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Boswellic acid inhibits cell migration and VEGF expression in mixed hemorrhoids *in-vitro*

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Abstract: Mixed hemorrhoids involve vascular hyperplasia, inflammation and angiogenesis, with VEGF playing a vital role. Current treatments mainly relieve symptoms, underscoring the need for therapies that target underlying mechanisms. This study investigates the potential of boswellic acid (BA) in treating mixed hemorrhoids. Human foreskin fibroblast (BJ) cells were treated with varying BA concentrations (0, 25, 50, 100 μM) for 24 and 48 h. BA did not significantly affect cell viability but markedly reduced VEGF mRNA and protein expression in a dose-dependent manner. Levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) also decreased, and cell migration was significantly inhibited. These results suggest that BA suppresses VEGF expression, attenuates inflammation, and limits fibroblast migration *in vitro*, supporting its potential as a therapeutic candidate for mixed hemorrhoids.

Keywords: Boswellic acid; Cell migration; Fibroblast cells; Mixed hemorrhoids; VEGF

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INTRODUCTION

Mixed hemorrhoids, characterized by both internal and external hemorrhoids, arise from factors such as venous dilation, vascular hyperplasia and chronic inflammation (Lohsiriwat, 2012, Sun *et al*., 2024). These processes lead to symptoms such as pain, bleeding and prolapse, which markedly impair patients' quality of life (Sun *et al*., 2024). Although various treatments exist, including lifestyle changes, medications and surgery, recurrence and complications like persistent bleeding remain common (Huang *et al*., 2024, Feng *et al*., 2021). Therefore, more effective therapeutic strategies are required to target these underlying mechanisms, reduce recurrence, and improve long-term outcomes.

Vascular endothelial growth factor (VEGF) serves as a critical mediator of angiogenesis, responsible for new blood vessel formation and the maintenance of vascular permeability (Pérez-Gutiérrez and Ferrara, 2023, Ghalehbandi *et al*., 2023). In hemorrhoidal tissues, VEGF overexpression results in the formation of fragile and leaky blood vessels, thereby exacerbating inflammatory responses and sustaining disease progression (Meng *et al*., 2022, Palumbo *et al*., 2023). By promoting angiogenesis, VEGF increases the blood supply to hemorrhoidal tissues. Although beneficial for tissue repair, this process also generates excessive vascular networks (JIA *et al*., 2023, Patel *et al*., 2023). These newly formed vessels are prone to rupture, leading to bleeding, a common symptom in patients with hemorrhoids (Fišere *et al*., 2023). Furthermore, VEGF increases vascular permeability, facilitating the leakage of fluid and proteins into surrounding tissues, which contributes to edema and an intensified inflammatory response (Nong *et al*., 2022). This inflammatory environment not only perpetuates swelling and discomfort but also attracts immune cells that release additional pro-inflammatory cytokines (Ross *et al*., 2021). The persistent inflammation weakens connective tissue, promoting remodeling and the progression of symptoms such as prolapse (Guler and Roovers, 2022). Recent studies have highlighted the critical role of VEGF in hemorrhoidal pathophysiology. For example, Porwal *et al*. (Porwal *et al*., 2021) reported that VEGF expression was significantly elevated in hemorrhoidal tissues, and treatment with the polyherbal formulation Anoac-H suppressed VEGF and RANTES levels, alleviating bleeding symptoms. Similarly, Zhou *et al*. (Zhou *et al*., 2023) further reviewed the role of Traditional Chinese Medicine (TCM) in hemorrhoid therapy and concluded that VEGF overexpression contributes to vascular congestion and microvascular dilation in anorectal tissues. Supporting this, Yu *et al*. (Yu *et al*., 2024) further demonstrated that Sanhuang ointment, a classical TCM compound, improved anorectal function in a rat hemorrhoid model by modulating VEGF and other cytokines involved in tissue repair. In addition, Liu *et al*. (Liu *et al*., 2025) showed that miR-143-3p regulates VEGF signaling and affects abnormal angiogenesis during hemorrhoidal development and postoperative healing. Collectively, These findings indicate that VEGF mediates angiogenesis and contributes to vascular and inflammatory abnormalities in hemorrhoidal disease. Given VEGF's central role in these processes, targeting VEGF signaling pathways may represent a potential therapeutic strategy for more effective hemorrhoid treatment.

Among the key cellular players in hemorrhoidal tissue, fibroblasts are particularly important. These stromal cells contribute to extracellular matrix remodeling, wound healing, and the regulation of inflammatory responses

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(Guler and Roovers, 2022). Notably, fibroblasts can secrete VEGF and pro-inflammatory cytokines under pathological conditions, thereby indirectly influencing angiogenesis and immune cell recruitment (Lee *et al*., 2021). Investigating the effects of therapeutic compounds on fibroblasts may thus offer insights into mechanisms by which they modulate key processes in hemorrhoidal disease.

Natural compounds have gained attention for their potential therapeutic benefits across various diseases, particularly due to their inhibition of both inflammation and angiogenesis (Lindell *et al*., 2022). Boswellic acid (BA), a pentacyclic triterpene isolated from the resin of Boswellia species, is one such compound with promising applications (Solanki *et al*., 2024). Studies have demonstrated that BA can inhibit key inflammatory pathways, reduce production of pro-inflammatory cytokines and suppress VEGF expression in various cell types (Ragab *et al*., 2024, Trivedi *et al*., 2023, Rajabian *et al*., 2023). However, its effects on fibroblast cells, which are crucial in wound healing and tissue remodeling in hemorrhoidal disease, have not been thoroughly explored. Understanding BA's impact on these cells could provide insights into its therapeutic potential in the management of hemorrhoids.

By elucidating the mechanisms through which BA exerts its effects on human foreskin fibroblast cells, this research aims to provide a scientific basis for developing novel, targeted therapies for hemorrhoidal disease. The findings could have important clinical implications, offering a new therapeutic approach targeting both symptoms and underlying mechanisms of the disease.

MATRIALS AND METHODS

Cell maintenance and treatment procedures

The human foreskin fibroblast cell line, designated BJ cells, was supplied by Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Method for BJ cell culturing and exposure to different test compounds was described previously (Al-Bahlani *et al*., 2020, Zhang *et al*., 2023). Briefly, cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Thermo Fisher Scientific, USA) containing 10% fetal bovine serum (FBS, HyClone, USA) and 1% penicillin-streptomycin (Gibco, Thermo Fisher Scientific, USA). Cell cultures were sustained in a humidified atmosphere of 5% CO_2 at 37°C. For treatment, cells were distributed onto appropriate culture plates or dishes and maintained overnight to ensure adhesion.

On the subsequent day, cells received interventions with diverse concentrations of BA (Sigma-Aldrich, USA) dissolved in DMSO (final DMSO concentration was 0.1% in all groups, including BA0 (vehicle control)). The treatment groups included BA0 (control, 0.1% DMSO vehicle without boswellic acid), BA25 (25 µM), BA50 (50 µM) and BA100 (100 µM). The cells were incubated with BA for 24 and 48 h, depending on the experiment.

CCK-8

The Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Japan) was used to assess cell viability. BJ cells were introduced into 96-well plates (5,000 cells/well) and subjected to overnight incubation for adherence. The next day, cells underwent BA treatment at multiple concentrations over 24- and 48-hour periods. After treatment, each well received CCK-8 solution (10 µL) and cell samples underwent another 2-h incubation at 37°C. A microplate reader (Bio-Rad, USA) was exercised for determining absorbance at 450 nm. All tests were conducted three times independently and a percentage-based evaluation of cell viability was made employing the control group as a reference.

Wound healing assay

A wound healing experiment was applied in the cell migration evaluation. BJ cells were introduced into 6-well plates and grown until they attained 90% confluency. The cell monolayer was scratched (wounded) via a sterile pipette tip (200 µL). Next, two washes with phosphate-buffered saline (PBS, Solarbio, China) were performed to eliminate non-adherent cells and debris. Cells received BA at 0 µM, 25 µM, 50 µM and 100 µM for treatment periods of 24 and 48 h. Using of an inverted microscope (Olympus, Japan), wound areas were photographed at 0, 24 and 48 h. The wound area measurement was attained through ImageJ software (NIH, USA) and the percentage of wound closure was calculated.

ELISA

Inflammatory cytokine levels, comprising tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-1 beta (IL-1β), were quantified through commercial ELISA kits (Nanjing Jiancheng, Nanjing, China), adhering to the operational standards specified by the manufacturer. After treatment, the supernatants were gathered and centrifuged at 1,500 g for 10 min to clear away residual cellular fragments. The absorbance was measured at 450 nm using a micro plate reader (Bio-Rad, USA) and TNF-α, IL-1β and IL-6 concentrations were estimated based on a standard curve.

qRT-PCR

Total RNA was separated from BJ cells by means of the TRIzol reagent (Invitrogen, Thermo Fisher Scientific, USA), referring to the manufacturer's procedure. RNA samples were evaluated for purity and concentration utilizing a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). The PrimeScript RT reagent kit (Takara, Japan) facilitated the generation of cDNA from total RNA (1 µg). Through the SYBR Green PCR kit (Takara, Japan), qRT-PCR was completed on the StepOnePlus Real-Time PCR System (Applied Biosystems, USA). The following primers were used for VEGF: Forward, 5′-TGTCTAATGCCCTGGAGCCT-3′; Reverse, 5′-TGCAACGCGAGTCTGTGTTT-3′. β-actin: Forward, 5′-CACCATTGGCAATGAGCGGTTC-3′; Reverse, 5′-AGGTCTTTGCGGATGTCCACGT-3′. The PCR program consisted of the following steps: a preliminary heating phase at 95°C for 5 min, succeeded by 40 cycles of 95°C for 15 s and 60°C for 30 s. Following normalization to β-actin, relative VEGF expression was assessed using the $2^{-\Delta\Delta Ct}$ technique.

Western blot analysis

RIPA buffer (Beyotime, China) comprising a protease inhibitor cocktail (Roche, Switzerland) was adopted for cell lysis. Protein determination was achieved with the aid of a BCA reagent kit from Beyotime (China). Following SDS-PAGE separation, 30 μg of protein per sample was transferred to PVDF membranes (Millipore, USA). Upon a 1-hour block at room temperature with 5% non-fat milk/TBST, membranes underwent an overnight incubation at 4°C with primary antibodies against VEGF (1:1000, Cell Signaling Technology, USA) and β-actin (1:2000, Cell Signaling Technology, USA). Post-washing, the membranes received another 1-h incubation with horseradish peroxidase-conjugated secondary antibodies (1:5000, Cell Signaling Technology, USA) at room temperature. ECL detection reagent (Millipore, USA) enabled band visualization and signal intensity was quantified with ImageJ software. Signal intensity was quantified using ImageJ software by measuring the grayscale density of each band. VEGF band intensities were normalized to their corresponding β-actin band intensities from the same lanes. Each band was selected using a rectangular selection of identical size and background intensity was subtracted using a nearby area of equal size. The normalized intensity (VEGF/β-actin) was calculated and used for statistical analysis.

Statistical analysis

All results were reported as the mean ± standard deviation (SD). Statistical analyses were performed via GraphPad Prism 8.0 software (GraphPad Software, USA). Two-way ANOVA was applied to evaluate the main effects of dose, time and their interaction, followed by Tukey's post hoc test for multiple comparisons. P-values lower than 0.05 were taken to imply statistical significance.

RESULTS

Impact of BA on cell viability

The chemical structure of BA was depicted in Fig. 1A. BA-induced alterations in BJ cell viability were quantified by CCK-8 assays at 24-h and 48-h intervals. Briefly, cell viability did not significantly differ between the control group (BA0) and the treatment groups (BA25, BA50, BA100) at both time points ($P > 0.05$, Fig. 1B).

BA inhibited cell migration

BJ cell migration after BA exposure was analyzed via a wound healing test. Representative images of cell migration at 0, 24 and 48 h are shown in Fig. 2. The treatment with BA markedly inhibited cell migration in a concentration-dependent manner. At both 24 and 48 h, the closure of the wound area was significantly reduced in BA-treated groups compared with the control (BA0) and additional differences were observed among the BA concentrations.

BA reduced inflammatory cytokine levels

BA-induced changes in the TNF-α, IL-1β and IL-6 secretion were assessed using ELISA. The concentrations of these inflammatory mediators in the conditioned media were markedly reduced in a concentration-dependent manner upon BA intervention (BA25, BA50, BA100) compared with the control group (BA0). At both 24 and 48 h, TNF-α, IL-1β and IL-6 levels significantly decreased in BA-treated groups, with additional differences observed among BA concentrations, reflecting its potent anti-inflammatory effect (Fig. 3).

BA inhibited VEGF expression

The effect of BA on VEGF expression in BJ cells was evaluated through qRT-PCR and Western blot analysis. As displayed in the Fig. 4A-B, BA significantly decreased the mRNA levels of VEGF in a concentration-dependent manner at both 24 and 48 h. The BA25, BA50 and BA100 groups exhibited a substantial decline in VEGF mRNA expression compared to the BA0 group (all $P < 0.01$).

Furthermore, Western blot analysis confirmed that BA also reduced VEGF protein levels in BJ cells. Representative blots of VEGF and β-actin (as a loading control) are shown in the Fig. 4C-D. The intensity of the VEGF bands decreased with increasing concentrations of BA, consistent with the qRT-PCR results. The quantification of the Western blot results further supported the conclusion that BA significantly inhibited VEGF protein expression in a concentration-dependent manner (all $P < 0.01$).

DISCUSSION

The current research provides insights into how BA influences human foreskin fibroblast (BJ) cells, particularly in the context of mixed hemorrhoid treatment. The reduction in VEGF expression and inhibition of cell migration observed in this study suggest that BA may interfere with key signaling pathways involved in angiogenesis and inflammation, consistent with previous reports (Lee *et al*., 2021). VEGF is well-known for its role in promoting angiogenesis and enhancing vascular permeability, both of which are critical in the progression of hemorrhoids, as described by Pérez-Gutiérrez and Ferrara and Ghalehbandi *et al*.

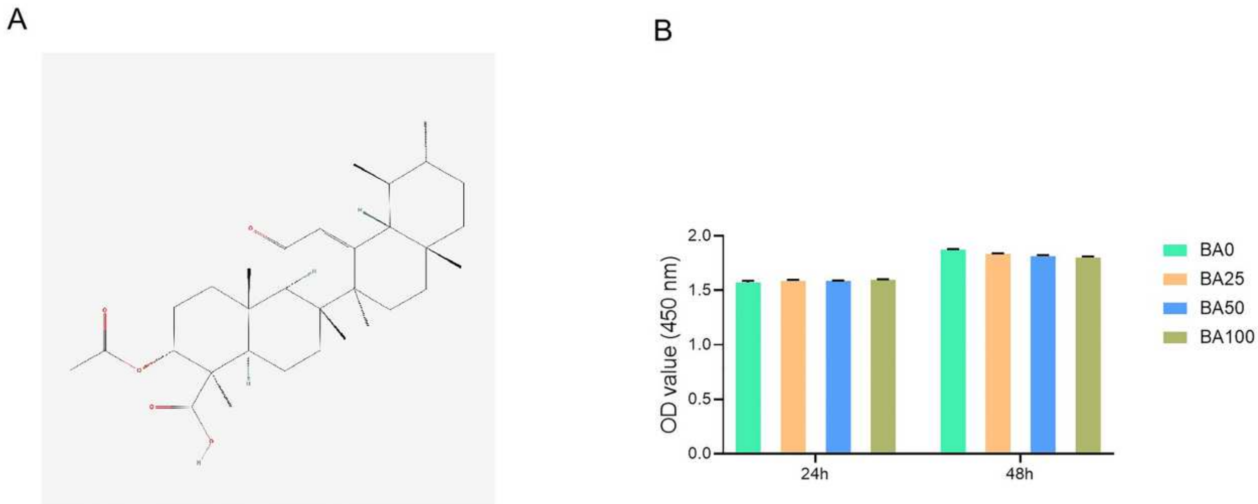

Fig. 1: Influence of BA on cell viability (n=3). (A) Chemical structure of BA. (B) Effect of BA on BJ cell viability at 24 and 48 h.

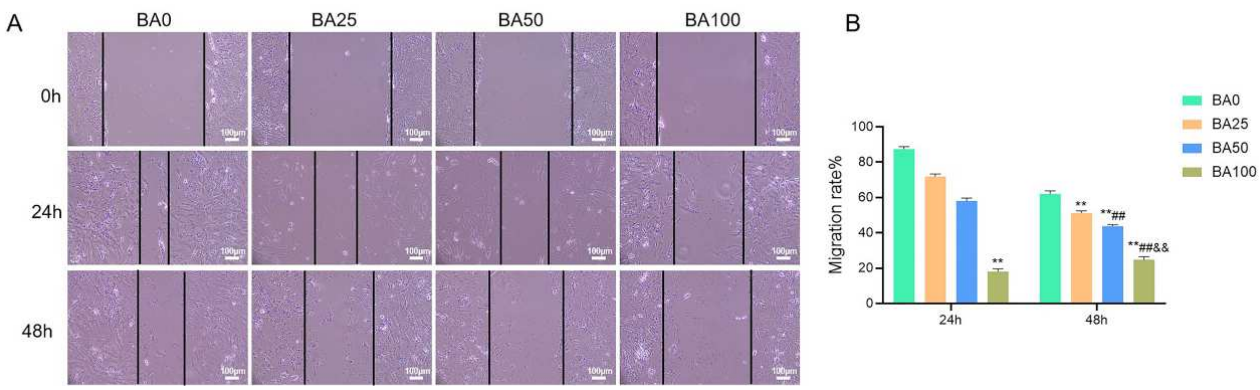

Fig. 2: BA inhibited BJ cell migration. (A) Representative images of wound healing at 0, 24, and 48 h. Scale bar = 100 μm. (B) Migration rate (%) of BJ cells at 24 and 48 h after BA treatment. **P < 0.01 vs. BA0 group; ##P < 0.01 vs. BA25 group; &&P < 0.01 vs. BA50 group.

(Ghalehbandi *et al*., 2023, Pérez-Gutiérrez and Ferrara, 2023) By inhibiting VEGF expression, BA could potentially limit the formation of new blood vessels and reduce the inflammatory response, thereby alleviating symptoms associated with hemorrhoids. This aligns with previous studies, such as those by Elnawasany *et al*.(Elnawasany *et al*., 2023) and Elmoslemany *et al*.(Elmoslemany *et al*., 2024) who stated the anti-angiogenic properties of BA in cancer models.

The data also suggest that BA treatment led to a downward trend in basal TNF-α, IL-1β and IL-6 levels in BJ fibroblasts, indicating potential cytokine-modulatory effects even in the absence of inflammatory stimulation. These cytokines are key mediators of the inflammatory process and are known to exert a significant function in hemorrhoid pathogenesis, as demonstrated by Ayun and Elya (Ayun and Elya, 2020). Siddiqui *et al*. (Siddiqui *et al*., 2021) claimed that BA reduced pro-inflammatory cytokine production in other inflammatory conditions, supporting our findings that it may function as an inflammation-suppressing agent with high efficacy. The relevance of this study to hemorrhoidal disease is evident through the clear demonstration of BA's effects on key cellular processes involved in the condition. In comparison to existing treatments that primarily focus on symptom relief, such as topical creams and surgical interventions, as discussed by Devi *et al*.(Devi *et al*., 2023). BA introduces a novel treatment approach by engaging the core mechanisms involved in the progression of disease. Gravina *et al*. (Gravina *et al*., 2021) have similarly explored the use of natural compounds, like flavonoids, in hemorrhoid therapy for their notable anti-inflammatory and vascular-protective properties, which corresponds well with our observations.

The findings of this research are supported by robust data that highlight the efficacy of BA in modulating key processes in hemorrhoid pathogenesis.

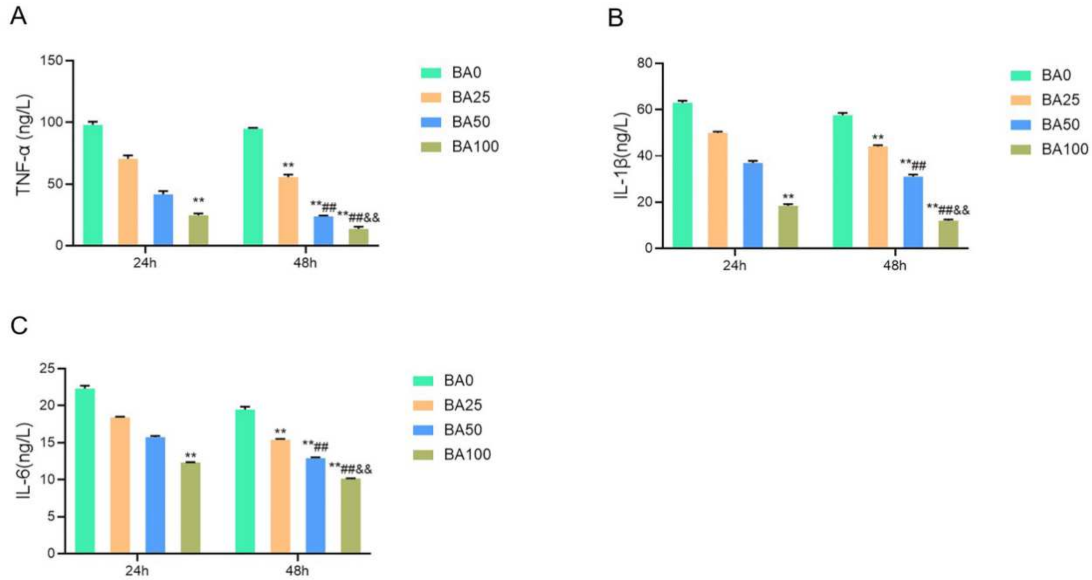

Fig. 3: BA reduced inflammatory cytokine levels. (A-C) ELISA was used for quantifying TNF-α, IL-1β, and IL-6 levels in the culture supernatants of BJ cells treated with BA (0 μM, 25 μM, 50 μM, 100 μM) for 24 and 48 h. **P < 0.01 vs. BA0 group; ##P < 0.01 vs. BA25 group; &&P < 0.01 vs. BA50 group.

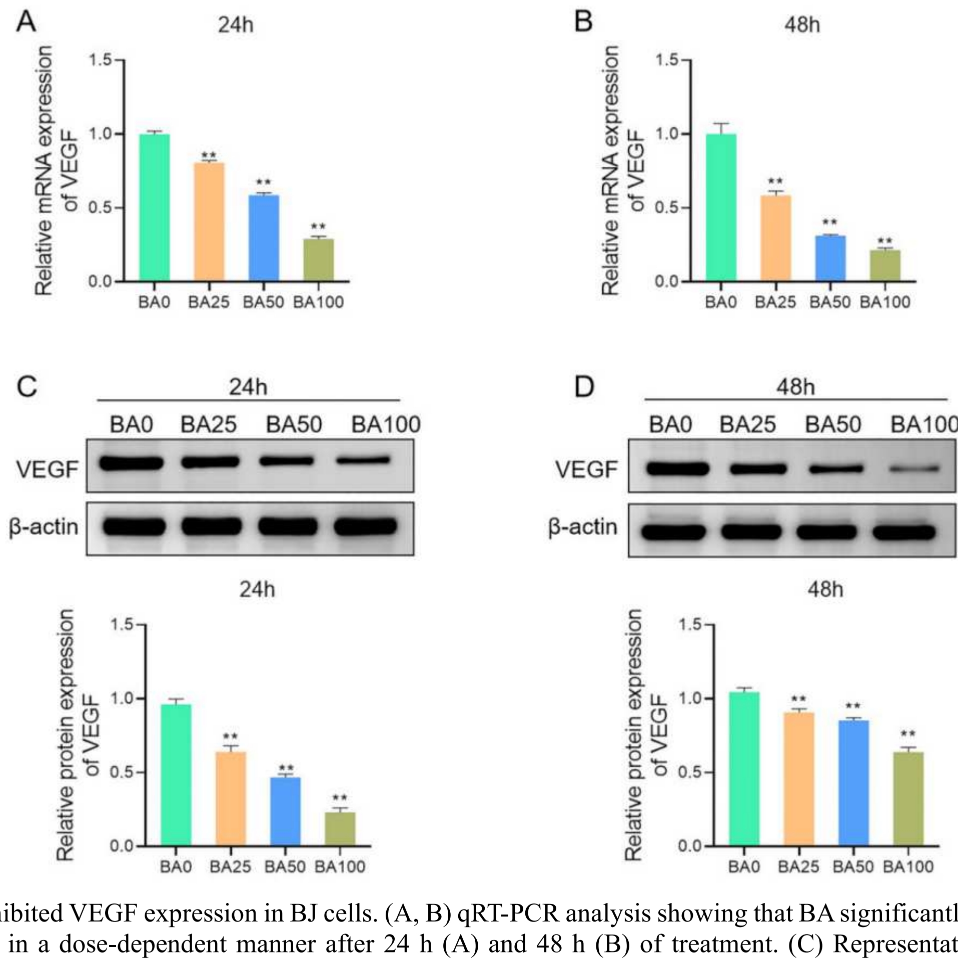

Fig. 4: BA inhibited VEGF expression in BJ cells. (A, B) qRT-PCR analysis showing that BA significantly reduced VEGF mRNA levels in a dose-dependent manner after 24 h (A) and 48 h (B) of treatment. (C) Representative Western blot images of VEGF and β-actin proteins in BJ cells treated with 0 μM, 25 μM, 50 μM and 100 μM BA for 24 h and 48 h. (D) Densitometric quantification of VEGF protein expression normalized to β-actin, confirming a concentration-dependent reduction following BA exposure.

The dose-dependent inhibition of cell migration and reduction in VEGF expression and inflammatory cytokines offer compelling evidence supporting the potential application of BA in treating hemorrhoids. The results also suggest that BA may have broader applications in other conditions characterized by excessive angiogenesis and inflammation, such as varicose veins and chronic venous insufficiency. Cicero *et al*. (Cicero *et al*., 2018) have also suggested that BA may have potential applications in such vascular conditions.

Nevertheless, acknowledging the methodological constraints of this study is important. First, the experiments were conducted in vitro, using human foreskin fibroblast cells, which might only partially reflect the complex environment of hemorrhoidal tissue *in vivo*. The effects of BA on other cell types, were not examined. Additionally, the sustained impacts of BA treatment on hemorrhoid recurrence and tissue repair were not assessed. These limitations highlight the need for future research, including in vivo studies and clinical trials, to confirm the findings and determine the therapeutic potential and safety profile of BA. In conclusion, this study demonstrates BA's potential utility as a novel intervention in the hemorrhoidal disease management. By targeting VEGF expression and inflammatory pathways, BA addresses key mechanisms underlying the condition, offering a promising alternative to existing treatments that focus primarily on symptom relief. BA could represent a valuable addition to the therapeutic arsenal for hemorrhoids, potentially improving patient outcomes and reducing the burden of this common condition.

CONCLUSION

This paper underscores the emerging therapeutic potential of BA for hemorrhoidal disease by targeting VEGF expression and inflammatory pathways, offering a unique approach beyond symptomatic relief. The findings demonstrate that BA inhibits fibroblast migration and lowers pro-inflammatory cytokine levels, making it a promising candidate for further research and clinical application. Clinically, BA could improve patient outcomes by addressing the underlying pathophysiological mechanisms of hemorrhoids, potentially reducing recurrence rates and offering a safer, more targeted treatment option. Validation through *in vivo* experimentation and long-term efficacy assessments should be prioritized in subsequent studies.

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Author's contributions
All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Data availability statement
The data used to support the findings of this study are available from the corresponding author upon request.

Ethical approval
Not applicable.

Conflict of interest
The authors declare that there are no conflicts of interest regarding the publication of this paper.

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