compared to the respective control group. However, 50 µg/ml treatment did not show any significant alterations in ATP production (Fig.3). Hence, at least 10 µg/ml treatment of SesZenBio™ shown to promote ATP production in DPCs, which may further contribute to DPCs proliferation and hair growth.

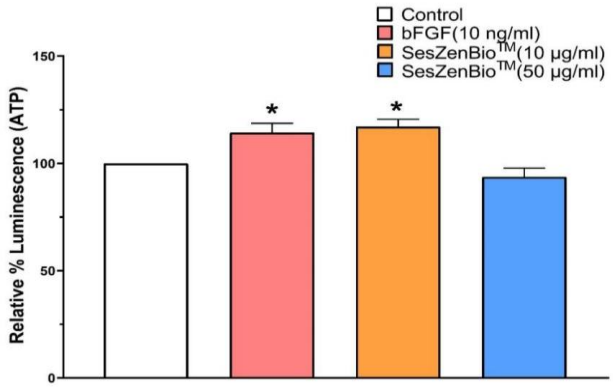

Figure3. Effect of SesZenBio™ on metabolic activity. 2 x 10^3 DP cells were seeded and treated for 24 h with different concentrations of SesZenBio™. The results are represented as the average of three independent experiments ± SEM performed in triplicates. Statistical significance (* $p \leq 0.03$) was calculated with respect to the control group.

SesZenBio™ treatment activated the Wnt/β-catenin signalling pathway

Wnt/β-catenin signaling is a master regulator of hair cell (such as hair matrix cell, outer root sheath cell (ORSC), and derma papilla cell) proliferation and promotes cell-cell interaction [28]. To determine the role of SesZenBio™ in Wnt/β-catenin signalling, RT-PCR was performed to check the expression levels of *Wnt10b, β-catenin*, and *Lef1* after treatment with 10 and 50 µg/ml concentration of SesZenBio™ for 24 h. The results of the RT-PCR analyses revealed dose-dependent increased expression of *Wnt10b*, and *β-catenin* in the SesZenBio™ - treated group (Fig.4). The 10 µg/ml treatment significantly ($p \leq 0.001$) increased the *Wnt 10b* expression 2.0 ± 0.3-fold and *β-catenin* ($p \leq 0.03$) 1.6 ± 0.1-fold as compared to control and 50 µg/ml treatment increase ($p \leq 0.001$) the expression 2.3 ± 0.2 and 2.4 ± 0.2-fold respectively. This indicates that SesZenBio™ helps in ORS cell differentiation and growth. On the contrary, the same treatment with SesZenBio™ for 24 h did not show any significant alteration in *Lef1* mRNA expression.

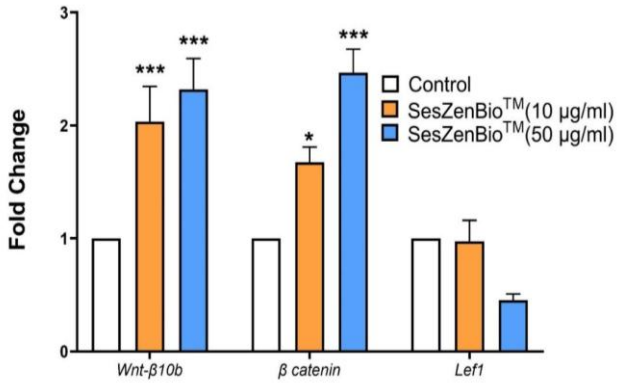

Figure.4: Effect of SesZenBio™ on Wnt/β-catenin signalling pathway. The ORSCs were treated with different concentrations for 24 h and relative mRNA expressions of *Wnt10b, β-catenin,* and *Lef1* were determined by RT-PCR. The results are represented as the average of three independent experiments ± SEM performed in triplicates. Statistical significance (***, $p \leq 0.001$, * $p \leq 0.03$) was calculated with respect to the control group.

SesZenBio™ promotes stem cell proliferation and differentiation

The CK15 is the most prominent marker for the ORS stem cell population and Sox 9 expression shown to play an important role in the hair cycle, hair formation, and maintenance of stem cell lineage [18,19]. The gene expression analysis by RT-PCR exhibited that after treatment with SesZenBio™, the expression of *CK15,* stem cell marker for ORSCs [29], was increased ($p \leq 0.001$) in a dose dose-dependent manner from 1.92 ± 0.1 to 3.69 ± 0.4-fold for 10 (non-significant) and 50 µg/ml ($p \leq 0.001$) as compared to the respective control group. This indicates that SesZenBio™ induces the proliferation of the stem cell population in ORSCs (Fig. 5). To further evaluate the differentiation potential, *Sox9* expression was also analyzed and as an evident treatment with 10 µg/ml SesZenBio™

significantly increased the *Sox9* expression by 2.83 ± 0.4-fold ($p \leq 0.002$); however, 50 µg/ml treatment shown only 1.47 ± 0.2-fold change (non-significant). The gene expression study reveals that SesZenBio™ helps in hair growth promotion by stimulating stem cell proliferation and differentiation in ORSCs.

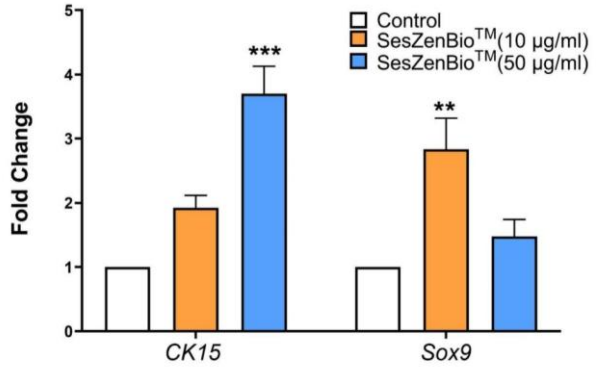

Figure.5. Effect of SesZenBio™ on stem cell proliferation and differentiation. The ORSCs were treated with different concentrations of SesZenBio™ for 24 h and relative mRNA expression levels of *CK15* and *Sox9* in human ORSCs, were determined by RT-PCR. The results are represented as the average of three independent experiments ± SEM performed in triplicates. Statistical significance (**, $p \leq 0.002$, *** $p \leq 0.001$) was calculated with respect to the control group.

SesZenBio™ increases hair shaft length in *ex vivo* hair growth model

To investigate whether SesZenBio™ promotes hair shaft elongation, the isolated intact hair was grown *in vitro* and treated with either 0.8 % SesZenBio™ or 1 % Minoxidil. After 10 days of treatment, hair shaft length was increased to 21.8 ± 2.5, 36.4 ± 3.5, and 41.4 ± 0.3 µm in control, minoxidil and SesZenBio™, respectively (Fig.6). A significant % ($p \leq 0.03$) increase in hair growth of 66.9 % and 89.9 % in the minoxidil and SesZenBio™ treated group was observed when compared to the control group.

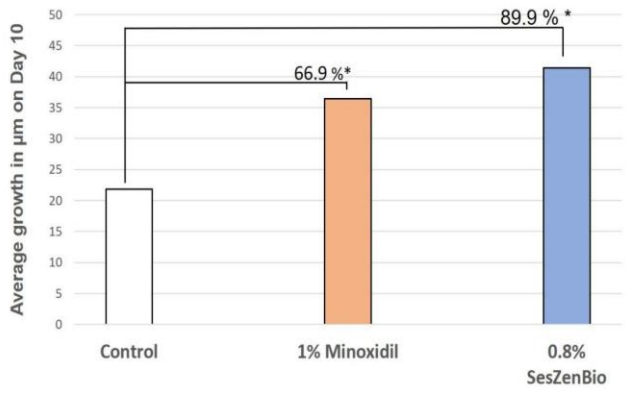

Figure.6: Hair growth in an *ex vivo* FC model. The results are plotted by hair growth in µm and as percentage differences in hair growth observed between day 0 and day 10, post-drug exposures. The results are represented as the Mean ± SEM. Statistical significance (* $p \leq 0.03$) was calculated with respect to the control group

DISCUSSION

The present study shows the potential of SesZenBio™, a trademark plant extract, as an effective alternative to conventional treatments for promoting hair growth and its impact on HF cells, specifically the ORSCs and DPCs, which play crucial roles in hair growth regulation [30].

The MTT-based cell viability results demonstrated that SesZenBio™ treatment significantly increases the viability of ORSCs in a dose-dependent manner. The upregulation of the anti-apoptotic *Bcl2* marker showed its potential role to protect ORSCs from apoptosis and further increased metabolic activity in DPCs, as indicated by the increased ATP production after treatment with SesZenBio™, which provides further evidence of its efficacy in improving overall survival of hair growth. The Wnt/β-catenin signaling pathway well-known regulator of hair follicle development and regeneration [31]. The extracts of several plants have been shown to increase the expression of WNTs and β-catenin levels [32,33]. The downstream, β-catenin is shown to interact with LEF1 and regulate the transcription of various target genes involved in hair growth [34]. The dose-dependent upregulation of *Wnt10b* and *β-catenin* gene expression suggests that SesZenBio™ can effectively stimulate HF cells to enter and remain in the growth phase, thereby promoting overall hair growth. Interestingly, while SesZenBio™ significantly increased the expression of these key signaling molecules, it did not affect the downstream *Lef1* expression, which demands further investigation of the downstream signaling pathway for a better understanding of the molecular mechanism. The study also demonstrated that SesZenBio™ promotes stem cell proliferation and differentiation in ORSCs, as evidenced by the increased expression of *CK15* and *Sox9*. These markers are indicative of stem cell proliferation and differentiation, which is essential for maintaining hair growth.

The plant extract from *Cucumis melo*, *Orthosiphon stamineus*, and *Panax ginseng* shown to promote hair shaft growth in *ex vivo* hair follicle organ culture providing further supportive evidence of plant-based extract in the promotion of hair growth [35,36]. In our study also, the *in vitro/ex vivo* hair growth model provided compelling evidence of SesZenBio™ efficacy. The significant increase in hair shaft length compared to both the control and minoxidil-treated groups highlights its potential as a treatment option for hair growth.

CONCLUSION

The results of this study suggest that SesZenBio™ is a promising candidate for hair growth treatment. Its ability to enhance cell survival and stem cell differentiation through activation of the Wnt/β-catenin signalling pathway positions it as a potent and potentially safer alternative to existing treatments. The present study findings and the previous relevant clinical data provide compelling evidence about the effectiveness of SesZenBio™ for hair growth as a treatment option.[6]

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Conflict of Interest: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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