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Article

Treatment with *Malva verticillata* seed extracts alleviates alopecia via activation of Wnt/β-catenin signaling

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Abstract: Hair loss attributed to excessive stress from work and lifestyle changes has become a growing concern, particularly among young individuals. However, currently used drugs such as minoxidil and finasteride impose a plethora of side effects. Therefore, natural substances as alternatives have garnered research interest. A recent study showed the efficacy of Malva verticillata seed extracts in alleviating hair loss via upregulation of the Wnt/β-catenin signaling pathway. In this study, the efficacy of M. verticillata seed extracts on alleviation of hair loss was further investigated. Further fractionation and purification of the seed extracts using silica gel column chromatography and preparative high-performance liquid chromatography helped identify linoleic acid (LA) and oleic acid as the major bioactive components. LA insufficiency is reported to cause hair loss. However, its mechanism of action is not clearly known. Here, we explored the efficacy of LA treatment on preventing hair loss and its underlying mechanism of action. We found that LA treatment activated Wnt/β-catenin signaling and induced dermal papilla cell (DPC) proliferation in cell proliferation assays. Moreover, it increased the expression of cell cycle proteins such as cyclin D1 and cyclin-dependent kinase 2. LA treatment also increased the expression of vascular endothelial growth factor, insulin-like growth factor-1, hepatocyte growth factor, and keratinocyte growth factor in a concentration-dependent manner and significantly inhibited that of DKK-1, induced by dihydrotestosterone. These findings suggest that LA treatment induces hair growth by increasing DPC proliferation and alleviates androgenic alopecia by activating Wnt/β-catenin signaling in DPCs.

Keywords: Malva verticillata; linoleic acid; proliferation; growth factor; dihydrotestosterone

1. Introduction

Good quality hair, in addition to protecting the body and scalp, enhances appearance. As society is being increasingly modernized, hair loss due to stress from work, incorrect eating habits, and exposure to harmful environments is becoming a serious issue, and interest in treating hair loss is also increasing [1]. Furthermore, it has been recently reported that excessive stress and use of chemical products lead to increased hair loss in individuals in their 20s and 30s [2]. Hair loss depends on how hair progresses through its growth cycle and falls out after growth has stopped [3]. Various factors, including genetic factors, excessive secretion of male hormones and sebum, and aging, are also involved in abnormal hair loss [4, 5].

Hair is produced in hair follicles and undergoes a repetitive growth cycle comprising the following four stages: anagen, catagen, telogen, and exogen [5-7]. The cycle duration varies

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depending on the site of hair production, but typically, it lasts for 2 to 8 years [8]. The hair growth cycle of a healthy person is repeated 10 to 15 times, and an average of 50–100 hairs are lost per day [9]. Dermal papilla cells (DPCs), mesenchymal cells located at the bottom of the hair follicle, are the key cells involved in hair growth and cycle regulation, supplying nutrients to hair follicles and regulating hair growth by inducing the expression of growth factors and inhibitors for hair proliferation [10, 11]. At the molecular level, the Wnt/◎-catenin signaling pathway is essential in maintaining the hair-inducing activity of DPCs [12].

Hair loss treatments using Food and Drug Administration-approved medications like minoxidil and finasteride impose several side effects [13-18]. Therefore, natural substances as alternatives and safe substitutes have garnered research interest. Of the several herbal resources, Malva verticillata seed extracts are used as laxatives and diuretics [19, 20]. In addition, a recent study showed their efficacy in alleviating hair loss via upregulation of the Wnt/◎-catenin signaling pathway [19].

Vegetable oils derived from the seeds of M. verticillata, flaxseeds, hemp seeds, and sesame seeds, along with nuts, sunflowers, corn, and soybeans, are rich in linoleic acid (LA) [21]. LA is an essential polyunsaturated fatty acid used for the biosynthesis of arachidonic acid and is primarily present in cell membranes [22]. It is known for its wound-healing and anti-cancer properties [23-25]. In addition, it offers lymphocyte protection and resistance against arteriosclerosis [26, 27]. Moreover, conjugated LA, a geometric isomer of LA, is known to be beneficial to the human body as it reduces body fat content, has anti-cancer properties, and suppresses arteriosclerosis and diabetes [28-30]. Thus, it is essential to consume LA through food intake. An insufficient amount of LA is reported to cause hair loss. However, its mechanism of action is not clearly known.

Therefore, in the present study, we isolated the bioactive components of M. verticillata using several organic solvents and investigated the mechanism of inhibition of hair loss by LA, identified as the major active compound of the M. verticillata seed extracts.

2. Results

2.1. M. verticillata seed extraction and active ingredient separation

Compounds 1 and 2 were obtained using preparative high-performance liquid chromatography (CH_3OH:H_2O = 70:30) for the MH2 fraction among the five fractions (MH1–5) fractionated using silica gel column chromatography on the n-hex fraction of dried *M. verticillata* seeds (Figure 1). The two separated compounds were structurally identified by comparing the reported literature [32, 33] and the spectrum data obtained through nuclear magnetic resonance analysis. For the purpose of re-confirm the compounds by co-eluting with the fatty acid standard, the retention times of compound 1 and 2 were finally identical to LA and oleic acid on HPLC chromatogram. Compounds 1 and 2 were identified as LA and oleic acid, respectively.

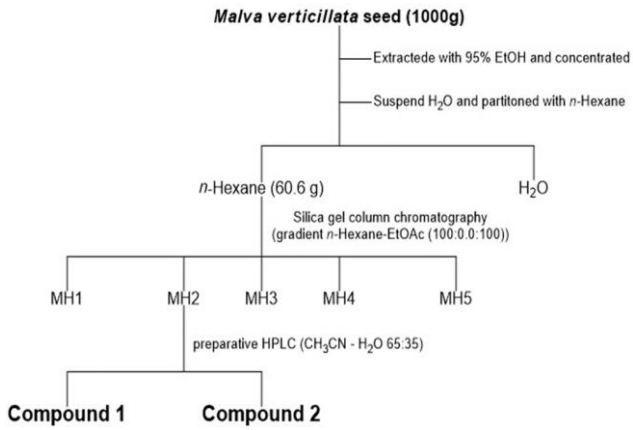

Figure 1. Isolated compounds from *Malva verticillata* seed

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2.2. Effects of M. verticillata extract and LA treatment on hair DPC proliferation

We next assessed the inhibitory effects of treatment with extracted M. verticillata and the *n*-hex fraction on the proliferation of HFDPCs. M. verticillata seed extract treatment significantly increased cell proliferation by 10.42% at 100 μg/mL concentration ($p < 0.5$), whereas the n-hex fraction showed significant effects at 30 μg/mL concentration and increased cell proliferation by up to 26.62% at 100 μg/mL ($p < 0.5$, Figure 2). Furthermore, LA treatment at 10 μg/mL showed a significant increase in cell proliferation, which was increased by up to 21.46% at 30 μg/mL concentration ($p < 0.5$, Figure 2). Additionally, there was no significant effect in response to 100 ug/mL of oleic acid (Figure 2). These findings suggest that the efficacy of *M. verticillata* seed extract in increasing hair cell proliferation is mediated by LA.

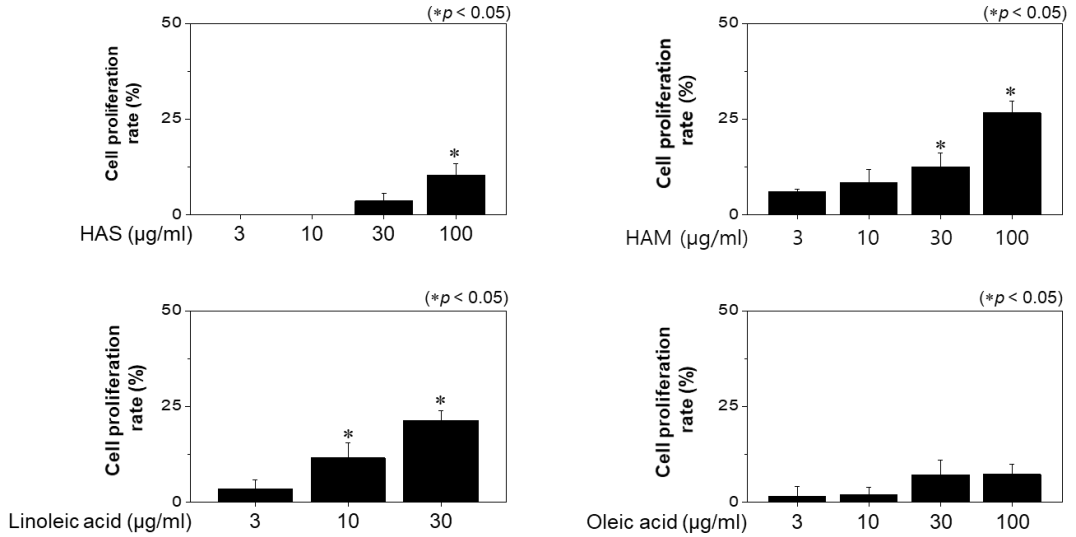

Figure 2. Effect on hair cell proliferation. HFDPC cells were treated with *M. verticillata* extract, hex fraction, compound 1, and compound 2 for 48 h. Cell proliferation was assessed by MTT assay and absorbance was measured by 550 nm (black bar). Significance was determined compared to untreated cells (*$p < 0.05$). All data are expressed as mean ±SD of three separate experiments performed in triplicate.

2.3. Effects of LA treatment on the Wnt/β-catenin pathway

The Wnt/β-catenin pathway regulates various physiological phenomena in cells and plays an important role in regulating the proliferation of DPCs [12]. Assessment of the effects of LA treatment on the Wnt/β-catenin pathway revealed a significant increase in the phosphorylation of GSK-3 at 10 μg/mL concentration (Figure 3A). LA treatment also sequentially increased β-catenin expression in the cytoplasm in a concentration-dependent manner (Figure 3A). Thus, it was inferred that LA might induce cell proliferation by activating the Wnt signaling pathway.

Activation of the Wnt/β-catenin pathway leads to the expression of various genes involved in the cell cycle, proliferation, and survival . The effects of LA treatment on the cell cycle were evaluated using RT-PCR. The cell cycle is controlled by cyclin–cyclin-dependent kinase (CDK) protein complexes. LA treatment increased the expression levels of cyclin D and CDK2, the two key proteins involved in regulation of the cell cycle, in a concentration-dependent manner (Figure 3B). These results suggest that LA regulates the proliferation of DPCs through modulation of the cell cycle.

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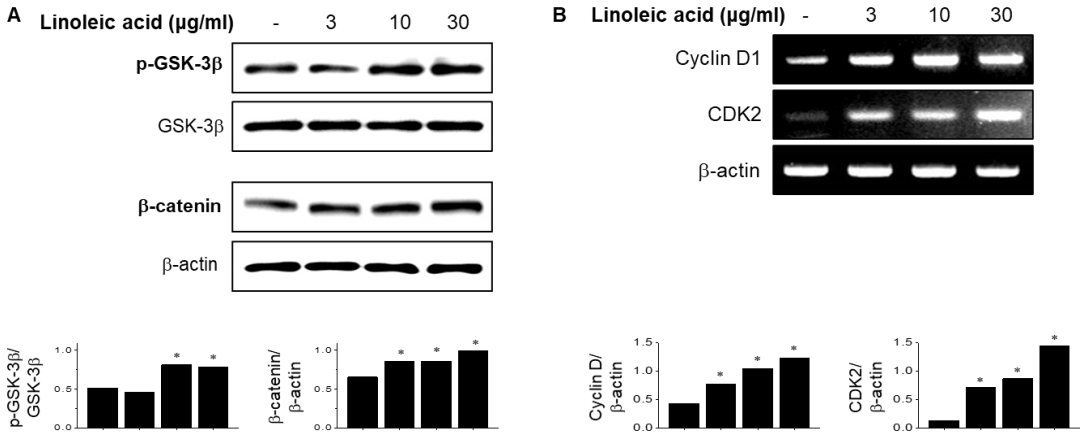

Figure 3. Effect of linoleic acid on Wnt/β-catenin signaling and cell cycle. (A) HFDPC cells were treated with linoleic acid for 6 h. Total cell extracts were blotted with GSK-3β and β-catenin antibodies. The mRNA levels of VEGF were measured using RT-PCR(B). Band intensities were quantified using ImageJ 1.47 software and normalized to non-phosphorylated form or β-actin. Significance was determined compared to untreated cells (*$p < 0.05$). All data are expressed as mean ±SD of three separate experiments performed in triplicate.

2.4. Effects of LA treatment on expression of hair growth factors

Growth factors such as vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), and fibroblast growth factor are involved in the growth and differentiation of hair papilla cells and regulate new hair formation. Fibroblast growth factor and IGF-1 promote hair growth by inducing follicle tissue growth and hair follicle cell proliferation, and VEGF promotes hair growth by inducing blood vessel formation to supply nutrients to hair follicle cells. RT-PCR and western blotting were performed to evaluate the effects of LA treatment on hair growth factors (Figure 4). LA significantly increased both the gene and protein expression levels of VEGF. Furthermore, the expression of IGF-1, hepatocyte growth factor, and keratinocyte growth factor was also increased in a concentration-dependent manner. These findings suggest that LA treatment promotes hair growth by inducing the expression of growth factors.

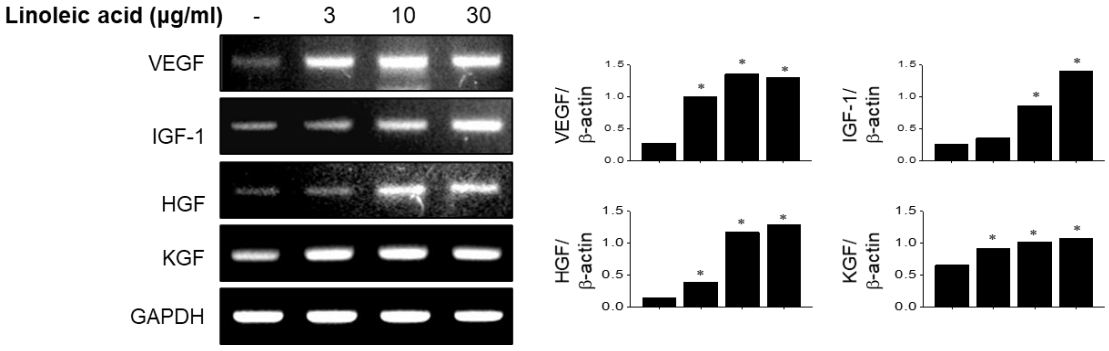

Figure 4. Effect of linoleic acid on growth factor expression. HFDPC cells were treated with various concentrations of linoleic acid for 6 h. Total cell extracts were blotted with VEGF and β-actin antibodies. The mRNA levels of growth factor were measured using RT-PCR. Band intensities were quantified using ImageJ 1.47 software and normalized to β-actin or GAPDH. Significance was determined compared to untreated cells (*$p < 0.05$). All data are expressed as mean ±SD of three separate experiments performed in triplicate.

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2.5. Inhibitory effects of LA treatment on androgenic alopecia

Androgenic alopecia is caused by excessive production of dihydrotestosterone, converted from testosterone, a male hormone, by 5β-reductase. It is caused by various factors such as genetic factors, stress, and lifestyle imbalance. When dihydrotestosterone binds to the androgen receptor, dickkopf-related protein (DKK)-1 is produced, inducing hair cell death and inhibition of Wnt/β-catenin signaling, which leads to hair loss. LA treatment significantly inhibited the expression of DKK1 increased by dihydrotestosterone treatment at a concentration of 30 μg/mL(Figure 5). DKK-1 protein encoded by the DKK1 gene antagonizes the Wnt/β-catenin signaling by inhibiting the Wnt coreceptors. Thus, it can be concluded that LA treatment could also be effective against hormone-induced androgen hair loss.

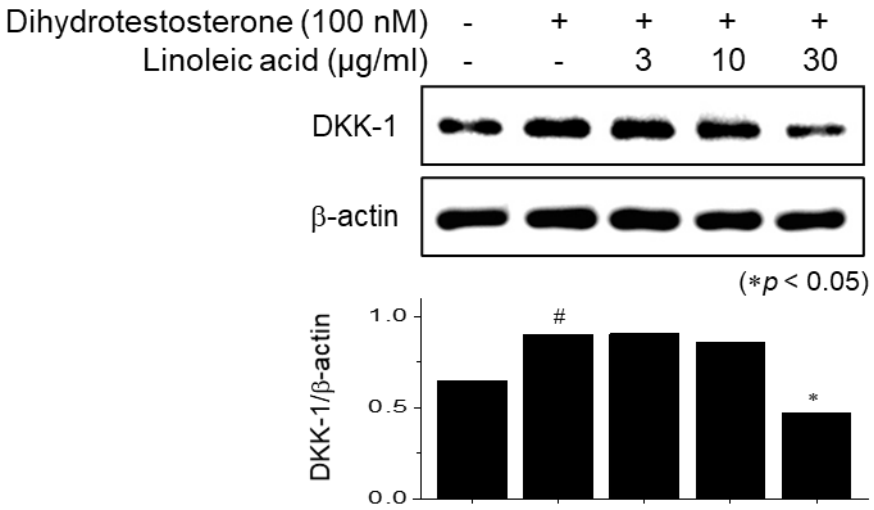

Figure 5. Effect of linoleic acid on dihydrotestosterone-induced hair loss mechanism. HFDPC cells were stimulated with dihydrotestosterone (DHT) for 2 h and treated with various concerntrations of linoleic acid for 6 h. Total cell extracts were blotted with DKK-1 and β-actin antibodies. Band intensities were quantified using ImageJ 1.47 software and significance was determined compared to DHT-treated cells (*$p < 0.05$). All data are expressed as mean ±SD of three separate experiments performed in triplicate.

3. Discussion

Hair loss is part of the normal growth cycle of hair and involves falling out of hairs that have completed their growth. Hair loss, which was traditionally a concern for the elderly, is now also often observed in younger age groups due to excessive stress, changes in dietary habits, and environmental factors. The most important mechanism underlying abnormal hair loss is dysregulation of the proliferation of DPCs that produce hair. DPCs are special mesenchymal cells that play a key role in hair growth. They exist in hair follicles and are supplied with nutrients by capillaries to activate hair matrix cells to promote hair growth. DPCs also regulate hair follicle development by secreting cytokines and growth factors or transmitting various signals through direct interaction. Mouse model studies suggested that the number of DPCs controls the size and shape of the hair. Moreover, hair follicles could not generate new hair if the number of DPCs were below a certain threshold value. Growing interest in treating hair loss has led to the quest for identifying alternative and safe and natural therapeutic sources. To this end, in the present study, we investigated the efficacy of treatment with the bioactive components of *M. verticillata*, particularly LA, in preventing hair loss.

Here, we showed that treatment with LA extracted from M. verticillata seeds significantly increased the proliferation of DPCs, suggesting that it could contribute to hair regeneration [11].

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Moreover, LA treatment could significantly induce activation of Wnt/◎-catenin signaling. In the Wnt/β-catenin signaling pathway, absence of external signals leads to phosphorylation of β-catenin by GSK-3, and β-catenin is then ubiquitinated and degraded. However, when the Wnt ligand binds to its receptor, the activity of GSK-3 is suppressed, inhibiting ◎-catenin degradation, following which, β-catenin moves into the nucleus. Subsequently, β-catenin is able to regulate the expression of genes such as those encoding cyclin D and *c-Myc*, which induces hair cell proliferation [34]. It has been reported that treatment with mixed herbal extracts of avocado, marshmallow, chamomile, thyme, rosemary, and sedge nettle increased the expression of cyclin D1 and CDK4 in DPCs, thereby increasing their proliferation [35]. LA treatment also induces cell proliferation by activating the cell cycle through increased expression of cyclin D1 and CDK2, which leads to activation of the Wnt/β-catenin pathway.

Androgen hair loss, another mechanism of hair loss caused by hormonal imbalance, occurs when dihydrotestosterone, converted from testosterone by 5α-reductase, is produced excessively. When dihydrotestosterone binds to the androgen receptor of DPC, the expression of DKK-1, which induces apoptosis, increases. This leads to the death of hair matrix cells, which is another cell type involved in hair loss. In a co-culture experiment of DPCs and outer root sheath keratinocytes, expression of DKK-1 in the DPCs was increased by dihydrotestosterone, and thus, the proliferation of ORS keratinocytes decreased [36]. Another important aspect of dihydrotestosterone-induced hair loss is reduced cell proliferation via inhibition of Wnt/β-catenin signaling. Studies have shown that LA activates Wnt/β-catenin signaling and effectively inhibits the expression of DKK1, which is increased in the presence of dihydrotestosterone.

4. Materials and Methods

4.1. Materials

Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and ◎-actin used Sigma-Aldrich (St.Louis, MO, USA). VEGF and DKK-1 antibodies used Santa Cruz Biotechnology (Santa Cruz, CA, USA) and GSK-3β, p-GSK-3β, and β-catenin antibodies used Cell Signaling Technology (Danvers, MA, USA). Phosphate-buffered saline (PBS) and penicillin/streptomycin used Gibco (Grand Island, NY, USA).

4.2. M. verticillata seed extraction and active ingredient separation

Dried *M. verticillata* seeds (1 kg) were extracted twice at room temperature using 95% ethanol (10 L). The ethanol extract was concentrated using a rotary evaporator (Basis Hei-VAP, Heidolph, Germany) and a vacuum pump (Rotavac valve control, Heidelberg, Germany). The concentrated product (408 g) was then suspended in 20% ethanol (5 L), and the product was fractioned according to the order of solvent polarity to obtain *n*-hexane (hex), dichloromethane, ethyl acetate, n-butanol, and water fractions. Among the fractions obtained, the n-hex fraction (60.6 g) was subjected to silica gel column chromatography, and a total of five fractions (MH1–5) were obtained using *n*-hex / EtOAc (100:0–0:100). Subsequent purification of the MH2 fraction using preparative high-performance liquid chromatography ($CH_3OH:H_2O$ = 70:30) led to the identification of compounds 1 and 2.

4.3. Cell culture

Human hair follicle DPCs (HFDPCs) were purchased from Promo Cell (Heidelberg, Germany). Follicle DPC growth medium (Promo Cell) was used for cultivation of the HFDPCs. The cells were passaged every 3–4 days and incubated at 37°C and 5% CO_2.

4.4. Cell proliferation assay

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HFDPCs were seeded at 2 × 10^4 cells/well in a 96-well plate and incubated for 1 day at 37°C and 5% CO_2. The cells were treated with the *M. verticillata* seed extracts and LA at various concentrations and incubated for 48 h. Subsequently, 20 μL MTT (5 mg/mL) reagent was added to each well, and the cells were incubated again for 3 h. The supernatant was removed, and 100 μL of DMSO was added to completely dissolve the formazan formed. Absorbance at 550 nm wavelength was measured using a microplate reader (SpectraMax i3x, Molecular Devices, San Jose, CA, USA).

4.5. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA from HDFPCs was extracted using TRIzol reagent, and cDNA was synthesized from 2 μg of the total RNA [31]. PCR premix (Bioneer, Daejeon, Korea) was used to perform the PCR; the primers used are listed in Table 1. The PCR products were analyzed using 2% agarose gel stained with eco dye, and the product band intensities were quantified using ImageJ 1.47 software (NIH, Bethesda, MD, USA).

4.6. Western blotting

HFDPCs were seeded at 2 × 10^5 cells/mL in a 6-well plate in 2 mL of medium and incubated for 24 h. The cells were treated with various concentrations of LA for 48 h. Next, they were washed once with 1× phosphate-buffered saline (PBS), and lysed using cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA). After centrifugation at 12,000 rpm and 4°C for 15 min, the supernatant was separated and used as protein solution. The protein content was quantified using bicinchoninic acid protein assay (Thermo Scientific, MA, USA), after which the proteins were resolved using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 95 V for 2 h. The separated proteins were then transferred to a polyvinylidene fluoride membrane and blocked for 1 h using 5% skim milk. The primary antibody dissolved in 5% bovine serum albumin was added to the membrane and reacted at 4°C for 24 h. Next, a secondary antibody was added and reacted for 2 h. A supersignal chemiluminescent substrates (Thermo Scientific, MA, USA) was used to identify the protein signals, which were quantified using ImageJ 1.47 software.

4.7. Statistical analysis

All experiments were repeated at least three times, and the results are expressed as mean ± standard deviation. Statistical analysis was performed using Microsoft Excel 2016 (Student's t-test, $p < 0.05$).

5. Conclusions

In summary, LA isolated from M. verticillata seeds activated Wnt/◎-catenin signaling to promote the cell cycle and growth factor secretion, inducing proliferation of DPCs and hair growth. Moreover, it alleviated androgenic alopecia, which is another cause of hair loss. This study presents a potential alternative and effective natural therapeutic agent against hair loss.

Author Contributions: Conceptualization, J.L. and Y.L.; methodology, H.R.; software, C.L.; validation, J.J., J.L. and S.P.; formal analysis, X.X.; investigation, H.R and J.J.; resources, C.L.; data curation, X.X.; writing—original draft preparation, H.R and J.L.; writing—review and editing, Y.L.. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Abbreviations

CDK	Cyclin-dependent kinase
DPC	Dermal papilla cells
LA	Linoleic acid
HFDPC	Human hair dermal papilla cells
DKK-1	Dickkopf-related protein-1

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