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The mechanisms of myricetin and quercetin in regulating miRNA-140 and MMP/TIMP signaling pathway in osteoarthritis treatment

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Abstract: **Objectives**: This study investigates the combined therapeutic effects of myricetin (Myr) and quercetin (Que) on miRNA-140 regulation and the MMP/TIMP signaling pathway involved in cartilage matrix remodeling in osteoarthritis (OA). **Methods**: *In-vitro* OA models were established using IL-1β-treated CHON-001 human chondrocytes, while *in-vivo* OA models were induced by monosodium iodoacetate (MIA) injection in male SD rats. Myr (10 μM *in-vitro*; 25 mg/kg *in-vivo*), Que (50 μM *in-vitro*; 10 mg/kg *in-vivo*), or their combination was administered. CCK-8 and TUNEL assays assessed cell viability and apoptosis, while qRT-PCR evaluated miRNA-140 expression. Western blot detected MMP/TIMP proteins and ELISA quantified IL-1β, TNF-α, IL-6 and PGE2 in supernatant and serum samples. **Results**: Myr and Que individually improved chondrocyte viability and reduced apoptosis, with the combination showing the strongest effect *in-vitro*. These effects were confirmed *in-vivo*, where combined treatment alleviated cartilage damage, restored miRNA-140 expression, suppressed MMP-3 and MMP-13 and upregulated TIMP-1 and TIMP-3. TNF-α levels were significantly reduced by co-treatment, while IL-1β and IL-6 showed moderate decreases, indicating synergistic anti-inflammatory and cartilage-protective effects. **Conclusion**: These findings suggest a miRNA-140-associated regulatory relationship between myricetin/quercetin treatment and MMP/TIMP pathway modulation.

Keywords: Myricetin; miRNA-140; MMP/TIMP signaling pathway; Osteoarthritis; Quercetin

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INTRODUCTION

Osteoarthritis (OA) primarily manifests as cartilage degeneration in joints, often causing pain, reduced mobility and stiffness (Corral *et al*., 2021; B. Li, Guan, Mei, Jiao, and Li, 2021). OA pathogenesis is mechanistically complicated, with numerous factors such as mechanical stress, aging and inflammatory mediators (He *et al*., 2020) In OA, Extracellular matrix (ECM) remodeling becomes dysregulated, with degradation surpassing synthesis (Peng *et al*., 2021). Inflammatory cytokines, particularly interleukin-1 beta (IL-1β), have a central influence on accelerating ECM breakdown via promotion of matrix metalloproteinases (MMPs) expression and concurrent downregulation of tissue inhibitors of metalloproteinases (TIMPs), the inhibitors that maintain matrix balance (Kacprzak and Stanczak, 2024; Molnar *et al*., 2021; Peng *et al*., 2021). Current treatments for OA aim mainly at alleviating symptoms, such as pain and inflammation, but they fail to address the underlying cartilage degeneration. Corticosteroids and nonsteroidal anti-inflammatory agents are frequently administered to patients; however, prolonged administration can result in notable adverse effects, such as gastrointestinal disturbances as well as immunosuppression (Eren, Armağan, and Talmaç, 2016). In advanced cases, joint replacement surgery is often required, but this option is invasive, costly and not always suitable for elderly or frail patients (Sloan, Premkumar, and Sheth, 2018). Thus, the need for symptom-relieving and disease-altering therapies is acute, as such interventions should not only alleviate symptoms but also target the core molecular processes involved in cartilage degradation.

MicroRNAs (miRNAs), comprising short non-coding RNA sequences, regulate gene expression through post-transcriptional mechanisms (Bartel, 2018) Among them, miRNA-140 is identified as a key factor in cartilage matrix homeostasis (Si *et al*., 2017), especially via modulation of MMP/TIMP signaling, which control the turnover of the ECM in cartilage. Dysregulation of miRNA-140 expression correlates with cartilage degradation in OA. Increased concentrations of MMPs, including MMP-3, MMP-9 and MMP-13 promote the breakdown of cartilage matrix, while reduced expression of TIMPs exacerbates this degradation (Hu and Ecker 2021). Therapeutic modulation of miRNA-140, aimed at maintaining the equilibrium of MMPs and TIMPs, could help halt or reverse the pathogenesis of OA. MiRNA-140 plays a crucial role in cartilage protection by regulating the expression of MMPs and TIMPs. Specifically, miRNA-140 has been shown to inhibit MMP-13 and other MMPs, which are central to cartilage degradation during OA (Si *et al*., 2017). Conversely, miRNA-140 upregulates the expression of TIMPs, which counteract the activity of MMPs, maintaining the structural stability of cartilage tissue (Al-Adlaan *et al*., 2025). MiRNA-140 expression is often downregulated in OA cartilage, contributing to an imbalance between MMPs and TIMPs and leading to

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excessive cartilage breakdown (S. H. Li and Wu, 2021). Several studies have demonstrated that restoring miRNA-140 expression in OA models leads to both MMP reduction and TIMP elevation, thereby protecting cartilage from degradation (Williams, 2018). Moreover, miRNA-140 also participates in regulating inflammation, a key factor in OA pathogenesis via inhibition of key cytokines, including IL-1β (Al-Modawi, Brinchmann, and Karlsen, 2019; Duan, Liang, Xu, Xiao, and Wang, 2020). This dual role of miRNA-140, both in cartilage degradation and inflammation regulation, makes it a promising target for OA therapy.

Sendeng-4 decoction, a traditional Mongolian herbal formulation, has long been used for treating inflammatory conditions, including OA. This decoction contains several medicinal herbs, among which myricetin (Myr) and quercetin (Que) are the key bioactive components. Due to their robust antioxidant and anti-inflammatory effects, Myr and Que have been widely investigated as potential therapeutic agents for OA. They are known to regulate key molecular pathways, including modulating miRNA expression, especially miRNA-140, which is vital for cartilage stability. By targeting miRNA-140, Myr and Que may modulate the MMP/TIMP signaling pathway, thereby restoring the balance between matrix metalloproteinases and their inhibitors and protecting cartilage from degradation (Pan *et al*., 2019; Si *et al*., 2017; H. Wang *et al*., 2023; Q. Wang, Ying, Wei, Ji, and Xu, 2022) Besides their roles in cartilage regulation, Myr and Que downregulate major inflammatory mediators, including IL-1β, TNF-α and IL-6, that contribute to OA pathogenesis (Al-Khayri *et al*., 2022; Pan *et al*., 2019; J. Zhang *et al*., 2020). This anti-inflammatory effect, combined with their ability to modulate cartilage metabolism, positions Sendeng-4 decoction as a promising therapeutic candidate for OA. The synergistic effects of Myr and Que, along with other herbal components in the decoction, may offer a comprehensive approach to managing OA by addressing both the inflammatory response and the metabolic disturbances involved in cartilage degradation.

This study aims to elucidate the interactive effects between Myr and Que, as components of Sendeng-4 decoction, on the regulation of miRNA-140 and their impact on the MMP/TIMP signaling pathway in OA. It is hypothesized that these compounds, acting together, can modulate miRNA-140 expression and restore the balance between MMPs and TIMPs, providing a potential natural therapeutic strategy for OA management.

MATERIALS AND METHODS

Model establishment and grouping

CHON-001 human chondrocytes (ATCC, CRL-2846) were maintained under standard culture conditions (37ºC, 5% CO_2) in DMEM supplemented with 10% fetal bovine serum and antibiotics (penicillin 100 U/mL; streptomycin 100 μg/mL). Cells were passaged around 80% confluence and those between passages 3 and 6 were used to ensure reproducibility (Table S1).

For the establishment of the OA model, IL-1β (10 ng/mL) served as an inducer of OA-like conditions in the CHON-001 cells (Fan, Ding, and Yang, 2021; Pan *et al*., 2019; J. Zhang *et al*., 2020). This cytokine was chosen for its ability to mimic the inflammatory environment observed in OA. It was administered in culture for a 24-hour period aiming to simulate the effects of inflammation and cartilage degradation characteristic of OA. A concentration of 10 ng/mL IL-1β, previously verified to provoke inflammation and cartilage matrix loss in chondrocytes, was applied. Following 24 h of stimulation, typical osteoarthritic alterations emerged, including heightened inflammatory cytokine release and catabolic activation causing ECM damage.

Following the induction of OA-like conditions with IL-1β, the cells were divided into 5 groups for subsequent studies. The normal group (NL) received no treatment, while the osteoarthritis model group (OA) was exposed only to IL-1β and served as the disease control group. The treatment groups had the cells treated with Que (50 μM; QN), Myr (10 μM; ML), or a combination of these two compounds for 24 hours. This treatment protocol was intended to evaluate the efficacy of Que and Myr on OA-related cellular injury by assessing changes in cell viability, apoptosis and the regulation of key molecular pathways involved in inflammation and cartilage degradation.

CCK-8

Cell viability was determined using the CCK-8 method (Beyotime, C0041, China). CHON-001 cells were seeded in 96-well plates at a rate of 5,000 per well and treated for 12 hours after adhesion. Subsequently, 10 μL of CCK-8 solution was added. After incubation at 37ºC and 5% CO_2 for 2 hours, the absorbance value was read at 450 nm. Each group contains 3 replica Wells and the experiment is repeated three times (Xiao *et al*., 2020).

Combination index (CI) analysis

To further evaluate the dose-response relationship between myricetin and quercetin and their possible synergistic effects, chondrocytes were treated with IL-1β alone or in combination with these compounds for 24 hours. Cell viability was assessed using the CCK-8 assay. The CI was calculated using the Chou-Talalay method with CompuSyn software (ComboSyn, Inc., USA). The CI < 1 indicates a synergistic effect, a CI = 1 indicates an additive effect and a CI > 1 indicates an antagonistic effect(N. Zhang, Fu, and Chou, 2016).

TUNEL staining

To evaluate apoptosis, TUNEL staining was carried out with the *In-situ* Cell Death Detection Kit (Roche, Switzerland). Cells were treated with 4% paraformaldehyde for fixation and 0.1% Triton X-100 for

permeabilization before detection. Apoptotic nuclei were observed under fluorescence microscopy (Nikon, Japan) and apoptotic index was calculated from TUNEL-positive populations(Xu, Zhi, Zhang, and Ding, 2024).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using RNAiso Plus (TaKaRa, Japan), following the supplier's recommendations. A NanoDrop spectrophotometer (Thermo Fisher, USA) was used to assess RNA purity and yield. Samples were adjusted to uniform concentrations and 1 μg RNA was utilized for cDNA synthesis using the PrimeScript RT kit (TaKaRa, Japan). Gene expression of miRNA-140, Bax, Bcl-2 and Cleaved Caspase-3 was determined with SYBR Premix Ex Taq II (TaKaRa, Japan) on a StepOne Plus qPCR platform (Applied Biosystems, USA). Primers used included: miRNA-140: Forward, 5'-ATGCATAGCTGAGCGAGGA-3'; Reverse, 5'-CGTTGACTTGGAAGACCAA-3'. Bax: Forward, 5'-GGGATGACAGGATGCGTGA-3'; Reverse, 5'-GTGGAGAGTGGTGGAGGTT-3'. Bcl-2: Forward, 5'-TGAACGGGGCATCGTG-3'; Reverse, 5'-GGACGGTGTGGAAGTGAAG-3'. Cleaved Caspase-3: Forward, 5'-GGAAGCTGGGAGTAAAGCAG-3'; Reverse, 5'-AGGCCACCAGGATTAGTCA-3'. Amplification involved 95ºC for 30 sec, followed by 40 cycles of 95ºC for 5 sec and 60ºC for 30 sec. Each sample was tested in triplicate, using GAPDH as the reference gene and expression was calculated via the $2^{-\Delta\Delta Ct}$ method(Ghafari, Moqadami, and Khalaj-Kondori, 2025).

Western blot

Proteins were isolated using RIPA buffer (Beyotime, P0013B, China) and their concentrations quantified via a BCA assay kit (Guangzhou Yujia, ST2222, China). Samples underwent SDS-PAGE (Beyotime, P0014A, China) and were transferred onto PVDF membranes (Beyotime, FFP39, China). Membranes were blocked with 5% non-fat milk for 1 hour at room temperature, incubated overnight with primary antibodies at 4ºC: Membranes were incubated in 5% milk blocker and probed with primary antibodies against MMP-3(ab52915), MMP-9 (ab283575), MMP-13 (ab39012), TIMP-1 (ab109125) and TIMP-3 (ab39184) (Abcam). Once washed, membranes were exposed to HRP-linked secondary antibodies for 1 hour at ambient temperature. Protein signals were detected via the ECL Western blotting substrate (Thermo Fisher, USA) on the ChemiDoc™ MP system (Bio-Rad, USA). Quantification was performed via ImageJ 6.0.1 software (NIH, USA)(Fu *et al*., 2022).

Experimental animals, grouping and management

30 healthy SPF male Sprague-Dawley rats (8 weeks old, 200-250 g) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Sample size was determined based on previous similar studies. Animals were housed at 22 ±2ºC and 50% - 60% humidity under a 12-h light/dark cycle, with unrestricted access to food and water. After a 1-week acclimation, the rats were randomly divided into five groups using a random number table (6 in each group): Sham, OA, QN, ML and CN.

OA was induced by injecting 40 μL of 3% MIA into the right knee joint, whereas the Sham group received sterile saline. This model was selected because it reliably mimics cartilage degeneration and inflammation observed in human OA. Beginning one day after induction, rats were treated orally once daily for 14 days: QN (10 mg/kg Que), ML (25 mg/kg Myr), or CN (10 mg/kg + 25 mg/kg mixture). Sham and OA animals were given saline. The selected dosage is based on the effective and non-toxic dosage range reported in previous experiments on inflammatory models in rodents (Hannan *et al*., 2023; Yang *et al*., 2018; Zhao, Hong, Dong, Meng, and Mu, 2013). After the administration period ended, all the animals underwent ELISA, qRT-PCR and Western blotting analyses. Outcome assessment and data analysis were performed by investigators blinded to group allocation.

ELISA

At the end of the treatment period, rats were euthanized for blood collection. To ensure profound anesthesia and a painless procedure, euthanasia was performed by an intraperitoneal injection of an overdose of sodium pentobarbital (150 mg/kg). Once the absence of pedal and corneal reflexes was confirmed (indicating surgical anesthesia), blood was collected via cardiac puncture. Samples were incubated at room temperature to clot, then centrifuged at 8000 rpm for 30 minutes to obtain serum. Commercial ELISA kits (Beyotime, Cat. PI303, PT516, PI328) were used to analyze IL-1β, TNF-α and IL-6 levels according to the manufacturer's protocol. After adding 50 μL of stop solution, absorbance was read at 450 nm using a microplate reader (Thermo Fisher Scientific, USA) and cytokine concentrations were determined using standard curves from reference samples(Xu *et al*., 2024).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.0.2. Data are presented as mean ± SD. Data normality and homogeneity of variance were assessed before applying parametric tests. Normality was assessed using the Shapiro-Wilk test. For comparisons among three or more groups, one-way ANOVA followed by Tukey's post hoc test was applied. For comparisons between two groups, an unpaired two-tailed Student's t-test was used. A p-value < 0.05 was considered statistically significant.

RESULTS

Dose-response effects of myricetin and quercetin on IL-1ß-induced chondrocytes

To determine whether myricetin and quercetin exert dose-dependent protective effects, IL-1β–stimulated chondrocytes were treated with increasing concentrations of each compound. As shown in Fig. 1, the viability of chondrocytes in the OA group was significantly decreased

relative to the NL group ($P < 0.001$), indicating successful establishment via the IL-1β-induced OA model. Both Myr and Que individually improved cell viability in a dose-dependent manner, with higher concentrations producing more pronounced protective effects (Fig. 1).

Synergistic effects of myricetin and quercetin evaluated by combination index analysis

To quantitatively evaluate whether the combined treatment produced a synergistic effect, a CI analysis was conducted. The results showed that at all tested effective concentration combinations, the CI values were all less than 1, indicating a synergistic effect of quercetin and myricetin in inhibiting the decline in cell viability caused by IL-1β (Fig 2). Based on this analysis and the feasibility of subsequent experiments, Que (50μM) and Myr (10μM) were selected (with a CI value of approximately 0.4, showing a significant synergistic effect) as the concentrations for the subsequent mechanism study.

Effects of myricetin and quercetin on chondrocyte viability and apoptosis

On the basis of the dose-response and synergistic interaction results, Que (50μM) and Myr (10μM) were selected for subsequent experiments. The results showed that treatment with Que or Myr could partially restore cell viability ($P<0.01$) and the cell viability level of the combined treatment group was close to that of the NL group (Fig. 3A).

TUNEL staining results (Fig 3B) further confirmed the protective effects of the treatments. The OA group exhibited markedly increased apoptosis relative to NL controls ($P < 0.01$), while Que and Myr provided measurable anti-apoptotic effects ($P < 0.01$). The CN group showed the greatest reduction, approaching values observed in NL ($P < 0.01$), indicating a synergistic protective effect of combined therapy.

Myricetin and quercetin regulate miRNA-140, apoptosis and inflammatory cytokines in OA chondrocytes

To investigate how Myr and Que protect OA chondrocytes, the expression of miRNA-140 was assessed, apoptosis-associated genes and inflammatory cytokines following treatment.

As shown in fig. 4A, miRNA-140 expression was markedly suppressed in OA cells relative to the normal group ($P < 0.01$), whereas treatment with either Que or Myr partially restored its expression ($P < 0.01$). The CN further elevated miRNA-140 expression to near-normal levels ($P < 0.01$).

Apoptosis-related markers were also modulated by treatment (Fig 4B). In OA chondrocytes, a clear apoptotic pattern emerged, characterized by upregulated Bax and Cleaved Caspase-3 and suppressed Bcl-2 ($P < 0.01$). Although Que and Myr treatments partially corrected these imbalances ($P < 0.01$), CN therapy demonstrated superior efficacy, reversing the expression of apoptotic markers more profoundly ($P < 0.01$).

In addition, inflammatory cytokine levels were assessed (Fig. 4C). In comparison with the NL group, OA group demonstrated pronounced increases in IL-1β, TNF-α, IL-6 and PGE2 ($P < 0.01$). Que and Myr treatments alleviated these pro-inflammatory responses, whereas CN induced the largest decrease ($P < 0.01$).

Regulation of MMP/TIMP signaling pathway by myricetin and quercetin

Western blot assays (Fig 5A-B) demonstrated a pronounced induction of MMP-3, MMP-9 and MMP-13, along with a marked inhibition of TIMP-1 and TIMP-3 in the OA group ($P < 0.01$). Treatment alone in the Que and Myr groups reduced MMP expression and partially restored TIMP protein levels but did not reach the levels of the normal group. Within the CN group, MMP-3, MMP-9 and MMP-13 levels were downregulated while TIMP-1 and TIMP-3 were upregulated markedly ($P < 0.01$), close to the normal cohort, indicating that the combined treatment effectively regulated the MMP/TIMP signaling pathway and slowed down the cartilage-degrading processes.

Functional validation of miRNA-140: Effects on cell viability, apoptosis and inflammation

To elucidate the functional role of miRNA-140 in OA pathogenesis, overexpression and knockdown experiments were conducted by transfecting chondrocytes with a miRNA-140 mimic (QM group) or inhibitor (MI group), alongside negative control (NC) and untreated control groups.

First, qRT-PCR results revealed that miRNA-140 expression was significantly increased in the QM group compared with the Control ($P < 0.01$), whereas MI exposure led to a significant reduction relative to NC ($P < 0.01$; Fig 6A). Functionally, the CCK-8 assay revealed that corresponding changes in cell viability, showing a notable increase in the QM group and decreases in the MI group ($P < 0.01$, Fig 6B).

In line with viability data, the TUNEL results indicated markedly less apoptosis in the QM group compared with Control ($P < 0.01$), while the MI group exhibited higher apoptotic activity than NC ($P < 0.01$) (Fig 6C). Western blot analysis corroborated these findings, showing downregulated Bax and Cleaved Caspase-3 but upregulated Bcl-2 in the QM group, opposite to that observed in MI ($P < 0.01$) (Fig 6D-F).

Additionally, according to ELISA results, QM-treated chondrocytes exhibited reduced TNF-α levels and slight elevations of IL-1β, IL-6 and PGE2 compared to NC. MI administration drastically amplified all four cytokines ($P < 0.01$) (Fig. 6G-J).

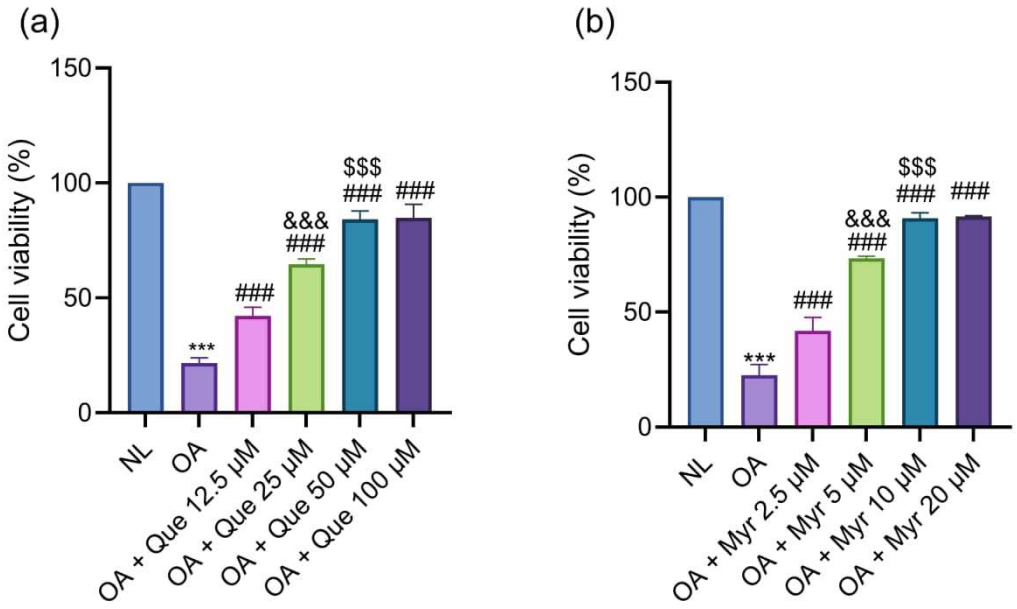

Fig. 1: Dose–response effects of Myr and Que on IL-1β–induced chondrocytes.
The CCK-8 assay method was used to evaluate the cell viability of chondrocytes treated with different concentrations of: (a) Que; (b) Myr for 24 h after being stimulated by IL-1β. Data are presented as mean ± SD (n = 3). ***P < 0.001 vs NL; ###P < 0.001 vs OA; andandandP < 0.001 vs OA + Que 12.5 μM, OA + Myr 2.5 μM; $$$P < 0.001 vs OA + Que 25 μM, OA + Myr 5 μM

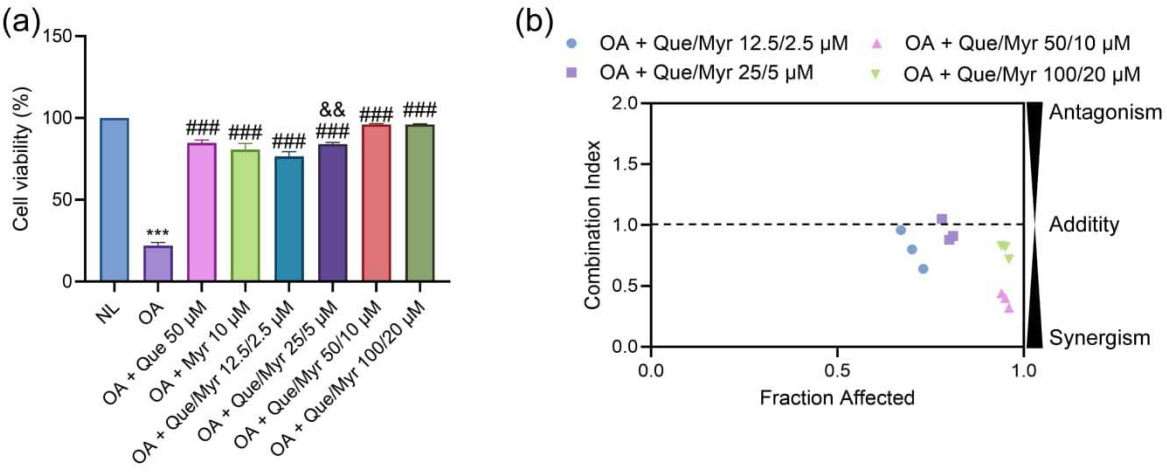

Fig. 2: Synergistic interaction between Myr and Que evaluated by combination index (CI) analysis.
(a) Cell viability was measured by CCK-8 assay following combined treatment with Myr and Que for 24 h; (b) Calculated CI values across different effective concentrations. Data are presented as mean ± SD (n = 3). ***P < 0.001 vs NL; ###P < 0.001 vs OA; andand P < 0.01 vs OA + Que/Myr 12.5/2.5 μM.

miRNA-140 regulates the MMP/TIMP signaling pathway

To further investigate whether miRNA-140 modulates the MMP/TIMP signaling pathway, protein expression profiles of MMPs (3, 9, 13) and TIMPs (1, 3) were evaluated under miRNA-140 mimic (QM) and inhibitor (MI) conditions.

As shown in Fig. 7A, MMP-3, MMP-9 and MMP-13 expression was substantially decreased in the QM group versus control (P < 0.01), while it was markedly upregulated in the MI group versus the NC (P < 0.01). Conversely, TIMP-1 and TIMP-3 expression were markedly upregulated under QM treatment and downregulated with MI treatment (P < 0.01) (Fig. 7B).

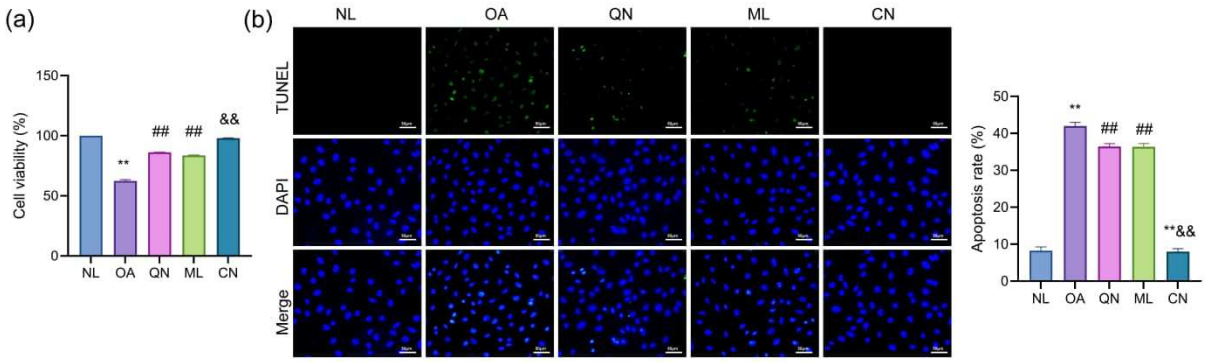

Fig. 3: Effects of Myr and Que on chondrocyte viability and apoptosis in IL-1β-induced osteoarthritis model.
(a) Cell viability was determined by CCK-8 assay; (b) TUNEL staining images and quantification of apoptotic cells. Data are presented as mean ± SD (n = 3). ** P < 0.01 vs NL; ## P < 0.01 vs OA; andand P < 0.01 vs QN, ML. Scale bar = 50 μm.

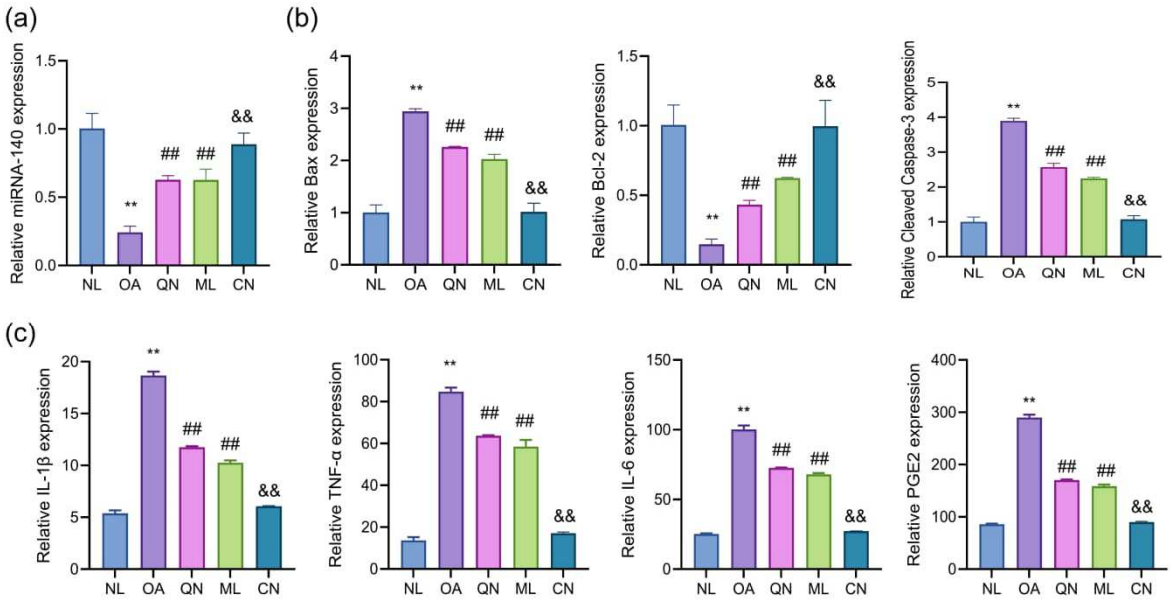

Fig. 4: Effects of Myr and Que on the expression of miRNA-140, apoptosis-related genes and inflammatory cytokines in OA chondrocytes.
(a) Relative miRNA-140 expression determined by qRT-PCR; (b) mRNA expression levels of Bax, Bcl-2 and Cleaved Caspase-3; (c) Levels of IL-1β, TNF-α, IL-6 and PGE2 measured by ELISA. Data are presented as mean ± SD (n = 3). ** P < 0.01 vs NL; ## P < 0.01 vs OA; andand P < 0.01 vs QN, ML.

Effects of quercetin and myricetin on inflammatory cytokines and miRNA-140 expression in OA rats

Based on the robust protective effects observed *in-vitro*, the therapeutic potential of combined myricetin and quercetin treatment was further evaluated in an MIA-induced OA rat model. As illustrated in fig. 8A, ELISA analysis showed significantly higher serum IL-1β, TNF-α and IL-6 levels in OA rats than in Sham controls (P < 0.01). Both Que and Myr treatments markedly decreased these cytokines compared with OA (P < 0.01), while the CN group produced the strongest anti-inflammatory response (P < 0.01). Notably, IL-1β concentration dropped from ~1500 pg/mL in OA rats to nearly baseline in the CN group, with TNF-α and IL-6 exhibiting comparable patterns, confirming the synergistic efficacy of co-administration.

Analysis via qRT-PCR indicated that OA cartilage exhibited significantly reduced miRNA-140 expression compared to Sham (P < 0.01), while both Que and Myr monotherapies elevated its expression to some extent (P < 0.01) (Fig 8B).

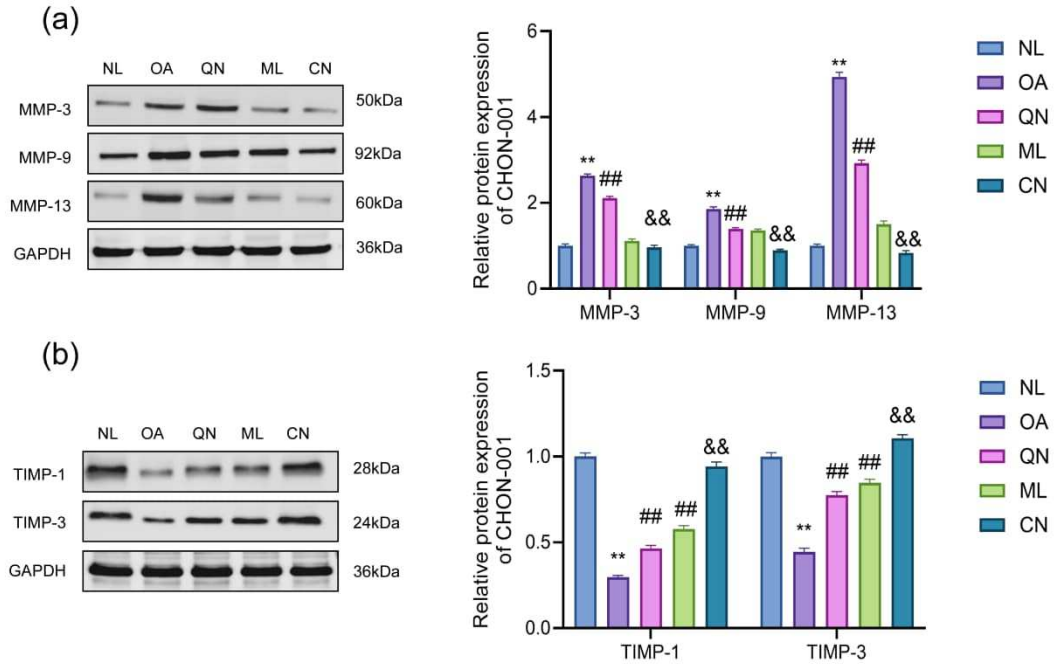

Fig. 5: Effects of Myr and Que on the expression of MMPs and TIMPs in IL-1β-induced OA chondrocytes.
(a) Western blot analysis and quantification of MMP-3, MMP-9 and MMP-13; (b) Western blot analysis and quantification of TIMP-1 and TIMP-3. GAPDH was used as loading control. Data are shown as mean ± SD (n = 3). **P < 0.01 vs NL; ##P < 0.01 vs OA; andandP < 0.01 vs QN or ML.

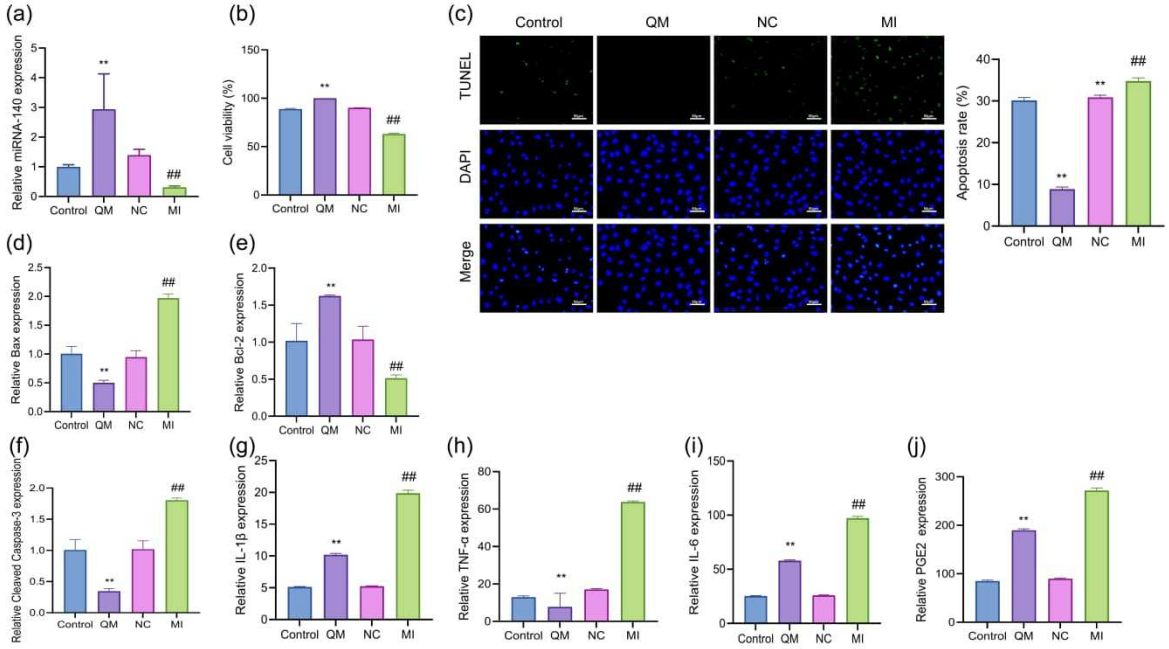

Fig. 6: Effects of miRNA-140 modulation on chondrocyte viability and apoptosis.
(a) Relative expression of miRNA-140 in Control, QM, NC and MI groups; (b) Cell viability was assessed by CCK-8 assay; (c) Apoptosis was evaluated by TUNEL staining and quantified; (d-f) Expression levels of Bax, Bcl-2 and Cleaved Caspase-3; (g-j) Relative mRNA levels of IL-1β, TNF-α, IL-6 and PGE2. Data are presented as mean ± SD (n = 3). **P < 0.01 vs Control; ##P < 0.01 vs NC. Scale bar = 50 μm.

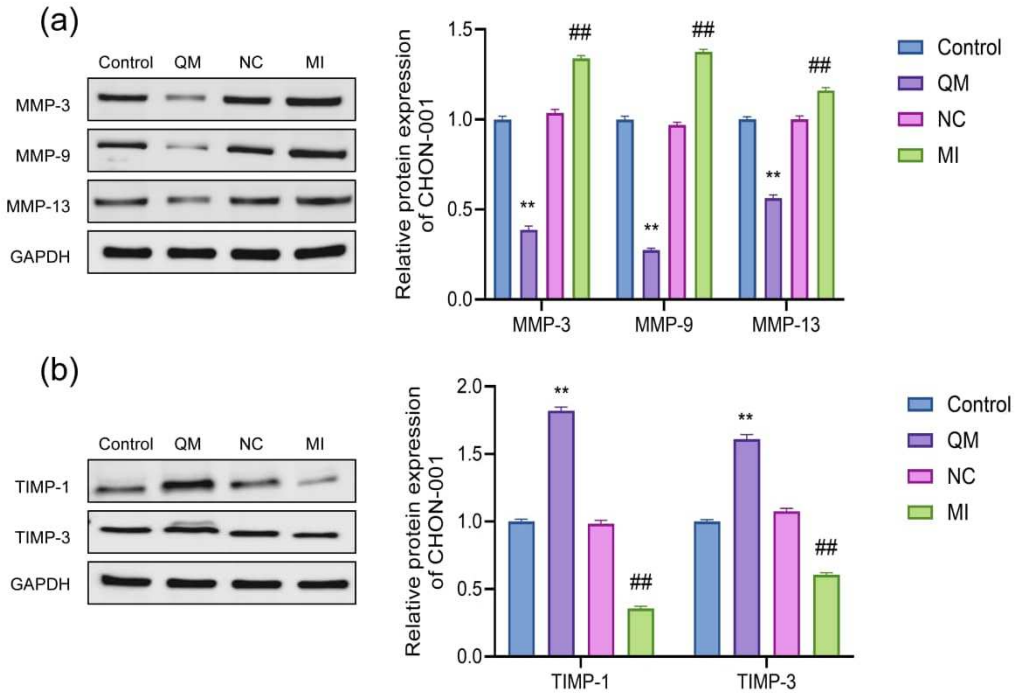

Fig. 7: Effects of miRNA-140 modulation on the expression of MMPs and TIMPs in IL-1β-induced OA chondrocytes. (a) Western blot analysis and quantification of MMP-3, MMP-9 and MMP-13; (b) Western blot analysis and quantification of TIMP-1 and TIMP-3. GAPDH served as loading control. Data are shown as mean ± SD (n = 3). **P < 0.01 vs Control; ##P < 0.01 vs NC.

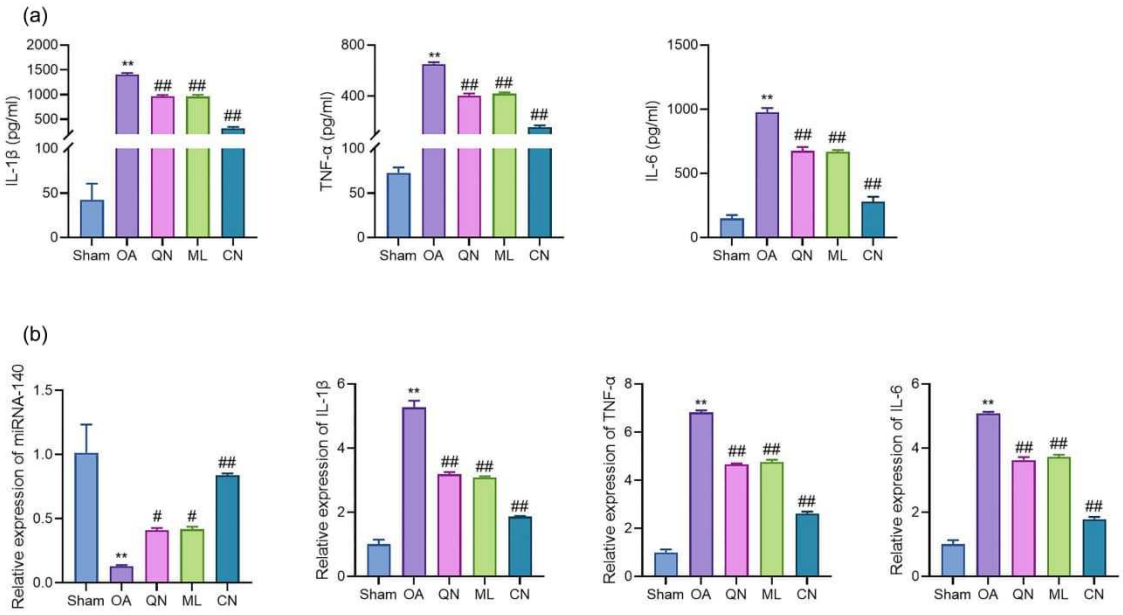

Fig. 8: Effects of Que and Myr on serum cytokine levels and gene expression in OA rats.
(a) Serum levels of IL-1β, TNF-α and IL-6 measured by ELISA; (b) miRNA-140, IL-1β, TNF-α and IL-6 mRNA expression in knee cartilage determined by qRT-PCR. GAPDH served as internal control. Data are presented as mean ± SD (n = 3). **P < 0.01 vs Sham group; ##P < 0.01 vs OA group.

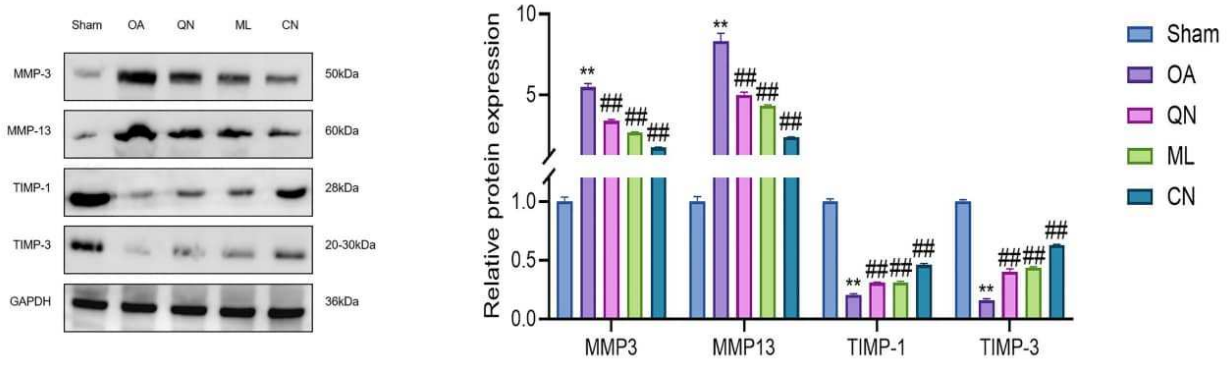

Fig. 9: Effects of Que and Myr on MMP and TIMP protein expression in OA rat cartilage tissues.
Western blot analysis and quantification of MMP-3, MMP-13, TIMP-1 and TIMP-3 in knee joint tissues. GAPDH was used as the loading control. Data are presented as mean ± SD (n = 3). **P < 0.01 vs Sham group; ##P < 0.01 vs OA group.

Notably, the CN group exhibited a more robust upregulation of miRNA-140, approaching levels observed in the Sham group. Compared with controls, IL-1β, TNF-α and IL-6 mRNA levels were markedly elevated in the OA group (P < 0.01), whereas treatment markedly reversed these increases, with the CN group showing the greatest inhibition (P < 0.01) (Fig 8B). This indicates that co-treatment with Que and Myr provides superior modulation of miRNA-140 and inflammatory responses than single-drug therapy.

Effects of quercetin and myricetin on MMP/TIMP protein expression in OA rats

According to Western blot analysis (Fig. 9), MMP-3 and MMP-13 expression were upregulated, whereas TIMP-1 and TIMP-3 expression declined significantly in the OA group compared with the Sham group (P < 0.01). Both Que and Myr monotherapies attenuated these changes to some extent (P < 0.01), showing decreased MMPs and increased TIMPs. The CN combination exerted the strongest regulatory effect, markedly reducing MMP-3 and MMP-13 expression while restoring TIMP-1 and TIMP-3 to near Sham grou*p-value*s (P < 0.01). These results indicate that Que and Myr, especially in combination, can effectively rebalance cartilage matrix metabolism in OA by modulating the MMP/TIMP axis.

DISCUSSION

Using both chondrocyte cultures and a rat model, this study examined the protective functions of Myr and Que in OA. In IL-1β-induced CHON-001 chondrocytes, treatment with either Myr or Que significantly improved cell viability and reduced apoptosis, with the combined treatment showing a stronger synergistic effect. This effect was characterized by increased miRNA-140, suppressed Bax and cleaved Caspase-3 and upregulated anti-apoptotic Bcl-2. These *in-vitro* effects were paralleled by findings in the MIA-induced rat OA model, where co-administration of the two compounds effectively reduced cartilage degeneration, restored miRNA-140 expression and modulated the expression of MMP-3, MMP-13, TIMP-1 and TIMP-3 in joint tissue. Reduced expression of key inflammatory cytokines (TNF-α, IL-1β, IL-6) was observed in both cell and animal models, implying that the combined intervention mitigates cartilage damage while attenuating inflammation. Such consistency between models enhances confidence in the findings and highlights the therapeutic value of flavonoid co-administration in OA.

These findings corroborate prior evidence that Que and Myr exert anti-inflammatory, antioxidant and anti-apoptotic functions (Rahmani *et al*., 2023). The therapeutic potential in OA is further confirmed by the results, and importantly, a synergistic effect is revealed when the two compounds are used in combination. Both *in-vitro* and *in-vivo*, co-treatment more effectively enhanced chondrocyte viability and suppressed apoptosis compared to monotherapies, suggesting that Que and Myr may act through complementary mechanisms to promote cartilage protection. This is in line with the observations of (Taheri *et al*., 2020)), who reported superior biological activities when Que and Myr were combined.

Distinct from prior studies, the regulatory role of miRNA-140 is emphasized in this work, which is associated with these protective effects. The combined treatment significantly restored miRNA-140 expression in both cell and animal models and inhibition of miRNA-140 reversed the therapeutic benefits, confirming its central role. miRNA-140 is known to maintain cartilage homeostasis by suppressing MMPs and enhancing TIMPs (Morya *et al*., 2024), which is consistent with the data showing that the combined treatment downregulated MMP-3, MMP-9 and MMP-13 while upregulating TIMP-1 and TIMP-3. Previous studies have likewise demonstrated that Que and Myr individually influence the regulation of MMPs and TIMPs, supporting the results (Mukherjee and Das, 2024; Qu, Li, Wu, and Chen, 2016).

Furthermore, it was revealed by the findings that combined Que and Myr treatment notably inhibited TNF-α expression *in-vitro* and *in-vivo*, while moderately downregulating IL-1β, IL-6 and PGE2. These outcomes further support their anti-inflammatory synergy. Compared with earlier studies focusing on single flavonoids or simplified *in-vitro* systems (T. Wang and He, 2018), more comprehensive evidence is provided by the present work, integrating inflammation, ECM remodeling and miRNA regulation in both cellular and animal models of OA.

In addition to the miRNA-140/MMP-TIMP axis, the pathogenesis of OA also involves other important signaling pathways, such as the NF-κB and Wnt/β-catenin pathways. The NF-κB signaling pathway plays a central role in regulating the inflammatory response of OA, being the main regulator of inflammatory cytokines (such as IL-1β, TNF-α) (Molnar *et al*., 2021) and can also affect the regulatory accumulation and remodeling of ECM proteins and have an indirect positive impact on downstream regulators of terminal chondrocyte differentiation (including β-catenin and Runx2) (Choi, Jo, Park, Kang, and Park, 2019; Yao *et al*., 2023) Moreover, the abnormal activation of the Wnt/β-catenin pathway has been considered to be related to the pathogenesis of OA. The Wnt/β-catenin signaling pathway plays a role in bone and joint pathology by directly influencing bone, cartilage and synovial tissues (Zhou, Wang, Hamilton, and Chen, 2017). Exosomes derived from BM-MSCs can limit IL-1β-induced chondrocyte damage by inhibiting the activation of the Wnt/β-catenin pathway (Dong, Li, Fang, and Zang, 2021). The observed reduction of inflammatory cytokines and improvement of matrix metabolism in the study suggest that the protective effects of myricetin and quercetin may also involve the regulation of these pathways. Future studies should directly detect changes in key mediators (such as p65 phosphorylation, β-catenin activity), to further clarify the comprehensive molecular mechanism by which myricetin and quercetin exert protective effects in OA.

Although coordinated changes were observed in miRNA-140 expression and MMP/TIMP signaling following myricetin and quercetin treatment, this study does not establish a direct causal interaction between miRNA-140 and specific MMP or TIMP targets. The observed effects, therefore, represent an associative regulatory pattern rather than definitive mechanistic proof. Direct regulation would require further experimental validation, such as miRNA-140 gain- or loss-of-function rescue assays, luciferase reporter analyses, or mutational confirmation of miRNA response elements within MMP or TIMP transcripts.

Several additional limitations of this study should be acknowledged. First, although both *in-vitro* and *in-vivo* experiments have shown encouraging protective effects, the treatment period is relatively short. The continuous therapeutic effects, cumulative toxicity and potential non-targeted effects of the combination of myricetin and quercetin, either alone or in combination, have not been evaluated. Future research should include longer-term animal experiments, systematic dose-response experiments, detailed toxicological evaluations and optimization of dosing strategies, in order to comprehensively assess the safety profile and long-term therapeutic potential of this flavonoid compound combination in the treatment of OA. Second, this study primarily focused on the miRNA-140/MMP–TIMP axis, whereas other signaling pathways known to influence OA progression, such as NF-κB, Wnt/β-catenin, oxidative stress–related pathways, or epigenetic regulatory mechanisms, were not explored. Third, pharmacokinetic parameters, including tissue distribution, bioavailability and serum or joint concentrations of myricetin and quercetin, were not assessed, which limits direct translation of these findings to clinical applications. Future studies incorporating comprehensive pharmacokinetic profiling, metabolic analysis and formulation optimization will be essential to better define the therapeutic window and translational feasibility of these flavonoids. Finally, the absence of a positive control using clinically established OA therapies precludes direct comparison with existing treatments. Although the main objective of this study was to investigate the biological effects and mechanism correlations of myricetin and quercetin (especially their combined effect) on miRNA-140 regulation and the downstream MMP/TIMP signaling axis, adding appropriate positive controls (such as non-steroidal anti-inflammatory drugs) in future studies will help to further strengthen the efficacy comparison assessment and clinical relevance.

CONCLUSION

This study demonstrated that the combined application of Myr and Que effectively modulates miRNA-140 expression and the MMP/TIMP signaling pathway, thereby enhancing chondrocyte survival and mitigating cartilage degradation in OA models. The treatment also exerted anti-inflammatory effects, particularly through the suppression of TNF-α. These findings provide robust preclinical evidence supporting the use of Myr and Que as promising natural candidates for OA therapy and offer a foundation for further clinical development of flavonoid-based combination strategies.

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Authors' contributions
H.Z. B: Conceptualization, investigation, methodology, writing - original draft, writing - review and editing; Y.H. X: Conceptualization, investigation, methodology, writing

- original draft, writing - review and editing; B.G. Z: Data analysis and interpretation, writing - review and editing; X. Q: Experimental procedures and data collection, writing - review and editing; J.J. M: Data analysis and interpretation., writing - review and editing; Z.D. H: Experimental support and data collection, writing - review and editing; S. C: Experimental support and data collection, writing - review and editing; G. J: Supervision and correspondence, writing - original draft, writing - review and editing. All authors have read and approved the final manuscript.

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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
This study was reviewed and approved by the Medical Ethics Committee of the Affiliated Hospital of Inner Mongolia Minzu University (Approval No. NM-LL-2023-03-10-03). All experimental protocols were followed the guidelines of the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996). This study was performed in adherence with the ARRIVE guidelines. See supplementary file for the ARRIVE checklist.

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

Supplementary data
https://www.pjps.pk/uploads/2026/05/SUP1780218815.pdf

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