Supporting Materials

Molecular insight of nanosized Ba-Hao herbal ointment in accelerating chronic wound healing

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1. Materials and Methods

1.1 Cell Culture

Normal human dermal fibroblasts (NHDF) were sourced from BeNa Culture Collection in Xinyang City, Henan Province, China. These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin, in a controlled environment at 37 °C and 5% CO_2. In the quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) studies, NHDF cells were segregated into one control group and three experimental groups. The experimental groups were exposed to varying concentrations of BHO (1, 10, and 100 μg/mL), while the control group received no BHO. For the protein expression analysis using Western Blot (WB) of Akt1/2/3 and phospho-Akt1/2/3, the cells were divided into a control group, which received no BHO, and an BHO group treated with 300 μg/mL BHO.

1.2 Cytotoxicity assay

Approximately 5,000 NHDF cells were plated in 96-well plates and incubated overnight to facilitate cellular adhesion. Subsequently, these cells were subjected to a range of BHO concentrations for a 48 hours treatment period. The BHO with 0 μg/mL concentration served as the control group, whereas the concentrations of 10, 100, 300, and 1000 μg/mL constituted the experimental groups. Post-treatment, cell viability was assessed through the addition of 10 μL of Cell Counting Kit-8 (CCK-8) solution (K1018, APExBIO, USA) and 90 μL of DMEM to each well, followed by a further incubation of 1 hour at 37 ℃. The absorbance at 450 nm was then quantitatively measured using a microplate reader, providing an index of cellular metabolic activity.

1.3 Construction of protein-protein interaction (PPI) network and identification of key targets

To elucidate the interactions among the intersected targets, these targets were uploaded to the STRING database (https://stringdb.org/) to construct a PPI network with a confidence threshold of 0.4. Subsequently, this network was imported into Cytoscape (v3.8.2) for advanced visualization and analysis. The significance of nodes within the network was evaluated using the CytoHubba plugin, employing the Maximal Clique Centrality (MCC) algorithm.

1.4 GO enrichment analysis and KEGG enrichment analysis

The intersected targets underwent Gene Ontology (GO) annotation and KEGG pathway enrichment analysis via the DAVID database (https://david.ncifcrf.gov/). The top 10 GO terms and top 20 KEGG signaling pathways associated with these targets were identified, using a significance threshold of $P < 0.05$.

1.5 qRT-PCR

The total RNA of NHDF was extracted using TRIzol and reverse transcribed into cDNA using the Prime-ScriptTMRT reagent kit (#RR047A) purchased from TaKaRa (Shiga, Japan). Moreover, qRT-PCR was performed using synthesized cDNA, specific primers (Table 1), Premix EX Taq, Novostart SYBR qPCR SuperMix Plus (#E096-01B)

purchased from Novoprotein (Shanghai, China). The relative mRNA expression of EGF and VEGFA was determined using the $2^{-\Delta\Delta Ct}$ method, normalized to the loading control GAPDH.

1.6 Western Blotting Assay

Total protein was extracted using RIPA buffer supplemented with PMSF and phosphatase inhibitors. Protein content was quantified using the BCA working solution (Beyotime, Shanghai, China), and the sample protein concentration was calculated accordingly. SDS-PAGE electrophoresis was performed, followed by transfer to a PVDF membrane. The membrane was blocked with 5% skim milk for 2 hours, then washed with TBST. Membranes were incubated overnight at 4 °C with the following primary antibodies: β-actin (1:1000, ZSGB-Biotech, Beijing, China), Akt1/2/3 (1:1000), and p-Akt1/2/3 (1:1000, Santa Cruz, California, USA). After washing with TBST, the membrane was incubated for 2 hours at room temperature with the secondary antibody goat anti-mouse (1:10000, ZSGB-Biotech, Beijing, China). Protein bands on the membrane were visualized using an ECL detection kit (Beyotime, Shanghai, China), and band intensities were analyzed using ImageJ (version 1.8.0) and normalized to the loading control β-actin.

1.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0. All data are presented as mean ± standard deviation ($\bar{x}\pm$ SD). Paired t-tests were used for comparisons between two groups, while one-way ANOVA was used for comparisons among multiple groups. A P-value of less than 0.05 was considered statistically significant.

Table S1 The list of active components in the top three for BHO

Rank	Name	Molecule ID	Molecule Name	Score
1	DY22	MOL000098	quercetin	142
2	DH6	MOL001880	Oxalis corniculata L (oxl)*	66
3	A1	MOL000358	beta-sitosterol	65

*Oxalis corniculata L is a main ingredient from oxalis and has active effect for hemorrhoids, burns, eczema and so on.

Table S2 Molecular docking results

Target	Target (PDB ID)	Compound	Affinity ($kJ \cdot mol^{-1}$)
VEGFA	4KZN	quercetin	-4.91
		oxl	-3.27
		beta-sitosterol	-6.47
TNF	2E7A	quercetin	-6.6
		oxl	-4.02
		beta-sitosterol	-9.22
IL-1β	1IOB	quercetin	-6.12
		oxl	-3.8
		beta-sitosterol	-7.7

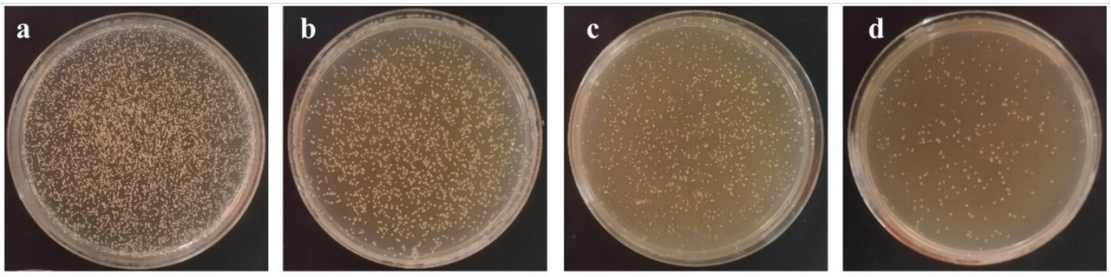

Figure S1 Bacterial colony growth of Staphylococcus aureus treated with the different concentration of BHO at (a) 0 μg/mL, (b) 50 μg/mL, (c) 100 μg/mL, (d) 300 μg/mL, respectively.

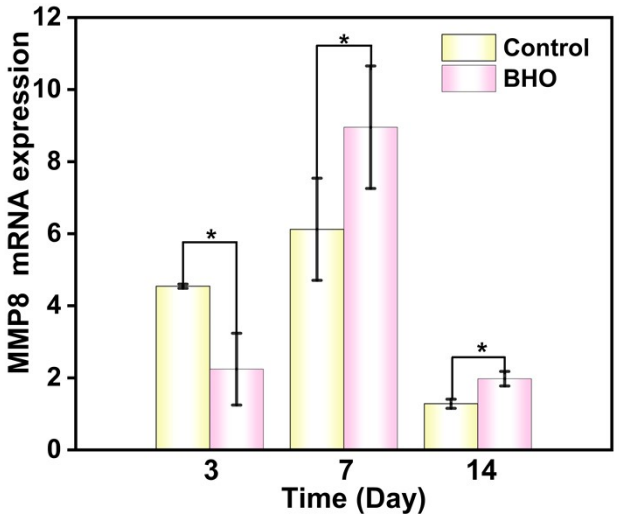

Figure S2 MMP8 mRNA expression levels. Results are expressed as mean ± standard deviation (n=5), *$p < 0.05$, vs. control, one-way ANOVA.

Table S3 Sequence of primers for Real-Time RT-PCR

Genes	**Forward primer**	**Reverse primer**
VEGFA	AATCGAGACCCTGGTGGACA	TGTTGGACTCCTAGTGGGC
EGF	TGAGAGTAAACAAGAGGACTGGC	CAAATATGTTCACAGCCTCCG
GAPDH	CGGATTTGGTCGTATTGG	GGTGGAATCATATTGGAACA