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RESEARCH ARTICLE

Diosmetin Suppresses *S. aureus*-Triggered Inflammation in Bovine Mammary Epithelial Cells by Modulating the NF-κb/iNos/No Cascade

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ABSTRACT

Diosmetin, is a bioactive flavonoid component of numerous traditional Chinese medicinal herbs such as *dandelion* and *honeysuckle*, which has clear anti-inflammatory properties. Based on this efficacy, we examined the anti-inflammatory potential of diosmetin using network pharmacology and in vitro validation. Network pharmacology analysis screened out 41 high-probability anti-inflammatory targets of diosmetin. Enrichment analyses showed that these targets were mainly related to reactive nitrogen metabolism, and the NF-κB cascade served as a dominant hub. In primary bovine mammary epithelial cells (bMECs) model, it was confirmed that diosmetin pre-treatment suppressed IκBα and p65 phosphorylation, blocked NF-κB/iNOS/NO cascade and dimimished the production of IL-1β, TNF-α and IL-6. Our results show that diosmetin exerts anti-inflammatory effect against *S. aureus* infection in bMECs by targeting the NF-κB/iNOS/NO signaling axis, providing a new mechanistic basis for its potential in clinical veterinary practice

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INTRODUCTION

Bovine mastitis is a common disease in dairy farming worldwide (Dhital *et al*., 2026). *Staphylococcus aureus* is known for causing persistent and difficult-to-eradicate subclinical infections that can lead to reduced milk production and quality, resulting in significant economic losses (Vidlund *et al*., 2024; Fotou *et al*., 2026). Antibiotics are an effective means of treating mastitis, but long-term use of antibiotics can lead to an increase in antibiotic-resistant bacteria (Li *et al*., 2026). Therefore, development of novel, effective and residue-free treatment strategies have become important in veterinary clinical practice (Xia *et al*., 2025; Zafar *et al*., 2025).

Traditional Chinese medicines (TCMs) has the advantages of abundant resources, low toxicity, and few side effects (Wu *et al*., 2023). Due to their anti-inflammatory and antibacterial activity, herbs with "heat-clearing and detoxifying" properties, such as dandelion and honeysuckle, have enormous potential in the prevention and treatment of mastitis in dairy cows (Yang *et al*., 2024; Fan *et al*., 2025). Diosmetin is the main active flavonoid in these herbs (containing up to 30% in honeysuckle extract) (Li *et al*., 2022). Therefore, clarifying its anti-inflammatory mechanism may be helpful in mastitis treatment.

bMECs are key cells of the mammary innate immunity system, recognizing pathogens through pattern recognition receptors and triggering host defense (Slepicka *et al*., 2021; Engler *et al*., 2025; Zhou *et al*., 2026). NF-κB signaling is critical for the inflammatory cascade. Under stimulation by *S. aureus*, signals converge on the IKK complex, leading to IκB phosphorylation and degradation, thereby releasing p65/p50 dimer, which translocates into the nucleus, where it induces the transcription of downstream mediators and inducible nitric oxide synthase (iNOS). thereby catalyzing the production of nitric oxide (NO). As a key inflammatory mediator NO can aggravate tissue oxidative stress and damage (Poma, 2020). Therefore, intervening in the NF-κB/iNOS/NO pathway may help in the treatment of bovine mastitis.

Based on the anti-inflammatory properties of diosmetin and the regulatory effects of NF-κB/iNOS/NO on inflammation, we hypothesized that diosmetin can inhibit this pathway and alleviate the inflammatory damage caused by *S aureus* in bMECs. To verify this scientific issue, this research systematically explored the diosmetin's anti-inflammatory efficacy through a

comprehensive strategy of network pharmacology prediction and targeted experimental verification. Specifically, first, predict the potential anti-inflammatory targets of diosmetin through network pharmacology analysis. Then, a cell model was established to experimentally verify this prediction, thus clarifying the application of diosmetin in the treatment of bovine mastitis from a pharmacological perspective.

MATERIALS AND METHODS

Network pharmacology analysis of diosmetin: Obtained the diosmetin structure from PubChem database. Its potential target genes were retrieved from TCMSP (v2.3; accessed on 21 December 2025; http://tcmspw.com/). Inflammation-related genes were systematically collected from OMIM and GeneCards via their official portals. GeneCards data were obtained from v5.26, while the OMIM database used the current version. Both databases were accessed on 21 December 2025. During the search the keyword was set to "inflammation". A Venn diagram was employed to visualize the overlapping genes.

PPI network building: Upload the overlapping genes to the STRING platform to build the PPI network. Set the species to "Bos taurus" to ensure biological relevance to our experimental model. Cytoscape (v3.9.1) was used to display the results. Use CytoHubba and NetworkAnalyzer in Cytoscape to identify hub genes.

GO function annotation and KEGG pathway analysis: Using the Metascape platform (https://metascape.org/) to perform GO annotation and KEGG analysis to elucidate the biological functions and related signals of intersection targets. The GO annotation covered three categories (cellular components, molecular functions, and biological processes), while KEGG mapping was conducted to identify their involvement in signaling pathways. $p<0.01$ and *enrichment factor>1.5* are considered statistically significant.

Isolation and cultivation of bMECs in vitro: Primary cells were isolated via surgical biopsy at healthy, mid-lactation Holstein cows from a commercial dairy farm in Shandong, China. The biopsy was performed under xylazine sedation and local lidocaine anesthesia, following aseptic surgical procedures. Approximately 0.5–1.0cm³ of mammary parenchyma was collected from the upper region of the gland using sterile instruments. The tissue was immediately placed in sterile PBS with antibiotics and transported on ice within 30minutes. bMECs were isolated using type I collagenase (0.1%), which was purchased from Sigma (USA). DMEM/F12 medium containing fetal bovine serum (10%) was used to culture the cells. The mediun and serum were all purchased from Gibco (USA). In order to promote cell growth, we added some additional growth factors: insulin (5μg/mL), hydrocortisone (1μg/mL) and EGF (10ng/mL), all purchased from Sigma (USA). The incubator was set at 37°C with 5% CO_2. Differential trypsinization was employed to remove fibroblasts in order to obtain a purified bMEC population. Cells from the first four generations were used for this study.

Preparation of *Staphylococcus aureus*: Culture *Staphylococcus aureus* strain ON138914 (a multidrug-resistant strain isolated from mastitic milk) in LB broth (Liu *et al*., 2022). Determine the bacterial concentration by serial dilution and adjust the multiplicity of infection (MOI) to 1:1 for subsequent experiments.

Assessment of diosmetin cytotoxicity: Screening the safe concentration of diosmetin using the methyl thiazolyl tetrazolium (MTT) method. Cells inoculation: 1×10^4cells/well in 96-well plates. Once cells had fully adhered, they were exposed to different concentrations of diosmetin (0-6.4μmol/L) for 12hours. After adding 20μL of MTT, the cells were incubated for an additional 4hours. Following incubation, we removed the culture medium and replaced with 150μL DMSO per well, The final DMSO concentration was less than 0.1%, it will not interfere with cell viability. Cell viability curves were curve based on the absorbance values measured at 570nm (Tecan Infinite M200 Microplate reader, Grödig, Austria).

Experimental design and grouping: Based on the results of MTT. 0.8μmol/L diosmetin was selected as a non-cytotoxic concentration for subsequent experiments. bMECs were grouped randomly into four experimental groups: Normal Group (CG): Cells grew in normal medium and did not receive any treatment; Diosmetin Group (DioG): Diosmetin (0.8μmol/L) was administered to cells for 12hours; Model Group (Mod): Cells were stimulated by *S. aureus* (MOI=1:1) for 30minutes; Diosmetin Pre-treatment Group (DioTG): Cells were first pre-treated with diosmetin for 12hours, and then stimulation by *S. aureus* (MOI=1:1) for 30minutes. These conditions have been consistently used in our previous studies on *S. aureus*-induced mastitis (Wang *et al*., 2018). After the respective treatments, cells and/or culture supernatants were collected for various analyses.

Protein expression of the NF-κB/iNOS cascade: Cells from each group were lysed in RIPA to extract total protein. The protein content was measured by bicinchoninic acid (BCA) protein assay kit (BioChain Institute Inc., Newark, CA, USA). The protein expression levels of NF-κB/iNOS cascade were analyzed by Western blot following the method established in previous study (Liu *et al*., 2024).

Measurement of NF-κB/iNOS pathway downstream products: Total RNA was prepared using TRIzol reagent. The RNA concentration and purity were assessed with a UV-Vis spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific), and RNA with an A260/A280 ratio between 1.8 and 2.0 was selected for subsequent studies. Use agarose gel electrophoresis to detect RNA integrity. Total RNA was synthesized to cDNA using the PrimeScript RT Reagent Kit (Takara, Japan), The qPCR assay was carried out on a conduct real-time PCR detection system (CFX, Bio-Rad, USA). Each PCR mixture contained: SYBR Green qPCR mix (10μL), primers (0.8μL each of F and R, 10μmol/L), cDNA (2μL), and nuclease-free water to a final volume of 20μL. The primer sequences and reaction efficiency were determined in previous studies (Wang *et al*., 2018; Table 1). The PCR cycling: 95°C for 30s; 40×(95°C for 5s, 60°C for 30s, 72°C for 30s); 72°C

(final extension). The target gene expression was normalized to β-actin using the 2^(-ΔΔCt) method. All reactions were run in triplicate.

Measurement of Nitric Oxide (NO) production: The Griess reagent method was used to determine the concentration of nitrite (NO_2^-) in cell culture supernatant to assess NO production. The specific steps are as follows: equal volumes of Griess Reagent I, cell-free supernatant and Griess Reagent II (50μL each, the reagent preparation methods are all configured according to the reagent kit instructions). to the 96-well plate in sequence (Beyotime Biotechnology, China). Incubated the mixture in the dark before detection (25°C, 10minutes). After incubation the absorbance of reaction solution at 540nm was recorded, and the nitrite production was calculated using a standard curve of known concentrations. Results were expressed as micromolar (μmol/L).

Statistical analysis: Data were presented as mean±SD from at least three independent experiments, and were analyzed with SPSS software (version23.0, USA). Two-tailed Student's t-test and One-way analysis of variance (ANOVA) were used for comparisons between two-groups and among multiple-groups respectively. Significance was set at $P<0.05$. Figs were plotted by GraphPad Prism software (version8.0, USA).

RESULTS

Impact of diosmetin on bMEC viability: Primary bMECs were successfully isolated by collagenase type I digestion. After approximately 12hours of adherent culture, the tissue explants were firmly attached to the surface of the culture dish (Fig. 1a). After 48hours of culture, a heterogeneous population of cells, mainly epithelial-like bMECs mixed with a smaller number of fibroblast-like cells, had migrated out of the explants and begun to proliferate (Fig. 1b). After purification by differential trypsinization digestion, a highly enriched bMECs population was obtained. These purified cells exhibited typical epithelial "paving stone"-like morphology and formed a unique, interconnected "garland-like" structure, while the remaining fibroblasts appeared as parallel rows of elongated spindle-shaped cells. To assess purity, ten fields were randomly selected at 200× magnification, and the percentage of epithelial cells (>90% purity) was calculated based on morphological characteristics (Fig. 1c). The cytotoxicity experiment of diosmetin is shown in Fig. 1d, bMECs were treated with diosmetin at a as high as 0.8μmol/L for 12hours.Relative to the untreated group, the cell viability was not significantly reduced ($P>0.05$). However, at higher concentrations (1.6μmol/L and above), diosmetin results in a marked decrease in cell viability in a dose-related manner. Therefore, 0.8μmol/L diosmetin was selected for subsequent research.

Identification of anti-inflammatory targets of diosmetin: The network pharmacology workflow yielded 43 potential pharmacodynamic targets of diosmetin from the TCMSP database. A comprehensive search for "inflammation" in the OMIM and GeneCards databases, after removal of duplicates, resulted in 15,161 unique inflammation-associated genes. Intersection analysis revealed 41 common genes between the diosmetin targets and the inflammation gene set, representing its putative anti-inflammatory targets (Fig. 2).

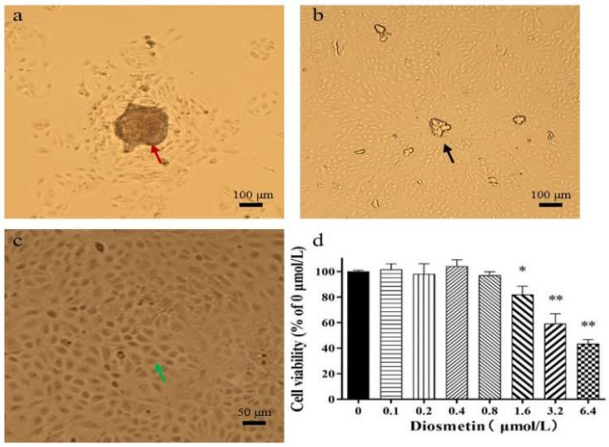

Fig. 1: Isolation of primary bovine mammary epithelial cells and cytotoxicity analysis of diosmetin. (a) After 12hours of type I collagensae digestion, the tissue block adheres to the surface of the culture plate (red arrow). (b) After 48hours of culture, Cells migrated out of the tissue block, mainly bMECs, accompanied by a small number of fibroblasts (black arrow). (c) Primary bMECs purified by differential trypsin digestion form a characteristic "garland-like" structure (green arrow). (d) Effect of diosmetin on bMECs viability.

Table 1: Details of oligonucleotide primers

Gene	F-Primer (5'→3')	R-Primer (5'→3')	Product Size (bp)
TNF-α	CTGCTGACGGGCTTTACC	GACTGCAATGCGGCTGAT	115
IL-1β	GCTATGAGCCACTTCGTGAGGAC	GATTGAGGGCGTCGTTCAGGAT	138
IL-6	TGATGACTTCTGCTTTCCCTACCC	ATCTTTGCGTTCTTTACCCACTCG	164
β-actin	ACATCCGCAAGGACCTCTA	CCATGCCAATCTCATCTCGTT	220

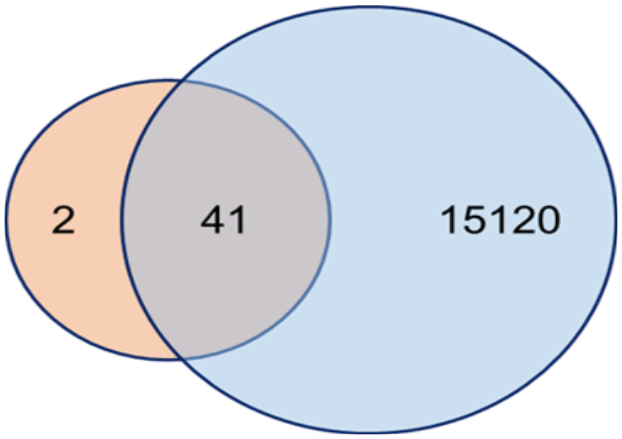

Fig. 2; Analysis of the intersection between the targets of diosmetin and inflammation.

The intersection of 43 predicted pharmacodynamic targets of diosmetin (from TCMSP database) and 15,161 inflammation-related genes (from OMIM and GeneCards databases). The overlap comprises 41 genes, representing potential anti-inflammatory targets of diosmetin.

Molecular docking analysis of diosmetin against anti-inflammatory targets: In order to strengthen the discovery of bioinformatics, four core targets including CALM1, DPP3, HSP90AA1, and NOS1 were randomly selected, and In silico docking analysis was conducted to further elucidate the docking poses and affinity of diosmetin against these core targets. The results are shown in Fig. 3. diosmetin exhibit good binding affinity with the above core targets, with biding energies of -8.27, -9.06, -8.44, and -9.35 respectively. The three-dimensional conformations revealed that the ligands are stably embedded into the active pocket of the core target proteins. These tight interactions further stabilize the structure of the ligand-protein complex and provide the molecular basis for its inhibition/activation mechanism.

PPI network building: The results show that the PPT network diagram contains 40 nodes and 109 edges (Fig. 4a). The network exhibited significant connectivity (PPI enrichment *p-value*$<1.0 \times 10^{-16}$). Topological analysis identified several hub genes with high node degrees, including estrogen receptor 1 (ESR1), prostaglandin E synthase 3 (PTGES3), nitric oxide synthase 3 (NOS3), and heat shock protein 90 (HSP90) (Fig. 4b). These hub genes are functionally implicated in steroid hormone signaling, prostaglandin metabolism, NO synthesis, and cellular stress response—processes intimately linked to inflammation regulation.

GO and KEGG enrichment analyses: Functional enrichment analysis offered comprehensive understanding of the anti-inflammatory potential of diosmetin. As shown in Fig. 5a, The top enriched biological processes (BP) included "RNS-MP," "NOS activity regulation," and "NO signal transduction." Key molecular functions (MF) were "nitric-oxide synthase regulator activity" and "heme binding." All terms were annotated with gene counts≥4 and FDR-adjusted P-values<0.01.KEGG pathway enrichment (Fig. 5b) identified inflammation-relevant cascades, with the "NF-κB cascade" ranking among the top enriched pathways. Other significant pathways included "IL-17 cascade," "NOD-like receptor cascade," and "TNF cascade." All data satisfied gene counts>3 and FDR<0.05. The dual enrichment in NO metabolism (GO) and NF-κB/inflammatory signaling (KEGG) indicated that diosmetin may play anti-inflammatory effects by regulating nitric oxide biosynthesis and suppressing NF-κB-driven transcriptional responses, a hypothesis that is now testable via in vitro validation.

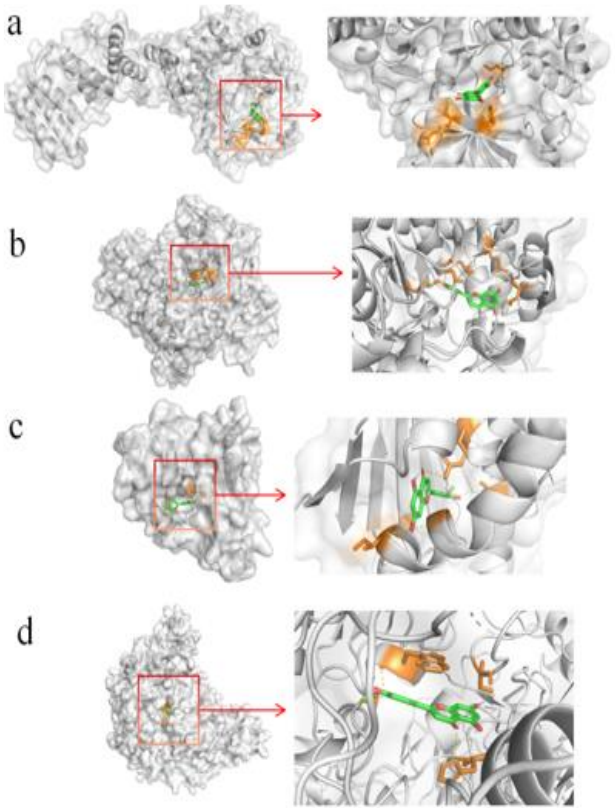

Fig. 3: Molecular docking analysis of diosmetin against anti-inflammatory targets. (a) The diosmetin binding pocket on CALM1 surface. (b) The diosmetin binding pocket on DPP3 surface. (c) The diosmetin binding pocket on HSP90AA1 surface. (d) The diosmetin binding pocket on NOS1 surface.

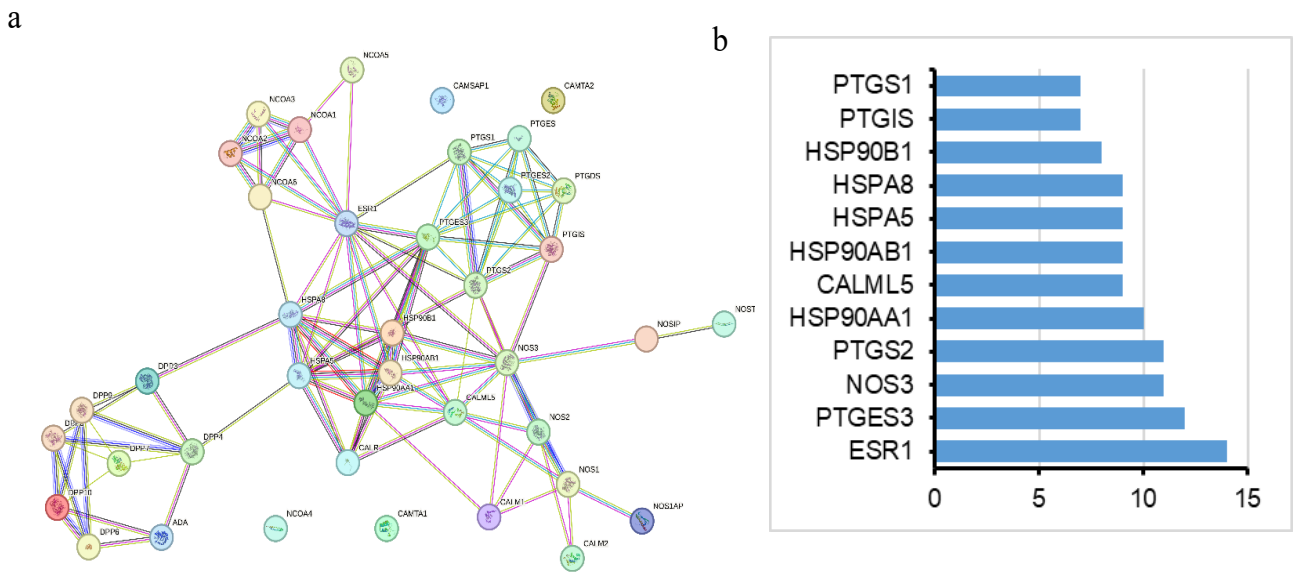

Fig. 4: PPI network and Topological analysis of core target. (a) PPI network. The mean node degree was calculated to be 5.45, and the *p-value* was found to be below the significance level. (b) Topological analysis of core target. Key genes include ESR1, PTGES3, NOS3, PTGS2, and HSPs.

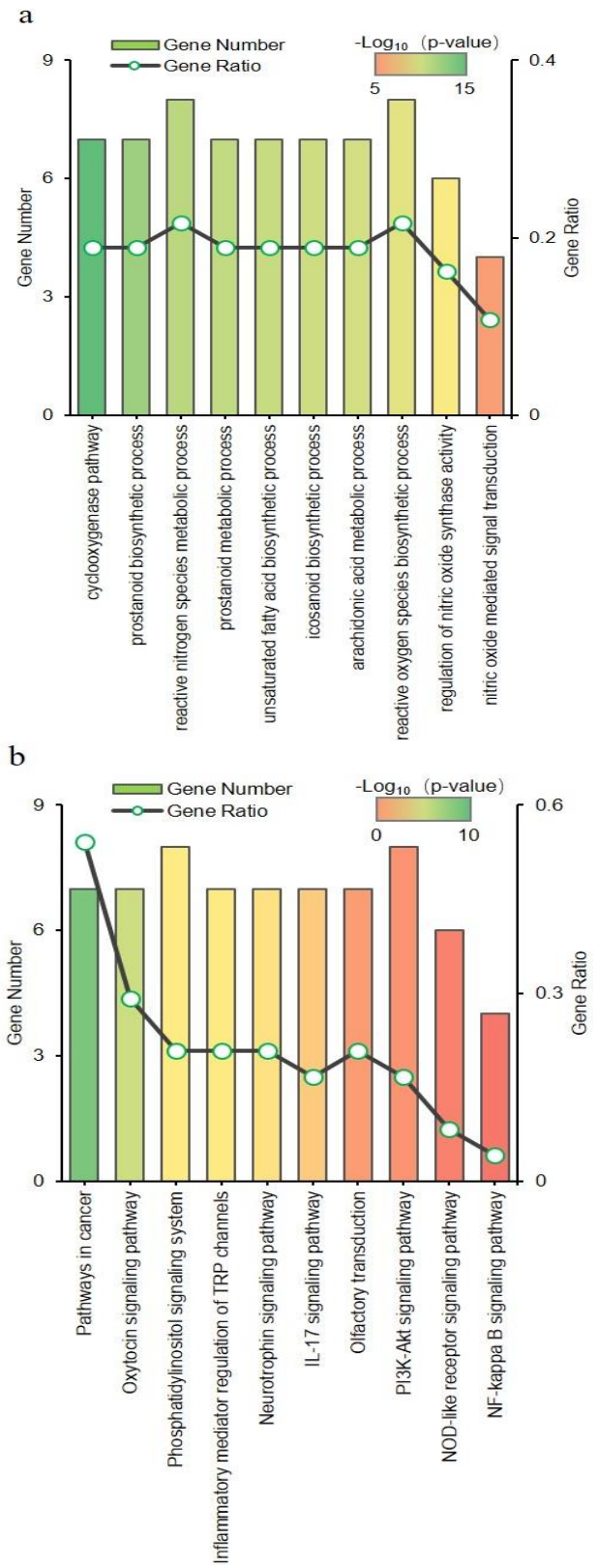

Fig. 5: GO and KEGG annotation. (a) GO annotation: Bars represent gene counts; colored bars indicate -$\log_{10}$(p-value) (green to red gradient); black line shows gene ratio. (b) KEGG annotation: Same visualization as (a). Top pathway "NF-kappa B signaling pathway" has $P=2.1\times10^{-6}$. All P-values are FDR-adjusted.

Diosmetin inhibits *S. aureus*-induced inflammatory damage in bMECs by regulating the signal transduction of NF-κB/iNOS pathway: The protein expression levels of NF-κB/iNOS cascade were analyzed by Western blot. The p-IκBα protein and p-P65 protein were normalized to β-actin. The pathway was activated after bMECs were infected with *S. aureus* for 30minutes (Fig. 6, compared with CG). This was evidenced by a marked increase in the phosphorylation levels of both IκBα (p-IκBα) and the p65 subunit (p-P65) in Mod vs CG. Pre-treatment with 0.8μmol/L diosmetin (DioTG) effectively mitigated this activation, significantly reducing the *S. aureus*-induced phosphorylation of IκBα and p65 back towards baseline levels. In DioG the pathway status was basically the same as CG. These results indicate that inhibits *S. aureus*-induced inflammatory damage in bMECs by regulating the signal transduction of NF-κB/iNOS cascade.

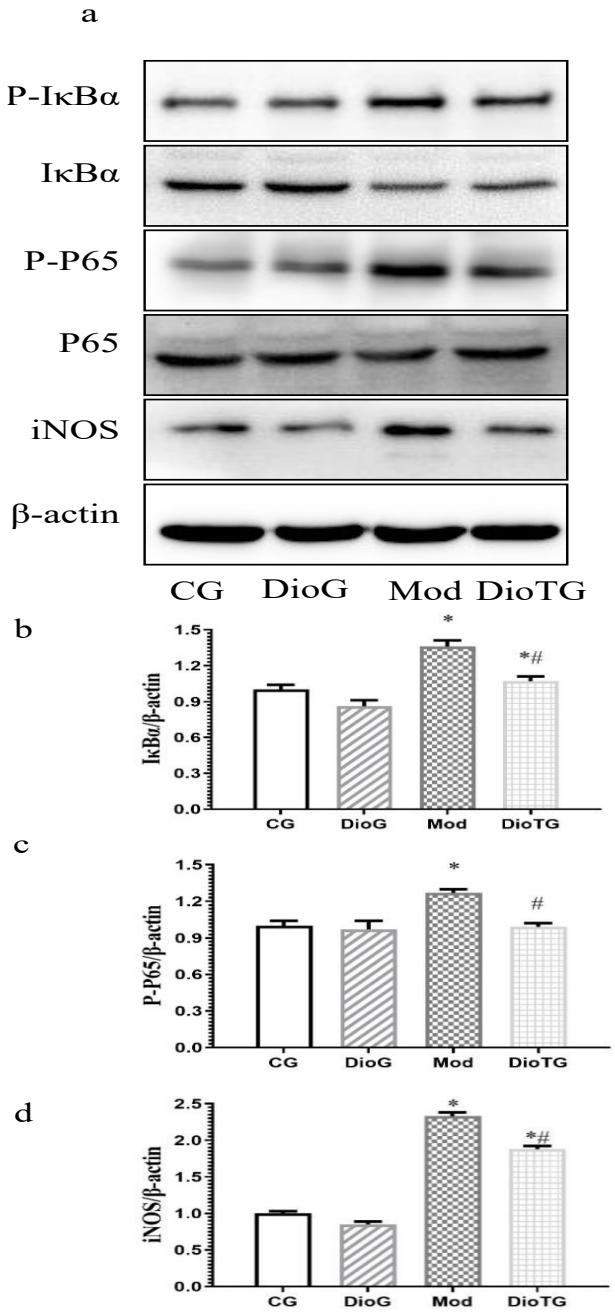

Fig. 6: Diosmetin inhibits *S. aureus*-induced inflammatory damage in bMECs by regulating the signal transduction of NF-κB/iNOS pathway. (a) The NF-κB/iNOS cascade-related proteins expression. (b-d) Quantitative expression of p-IκBα protein (b), p-p65 protein (c), and iNOS protein. (d). bMECs were pretreated with or without diosmetin followed by infection with *S. aureus*. CG, control group; DioG, diosmetin-only group; Mod, model group (*S. aureus* only); DioTG, diosmetin pretreatment+*S. aureus* group.

Diosmetin suppresses inflammatory cytokine expression and NO release: Activated NF-κB transcribes a broad array of genes encoding pro-inflammatory mediators. The mRNA expression of three cytokines was significantly upregulated in the MG vs CG (P<*0.05*). Diosmetin pretreatment markedly downregulated these cytokines in the DioG vs MG (P<*0.05*; Fig. 7a-c). At last, the functional consequence of iNOS inhibition was assessed by measuring NO release. *S. aureus* stimulation caused a dramatic surge in NO concentration in the culture supernatant (30.68±2.15μmol/L) in the MG vs CG (6.11±0.87μmol/L) (P<0.05). Pre-treatment with diosmetin significantly blunted this NO release, reducing it to 22.95±1.76μmol/L in the DioG vs Mod (P<0.05; Fig. 6d).

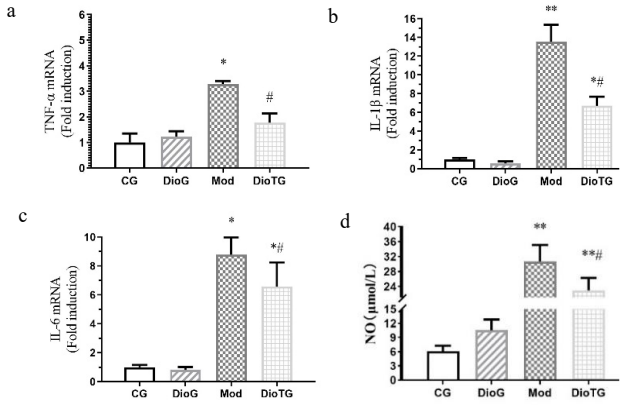

Fig. 7: Diosmetin downregulated the expression of these three cytokines and NO release. (a-c) TNF-α transcripts (a), IL-1β transcripts(b), and IL-6 transcripts(c). (d) NO production. Experimental groups and treatments are the same as described in Fig. 5.

DISCUSSION

The emergence of antibiotic-resistant bacteria and the need for clean dairy products have spurred the search for natural medicines (Ahmad *et al*., 2024). TCM, with its advantages of multiple targets and multiple pathways, provides a source of such alternatives (Hu *et al*., 2020; Liu *et al*., 2022; Javed *et al*., 2024). In this work, we assessed the anti-inflammatory properties of diosmetin, a key flavonoid derived from anti-mastitis herbs such as dandelion and honeysuckle, and elucidated its underlying molecular mechanism in bMECs.

Firstly, we applied network pharmacology to screen 41 candidate anti-inflammatory modules. Enrichment analysis of the above targets showed that nitric oxide (NO) biology and NF-κB signaling pathway may be its potential anti-inflammatory pathways. To further corroborate these predictions, we employed docking assays to dock diosmetin into the binding pockets of the hub proteins screened in the network pharmacology. The docking results show that diosmetin exhibits strong binding affinity to these targets, and its binding energy is comparable to or better than the respective original ligands, suggesting the existence of favorable molecular interactions at the active sites. This calculation prediction is extremely accurate, because our cell experiments also confirmed that the effect of diosmetin is indeed concentrated on the NF-κB/iNOS/NO axis. This result validates the accurate and effectiveness of network pharmacology in drug development.

The NF-κB cascade is a canonical transcription factor during bovine mastitis (Ma *et al*., 2019). Our data clearly show that diosmetin can regulate this pathway by inhibiting the p-IκBα expression. By stabilizing this pathway, diosmetin further prevents the release and nuclear translocation of p65/p50 dimers, effectively silencing the NF-κB transcriptional program. This mechanism is crucial because it will further affect the release of pro-inflammatory mediators. The downstream consequences of NF-κB inhibition are clear. First, diosmetin significantly reduced the TNF-α, IL-1β, and IL-6 expression (Simon *et al*., 2015; Zhuang *et al*., 2020; Chen *et al*., 2022). These cytokines are not only markers of inflammation but also powerful drivers of the inflammatory cascade, recruiting immune cells and inducing tissue damage (Vitenberga-Verza *et al*., 2022; Xiao *et al*., 2025). Therefore, reducing their production is a key therapeutic goal during mastitis treatment. Secondly, diosmetin effectively inhibited iNOS expression and NO overproduction. While physiological levels of NO play a role in vasodilation and microbial killing, during sever inflammation, excessive and sustained NO production catalyzed by iNOS peroxynitrite ($ONOO^-$), a strong oxidant that will lead to various forms of cell damage. Therefore, the ability of diosmetin to inhibit this "NO burst" predicts its potential as a key cytoprotective agent to limit oxidative stress-mediated damage to breast tissue. These findings are particularly promising for treating mastitis in dairy cows. Notably, a recent in vivo study showed that diosmetin significantly alleviated *S. aureus*-induced mastitis in mice by reducing inflammation and ferroptosis (Zhao *et al*., 2023). These results in turn strengthen the rationale for developing diosmetin as a natural multi-target drug for the prevention and control of bovine mastitis.

It is particularly critical to place the effects of diosmetin within a broader pharmacology context. Recent studies have shown that diosmetin also has other beneficial properties, including antioxidant activity through the Nrf2 pathway, anti-apoptotic effects, and modulation of the gut microbiota (Favarin *et al*., 2024). Although this study focused on one key pathway, its benefits in complex diseases such as mastitis may be pleiotropic. The network pharmacology results hint at potential interactions with other pathways such as other signaling pathways, which are also critical in inflammatory defense. Future studies should explore these additional dimensions and identify potential synergistic effects with low-dose of antibiotics or other chemicals, and more importantly, build on these in vitro findings and further validate them in *in vivo* models.

This study confirms that diosmetin can reduce the inflammatory damage in *S. aureus* infected bMECs by regulating the NF-κB signaling pathway, down-regulating iNOS expression and NO production, and reducing cytokines expression. These findings elucidate the modern pharmacological basis of diosmetinn and pinpoint a potential target for managing bovine mastitis.

Conclusions: This study combines research in network pharmacology and molecular cell biology to elucidates the mechanistic basis of diosmetin's anti-inflammatory effect on *Staphylococcus aureus* in bMECs, and identifies the

NF-κB/iNOS/NO signaling axis as a key therapeutic target. From a clinical perspective, diosmetin represents a promising active ingredient of Chinese herbal medicine for the development of novel preventive or adjunctive therapies for bovine mastitis and has the potential to reduce reliance on traditional antibiotics. At the methodological level, the high agreement between anti-inflammatory target prediction and experimental validation demonstrates the value of integrating network pharmacology with in vitro studies as a framework for reproducible research in veterinary pharmacology. However, given that the present study were in vitro experiments, more validation experiments are necessary to supplement the results to fully reveal the function of diosmetin in veterinary practice.

Conflict of Interest: The authors have no competing interests to declare.

Ethics Statement: All animal experiments were approved by the Institutional Animal Care and Use Committee of Linyi University and Hebei Agricultural University, The protocol was designed to minimize animal suffering and the number of animals used.

Authors Contribution: CLB designed the experiments and revised the paper; JJL performed the experiments and wrote the paper; ZNW, RJL, and MQC analyzed the data; JXX and ZQH provided materials and analytical support.

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