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"Preparation and characterization of biota orientalis leaf exosome-like nanovesicles"

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1. Introduction

Biota orientalis leaf contains a variety of bioactive compounds, such as quercetin, kaempferol, and flavonoids[1]. These compounds exhibit various pharmacological activities, including antioxidant, anti-inflammatory, and antimicrobial properties. biota orientalis leaf is capable to stimulate the proliferation of human dermal papilla cells (hDPCs), especially extracts with higher concentrations of quercetin, which show greater cell proliferation activity, indicating that biota orientalis leaf extracts probably promote hair growth by enhancing the growth and metabolism of hair follicle cells. A study by Jaeyoon Kim et al. found that Biota orientalis leaf extracts can promote the proliferation of hair follicle cells through the non-receptor tyrosine kinase ACK1 pathway[2]. With significant anti-inflammatory effects, biota orientalis leaf extracts could significantly inhibit the production of interleukin-6 (IL-6) and the formation of leukotriene B4 (LTB4) as well as 5-hydroxyeicosatetraenoic acid (5-HETE)[3]. These anti-inflammatory properties help reduce scalp inflammation and alleviate symptoms such as scalp itching and redness.

Plant-derived extracellular vesicles (PDEVs) are nanoscale vesicles secreted by plant cells, carrying lipids, proteins, RNA, and secondary metabolites. In contrast to plant extracts with poor bioavailability and complex compositions, PDEVs exhibit higher bioavailability and are protected by a natural lipid membrane structure,enhancing resistance to acid and enzymatic degradation[4]. In this study, size-exclusion chromatography combined with ultrafiltration was utilized to isolate PDEVs fromi biota orientalis leaves. The study demonstrated their effects on promoting hair follicle regeneration, controlling sebum production, and inhibiting inflammatory factors. In the results, biota orientalis leaf exosome-like nanovesicles (BOLELNVs) have been proved for their potential applications in scalp care.

2. Materials and Methods

2.1 Preparation and characteristics of BOLELNVs

Biota orientalis leaves used for the isolation of extracellular vesicles were sourced from the Qinling Mountains in Shaanxi, China. Firstly, these collected leaves were washed with distilled water to remove dust and soil. Subsequently, the leaves were homogenized in a blender at a ratio of leaves: PBS buffer (Wuhan Servicebio Technology, China)=1 g: 20 mL. The homogenate was then centrifuged sequentially at 700g for 10 minutes, 2,000g for 20

minutes, and 12,000×g for 30 minutes respectively to remove large plant debris. Consequently, a centrifugal filter (Millipore, USA) was used to concentrate the extract, followed by the isolation of extracellular vesicles using a size-exclusion chromatography column (Beijing Enzecan Technology, China). Finally, the isolated BOLELNVs were resuspended in PBS, sterilized by filtration through a 0.45μm filter (Merck Millipore, USA), and stored at -80°C for latter use.

The particle size and concentration of BOLELNVs were measured by Resistive Pulse Sensing (RPS, Zhuhai Truth Optics Technology, China), and their morphology was observed using Transmission Electron Microscopy (TEM, Hitachi, Japan).

2.2 Measurements of total flavonoid and polyphenol content

The total flavonoid content was determined by using the Rutin (purity ≥ 97%, National Institutes for Food and Drug Control, China;) method, which is based on spectrophotometry. Under alkaline conditions, colored complexes will be produced when flavonoid compounds acting with aluminum ions (e.g., $Al(NO_3)_3$), exhibiting a maximum absorption peak at 510 nm[5]. The total flavonoid content (mg/g) was calculated with the formula, content of total flavonoid (mg/g)=(C×V×F×100)/(M×1000), where C: Conc. calculated from the standard curve (μg/mL), V: Volume of the sample solution (mL), F: Dilution factor and M: Mass of the sample (g).

The Folin-Ciocalteu method is a commonly used colorimetric analysis for determining the total phenolic content in plant, food, and beverage samples. The principle is based on the reduction of Folin-Ciocalteu reagent (phosphomolybdic and phosphotungstic acids, Aladdin, China) by phenolic compounds under alkaline conditions, resulting in the formation of a blue complex (molybdenum blue and tungsten blue). The absorbance is measured at 760 nm, and the total phenolic content (mg GAE/g) is calculated with the formula, content of total polyphenol (mg GAE/g)=(C×V×D)/W, where C: Conc. obtained from the standard curve (μg/mL), V: Volume of the sample (mL), D: Dilution factor and W: Mass of the sample (g)[6].

2.3 Cell viability

Cell Counting Kit-8 (CCK-8, Suzhou Xinsai Mei Biotechnology, China) is a cell proliferation and cytotoxicity assay based on the water-soluble tetrazolium salt (WST-8), commonly used to evaluate the cytotoxicity of drugs, compounds, or environmental factors on human dermal papilla cells (hDPCs, Chinese Academy of Sciences, China). OD values were obtained with the Multiscan Spectrum (Molecular Devices, USA). Meanwhile, cell viability (%) was calculated with the formula, Cell Viability (%) = $(OD_{experimental}-OD_{blank})/(OD_{control}-OD_{blank})$, where the blank group represents medium + CCK-8 (without cells), and the control group represents cells + CCK-8 (without tested samples)[7].

2.4 Wound healing assay

Human dermal papilla cells (hDPCs) were used for the wound healing assay. hDPCs (10^6 cells per well) were seeded into 6-well plates and cultured for 1-2 days until confluence was reached. The confluent monolayer of hDPCs was scratched with a pipette tip. Consequetly, the detached cells and debris were removed by washing with PBS. The cells were then treated with or without the sample BOLELNVs in DMEM containing 5% fetal bovine serum. Finally, images of the scratch area were captured at 0 h and 24 h after scratching and were quantified using ImageJ software. The percentage of wound closure was calculated based on the remaining size at 0 h[8].

2.5 Reactive Oxygen Species (ROS) inhibition assay

hDPCs were seeded in appropriate culture dishes or plates and cultured until the cell confluence reached 70%-80%. Meanwhile, cells were treated with 0.1 mM H_2O_2 (30%, Shanghai Kanglang Biotechnology, China) as a positive control. DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate) was used as a fluorescent probe, diluted to a working solution of 10 μM and added to the cell culture medium, followed by incubation at

37°C in the dark for 30 minutes. Next, the cells were washed 1-2 times with PBS to remove the unincorporated dye. Finally, the intracellular ROS levels were detected using a fluorescence microscope with an excitation wavelength of 488 nm and an emission wavelength of 530 nm[9].

2.6 mRNA expression of hair follicle-related growth factors

Vascular endothelial growth factor (VEGF) is an important factor for hair follicle growth, promoting angiogenesis to provide sufficient nutrients and oxygen to the hair follicles. The proliferation of hDPCs is usually accompanied by an increase in VEGF expression. β-catenin is a key molecule in the Wnt signaling pathway, which plays a central role in hair follicle regeneration and hair growth. Proliferative effects generally activate the Wnt/β-catenin pathway, leading to increased expression. The Wnt/β-catenin signaling pathway has been proven to be crucial for hair follicle development and hair growth. Activation of this pathway in hDPCs promotes the proliferation and differentiation of hair follicle cells, initiating the anagen phase of the hair cycle. Transforming growth factor β-1 (TGF-β1) is an inhibitor of hair growth. Under conditions that promote hair growth, its expression is usually downregulated to reduce the inhibitory effect on hair follicle growth[10].

hDPCs were cultured in vitro, and samples were added at certain concentrations based on cytotoxicity results. Cell proliferation was detected using the Edu method. hDPCs were treated with 10 μM Edu (Elabscience, China) for 2 hours, with proliferating cells stained green and all cells counterstained with DAPI (blue). After experimental treatment, 1 mL of Trizol (a novel total RNA extraction reagent, Toyobo, Japan) was added, and cells were lysed by vigorous shaking. Total RNA was extracted using the phenol-chloroform extraction method. cDNA was synthesized using the ReverTra Ace qPCR RT Master Mix with gDNA Remover kit according to the primer sequences in Table 1, and gene expression levels were detected using the SYBR® Green Realtime PCR Master Mix kit (Thermo Fisher Scientific, USA). The relative expression of collagen genes was analyzed using the $2^{-\triangle\triangle CT}$ method (the relative content of the blank group was set to 100%)[11].

Table 1. Primer sequence

Primer	Sequenc(5'to3')	Nucleotides/ Base Pairs
VEGF：F	ATCGAGTACATCTTCAAGCCAT	22
VEGF：R	GTGAGGTTTGATCCGCATAATC	22
β-Catenin：F	TGGATTGATTCGAAATCTTGCC	22
β-Catenin：R	GAACAAGCAACTGAACTAGTCG	22
TGFβ1：F	GCAAAGTTGTGAAAACAAGAGC	22
TGFβ1：R	ATCCCAGGTTCCTGTCTTTATG	22

2.7 Oil red O staining assay

Oil Red O staining is a commonly used method for detecting intracellular lipid accumulation, particularly suitable for sebaceous gland cells and other lipid-rich cell types. The efficacy of the samples was tested by examining the changes in lipid content in sebocytes stimulated by linoleic acid (LA)[12]. LA is an essential unsaturated fatty acid that can be absorbed and utilized by human cells. It binds to peroxisome proliferator-activated receptors (PPARs) and regulates genes involved in lipid metabolism within peroxisomes, microsomes, and mitochondria, thereby promoting the differentiation, proliferation, and sebum synthesis of sebaceous glands. Sebocytes can utilize linoleic acid to synthesize sebum, which is a significant factor influencing sebum metabolism. Oil Red O is a lipophilic dye that specifically stains neutral fats such as triglycerides in tissues. To some extent, the more and larger the red lipid droplets, the stronger the lipid synthesis and secretion[13]. After culturing LA-stimulated sebocytes for a period, Oil Red O staining was used to detect changes in

cellular lipid synthesis. The results of Oil Red O staining were presented in photographic form, with red dots representing lipid droplets.

2.8 mRNA expression of inflammatory cytokine

Interleukin-6 (IL-6) may activate signaling pathways such as NF-κB, inhibiting the proliferation of human dermal papilla cells (hDPCs) and thereby affecting hair follicle growth. It can also induce hDPCs to produce more inflammatory mediators, such as tumor necrosis factor-α (TNF-α) and IL-1β, forming an inflammatory cascade that further exacerbates the inflammatory environment around the hair follicles. Persistent high expression of IL-6 may prematurely push hair follicles from the anagen phase into the catagen phase, leading to follicular atrophy and hair loss. TNF-α can bind to the TNF receptor, activating intracellular signaling pathways that reduce the viability of hDPCs, inhibit their proliferation and metabolism, and induce apoptosis in hDPCs, decreasing the number of dermal papilla cells and thus affecting the normal function of hair follicles. TNF-α can also induce the expression of adhesion molecules on vascular endothelial cells surrounding the hair follicles, promoting the infiltration of inflammatory cells and further exacerbating the inflammatory response. Interleukin-1β (IL-1β) can bind to the IL-1 receptor, activating the NF-κB signaling pathway and promoting the expression of inflammation-related genes. IL-1β can induce hDPCs to release various inflammatory mediators, such as IL-6 and TNF-α, forming an inflammatory cascade. IL-1β can also affect the extracellular matrix metabolism of hDPCs, disrupting the stability of the follicular microenvironment and thereby affecting hair follicle growth [15]. Lipopolysaccharide (LPS) is a common inflammatory inducer that can activate the inflammatory response in hDPCs, leading to significant upregulation of mRNA expression of various inflammatory cytokines (such as IL-6, IL-1β, TNF-α)[14]. In the experiment, hDPCs were divided into control and LPS-stimulated groups. The LPS-stimulated group was treated with 100 ng/mL LPS for 6 hours. The mRNA expression levels of inflammatory cytokines (IL-6, IL-1β, TNF-α, etc.) were detected using qRT-PCR. The primer sequences listed in Table 2 were used for reverse transcription to synthesize cDNA and to detect gene expression levels. The relative expression of genes was analyzed using the $2^{-\triangle\triangle CT}$ method.

Table 2. Primer sequence

Primer	Sequenc(5'to3')	Nucleotides/ Base Pairs
IL-6：F	CCAAATGGCCTCCCTCTCAT	20
IL-6：R	GGTGGTTTGCTACGACGTGG	20
TNF-α：F	CGAGTGACAAGCCTGTAGC	19
TNF-α：R	CCTTCTCCAGCTGGAGAGC	19
IL-1β：F	CCTGTGGCCTTGGGCCTCAA	20
IL-1β：R	GGTGCTGATGTACCAGTTGGG	21

2.9 Statistical analysis

Data analysis was performed using SPSS 28.0. Results are presented as the mean ± standard deviation (SD). Comparisons between groups were analyzed using paired sample t-tests, with all statistical analyses being two-tailed. A significant difference was considered when $P<0.05$, indicated by "*" in the figures; when $P\geq0.05$, no significant difference was observed, indicated by the symbol "n.s." in the figures.

3. Results

3.1 Morphology and particle size of BOLELNVs

After diluting BOLELNVs 100 times with PBS buffer solution, the average particle size was measured to be approximately 71 nm, with a calculated concentration of 5.57×10^{10} particles/mL (Figure 1, right). The plant-derived extracellular vesicles exhibit a vesicular structure with a lipid bilayer membrane, appearing as saucer-shaped or cup-shaped, similar to exosomes, which are composed of a phospholipid bilayer (Figure 1, left).

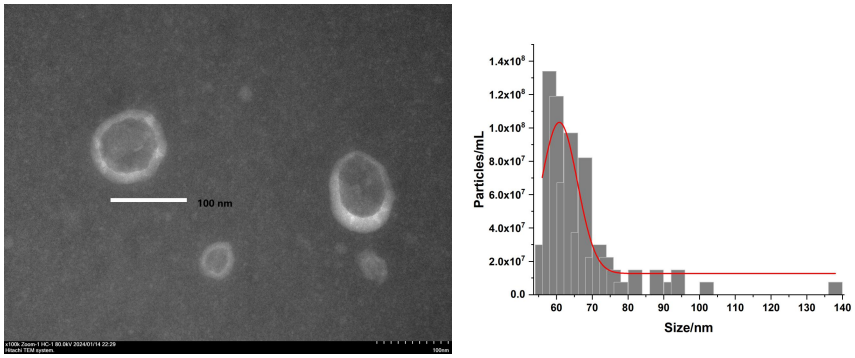

Figure 1. TEM microstructure (left) and particle size distribution of BOLELNVs (right).

3.2 Total flavonoids and polyphenols content

Standard curves for rutin and gallic acid were established (Figure 2). The total flavonoid and phenolic contents in the BOLELNVs samples were determined by referencing these standard curves, with the results showing 109.31 ppm of flavonoids and 224.66 ppm of phenolics. This indicates that phenolics are more abundant in the aqueous solution of BOLELNVs.

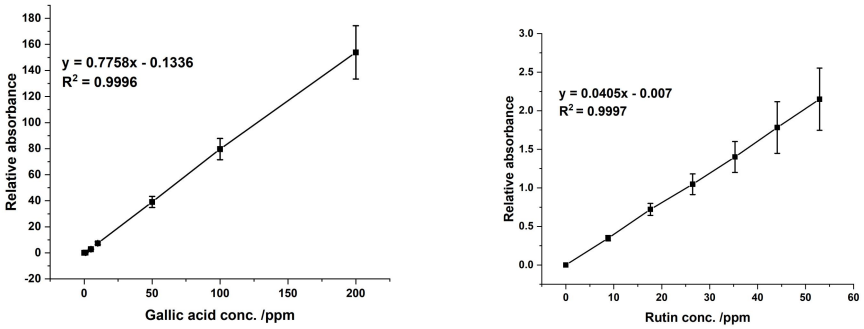

Figure 2. Standard curves for rutin (left) and gallic acid (right).

3.3 CCK-8 Cytotoxicity

Within the concentration range of ≤ 5.57 × 10^{10} particles/mL, the cell viability of hDPCs was concentration-dependent (Figure 3). When the concentration of BOLELNVs was ≥ 1.14 × 10^{8} particles/mL, cell viability significantly increased compared to the blank control group ($P < 0.05$). However, at lower concentrations (≤ 5.57 × 10^{7} particles/mL), although non-toxic, there was no significant difference in cell viability ($P > 0.05$). Therefore, within this concentration range, subsequent cell efficacy tests can be conducted.

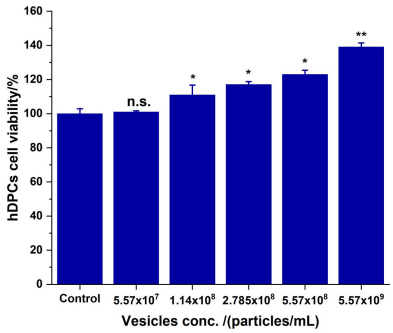

Figure 3. Schematic diagram of the CCK-8 cytotoxicity test.

3.4 Wound healing rate

As shown in Figures 4 and Figure 5, compared to 0 h, the control group hDPCs exhibited baseline migratory ability (10% closure) at 24 h. The experimental groups with different concentrations of BOLELNVs vesicles showed significantly higher migration rates than the control group (P < 0.001), indicating that BOLELNVs may promote hDPCs migration in a concentration dependent manner.

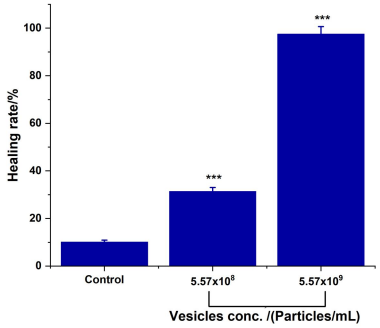

Figure 4. Comparison of cell scratch before and after 24 h.

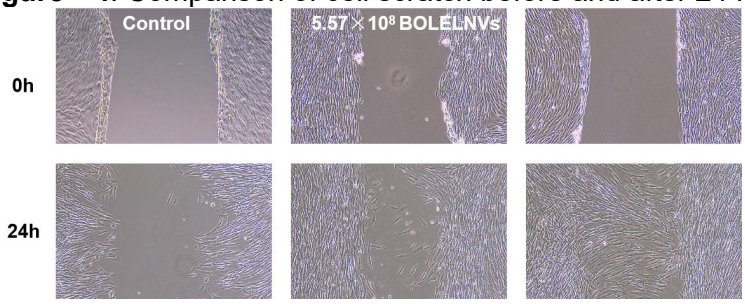

Figure 5. Comparison of cell wound healing before and after 24 h at different vesicle conc.

3.5 ROS inhibition

Under fluorescence microscopy (Figure 6), compared to the positive control group induced by H_2O_2, the experimental groups with different concentrations of BOLELNVs vesicles exhibited reduced fluorescence, indicating quenching of ROS in a concentration dependent manner.

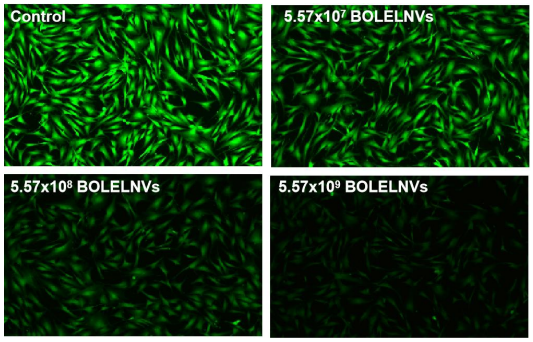

Figure 6. Fluorescence microscopy image of ROS.

3.6 mRNA expressions of VEGF, β-Catenin, and TGF-β1

As shown in Figure 7, when the concentration of BOLELNVs vesicles was ≥ 5.57 × 10^8 particles/mL, the expression of VEGF and β-catenin significantly increased compared to the control group (P < 0.05), indicating that BOLELNVs promote the expression of these growth factors at certain concentrations. Conversely, the expression of TGF-β1 significantly decreased with increasing vesicle concentration, suggesting that BOLELNVs inhibit TGF-β1 gene expression, which may reflect potential hair growth-promoting effects.

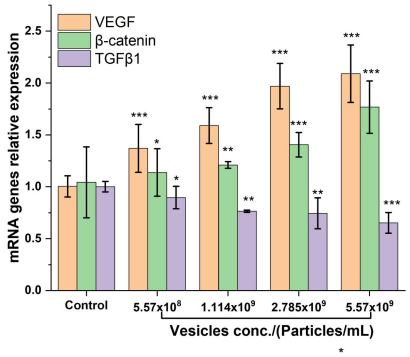

Figure 7. Relative expression levels of follicle-related mRNAs under different vesicle conc.

3.7 Oil red O Staining

As shown in Figure 8, when the concentration of BOLELNVs vesicles was ≥ 5.57 × 10^8 particles/mL, the density of lipid droplets in adipocytes visibly decreased compared to the control group. This indicates that BOLELNVs inhibit lipid secretion by adipocytes at certain concentrations, reflecting potential sebum control effects.

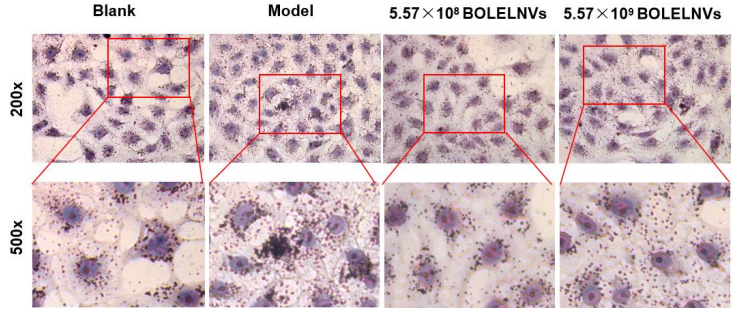

Figure 8. Electron microscopy image of Oil Red O staining experiment.

3.8 mRNA expressions of IL-6, TNF-α, and IL-1β

As shown in Figure 9, compared to the blank control group, the expression levels of three inflammation-related genes significantly increased in the LPS (100 ng/mL)-stimulated hDPCs model group ($P < 0.001$), confirming successful modeling. When the concentration of BOLELNVs vesicles was ≥ 5.57 × 10^8 particles/mL, the expression of IL-6, TNF-α, and IL-1β significantly decreased compared to the control group ($P < 0.001$), indicating that BOLELNVs inhibit these genes at certain concentrations, reflecting potential anti-inflammatory effects.

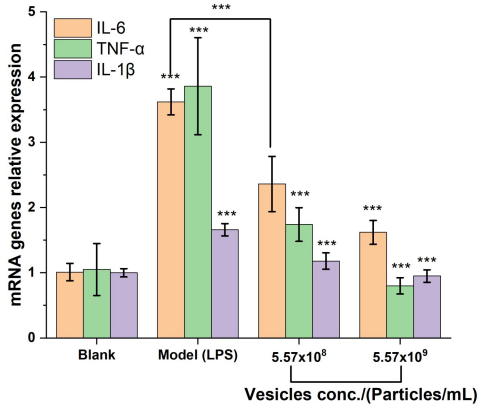

Figure 9. Relative expression levels of inflammatory cytokine mRNAs at different conc.

4. Discussion

Extracellular vesicles (EVs) were extracted and separated from dried biota orientalis leaves sourced from the Qinling Mountains in Shaanxi using ultracentrifugation and size-exclusion chromatography. The resulting vesicles exhibited a liposome-like bilayer phospholipid structure with a particle size of approximately 71 nm, classifying them as nanovesicles. The total flavonoid and phenolic contents were determined using the rutin method and the Folin-Ciocalteu method, respectively. The results indicated that the total flavonoid content was 109.31 ppm, while the total phenolic content was 224.66 ppm. Given that the extraction process utilized PBS buffer as a solvent, the higher proportion of water-soluble phenolics compared to flavonoids can be attributed to the physical extraction method. Some flavonoid mixtures remained lipophilic and could not be obtained in large quantities through this extraction method. A series of in vitro cellular experiments were conducted, including cytotoxicity assays on human dermal papilla cells (hDPCs), scratch assays, reactive oxygen species (ROS) inhibition, follicle-related growth factor gene expression, lipid accumulation in adipocytes using Oil Red O staining, and the expression of inflammation-related genes. The results demonstrated that the plant-derived extracellular vesicles (BOLELNVs) not only lacked cytotoxicity but also promoted cell proliferation in a concentration-dependent manner when the vesicle concentration was ≤ 5.57×10^{10} particles/mL. Specifically, when the BOLELNVs concentration was ≥ 1.14×10^{8} particles/mL, a significant increase in cell viability was observed compared to the blank control group ($P < 0.05$). This may be due to the superior biocompatibility and low immunogenicity of plant-derived EVs, which are less likely to cause immune reactions and toxicity[15]. In the scratch assay, BOLELNVs significantly enhanced the migration of hDPCs compared to the blank control group ($P < 0.05$) in a concentration-dependent manner. This effect may be attributed to the provision of essential nutrients and a basis for material exchange by BOLELNVs, which accelerates the healing process and creates a favorable environment for cell migration and proliferation. Additionally, BOLELNVs exhibited a significant inhibitory effect on ROS compared to the positive control group. This is likely due to the presence of antioxidant compounds, such as phenolics and flavonoids, which can significantly reduce intracellular ROS levels and mitigate oxidative stress-induced cellular damage[16]. In the qPCR analysis of mRNA expression of VEGF, β-catenin, and TGF-β1, which are related to hair follicle growth, the relative expression of VEGF and β-catenin increased with the concentration of BOLELNVs, while the expression of TGF-β1 was significantly downregulated. This may be because microRNAs or lipid components in BOLELNVs target the transcription or translation of VEGF, thereby enhancing its expression level[17]. The activation of VEGF may further promote angiogenesis and cell proliferation, accelerating tissue repair. β-catenin, a core transcription factor in the Wnt signaling pathway, is regulated by the interaction of Wnt ligands and receptors. BOLELNVs may stabilize β-catenin and promote its nuclear translocation by carrying Wnt ligands or inhibiting β-catenin degradation-related proteins (e.g., GSK-3β), thereby activating downstream target genes[18]. The activity of TGF-β1 is typically regulated by the interaction of its latency-associated protein and integrins. Components in BOLELNVs (e.g., specific lipids or proteins) may interfere with the binding of TGF-β receptors and ligands or inhibit the phosphorylation of downstream SMAD proteins, thereby reducing the transcriptional activation of TGF-β1. Additionally, BOLELNVs may carry proteases targeting TGF-β (e.g., plasmin), accelerating its degradation and reducing intracellular TGF-β1 levels[19]. In the Oil Red O staining of adipocytes, BOLELNVs significantly reduced lipid droplet density compared to the oleic acid-induced model group, with a visible decrease in lipid secretion. Adipocytes in the model group take up free fatty acids (FAs) from the circulation or synthesize FAs de novo from glucose. These FAs are esterified into triglycerides (TAGs) by diacylglycerol acyltransferase (DGAT) in the endoplasmic reticulum and stored in

lipid droplets, with the added oleic acid further promoting lipid droplet secretion. BOLELNVs contain various bioactive molecules, including proteins, lipids, and microRNAs (miRNAs), which play important roles in intercellular signaling. BOLELNVs can regulate lipid metabolism and signaling pathways in adipocytes, thereby inhibiting the formation and secretion of lipid droplets[20]. In the qPCR analysis of mRNA expression of three inflammatory cytokines, BOLELNVs significantly reduced the expression of these cytokines ($P < 0.001$) in a concentration-dependent manner. BOLELNVs may carry anti-inflammatory factors (e.g., IL-10) or regulatory non-coding RNAs (e.g., microRNAs) that target and inhibit pro-inflammatory signaling pathways such as NF-κB and JNK, thereby reducing the transcriptional activity of IL-6, TNF-α, and IL-1β. Additionally, specific microRNAs in BOLELNVs (e.g., miR-145) may directly target the mRNA of IL-6, TNF-α, or IL-1β, inhibiting their translation or promoting their degradation through base complementarity, thereby reducing expression levels. Moreover, BOLELNVs may promote the secretion of anti-inflammatory cytokines (e.g., IL-10) or inhibit the release of pro-inflammatory cytokines, disrupting the balance of the inflammatory microenvironment and indirectly suppressing the expression of IL-6, TNF-α, and IL-1β. Based on these findings, BOLELNVs show promise as a novel material that could potentially replace traditional plant extracts in personal care products.

5. Conclusion

Through the preparation of biota orientalis leaf plant-derived extracellular vesicles (BOLELNVs), vesicles with an average particle size of 71 nm and a concentration of 5.57×10^{10} particles/mL were obtained. These vesicles exhibited a distinct lipid bilayer membrane structure and a cup-shaped morphology. In physicochemical tests, the total polysaccharide and total phenolic contents were determined to be 109.31 ppm and 224.66 ppm, respectively, using the rutin method and the Folin–Ciocalteu method. In the CCK-8 cytotoxicity and scratch wound healing assays, the proliferation and scratch repair effects of BOLELNVs were concentration-dependent. In the ROS inhibition test, BOLELNVs significantly quenched intracellular ROS levels in a concentration-dependent manner. In the qPCR experiment, BOLELNVs significantly upregulated the mRNA expression of VEGF and β-Catenin while downregulating TGF-β1 expression ($P<0.05$). In the Oil Red O staining test, BOLELNVs significantly inhibited lipid secretion in adipocytes. For the expression of inflammatory factors IL-6, TNF-α, and IL-1β, the inhibitory effects increased with the concentration of BOLELNVs ($P<0.001$). These results suggest that BOLELNVs have potential therapeutic applications in hair care products, including promoting hair growth, inhibiting sebum secretion, and reducing inflammation.

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