Anti-inflammatory, and Antioxidant Effect of *Akebia quinata* D. Leaves Aqueous Extract against Osteoarthritis in Mouse Chondrocytes and in Rat Model of Osteoarthritis: *in Vitro* and *in Vivo* Studies

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Osteoarthritis (OA) is characterized by inflammation of the synovial joints. However, the drugs used to treat OA are insufficient due to multifactorial pathogenesis of the disease. The present study determined the anti-inflammatory and antioxidant effect of *Akebia quinata* D. aqueous extract (AQDE) against osteoarthritis using *in vitro* and *in vivo* models of OA. Initially, the effect of AQDE was identified to determine its effect on the various biomarkers of the TNF-α mouse chondrocytes. The results suggest that AQDE causes significant ACGAN and COL2 and reduction in IL-1B, IL-6, MMP-13 and PTGS2. It also causes induction of the expression of Bcl2, CDK1, CCND, and reduction in the rate of apoptosis of mouse chondrocytes. In the *in vivo* rat model, AQDE improved mechanical allodynia in the Von Frey test and reduced knee joint swelling. Micro-CT scanning showed improvement in bone erosion. The level of oxidative stress (MDA, SOD, and GSH) and production of pro-inflammatory cytokines (IL-1B, and IL-6) was restored to near-normal levels in a dose-dependent manner. AQDE reduced the mRNA expression of COX-2 and NF-kb in both *in vitro* and *in vivo* study. The results demonstrated the significant pharmacological activity of AQDE against osteoarthritis in both *in vitro* and *in vivo* model.

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INTRODUCTION

Osteoarthritis (OA) is a common disorder of the bones and joints that is characterized by inflammation of the synovial joints, sclerosis of the subchondral bone, and chronic degradation of articular cartilage (Sun *et al*. 2019b). The repercussions include impairments and restrictions on one's own activities, in addition to having a big financial impact on the entire world (Wood *et al*. 2022). Numerous factors, such as age, weight, genetic inheritance, and prior joint damage or deformity, increase the chance of developing osteoarthritis (OA). Chondrocytes are the only cells found in articular cartilage, and the stability of the cartilage depends on their complex biosynthetic activities. Secondary data suggests that the degeneration of cartilage is brought on by chondrocyte mortality as a

consequence of pro-inflammatory cytokine release, such as TNF-α and interleukin-1β (IL-1β) (Katturajan and Sabina 2021). According to experimental data, chondrocytes can be directly stimulated by inflammation cytokines to secrete catabolic enzymes that aid in the breakdown of the extracellular matrix (ECM), such as matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) (Eccleston 2023). Furthermore, prior research has demonstrated that chondrocyte survival is significantly impacted by persistently elevated levels of TNF-α and IL-1β. The continuous inflammatory processes prevent proliferative chondrocytes from developing in the cartilage tissue, which makes it unable to repair the injured cartilage tissue. Thus, degenerative arthritis gets worse as a result of this process (Thudium *et al*. 2019). Therefore, inhibiting these inflammatory reactions could slow the progression of osteoarthritis.

Contemporary therapy for osteoarthritis (OA) mostly involves the use of glucocorticoids and non-steroidal anti-inflammatory medications (NSAIDs), which suppress the mechanisms responsible for inflammatory responses (Grässel and Muschter 2020; Lin *et al*. 2023). Nevertheless, these drugs are unable to change the pattern of disease development. In addition, these drugs have too much linkage to significant adverse effects when used for an extended period. Multiple studies have documented the therapeutic advantages of certain traditional Chinese herbs in reducing the effects of osteoarthritis (OA) by inhibiting the functioning of matrix metalloproteinases (MMPs) and interleukins (ILs), hence effectively reducing OA generated by monosodium iodoacetate (MIA) (Lee *et al*. 2022b).

The creeping woody vine *Akebia quinata Decaisne* (Lardizabalaceae) is extensively found in East Asia, encompassing Korea, China, and Japan (Scholler and Gams 1998; "*Akebia quinata* (five-leaf akebia)" 2022). Historically, the extract of *A. quinata Decaisne* (AQE) has been suggested to alleviate heat caused by stress and fatigue-induced depression, enhance fatigue, promote mental relaxation, facilitate the excretion of feces and urine, and suppress thirst (Park *et al*. 2018). The desiccated stem of the plant most commonly serves as a diuretic for the treatment of hypothermia and rheumatic pain (Han *et al*. 2012). Furthermore, this plant showed antipyretic and mild analgesic properties, as well as promoting intestinal motility and control ileal contractility (Bensky *et al*., 2004). It also showed protein tyrosine phosphatase 1B inhibitor activity. However, the impact of *A. quinata* Decaisne aqueous extract (AQDE) on osteoarthritis has remained undetermined. The present study determined the pharmacological effect and mechanism of *A. quinate Decaisne* (AQDE) leaves aqueous extract in osteoarthritis in mouse chondrocytes.

EXPERIMENTAL

Preparation of Extract

A. quinate Decaisne leaves were obtained from the commercial supplier and authenticated by an institutional botanist. The midribs of the several leaflets (usually five to seven) shoot outward from a single point on the leaf. The leaves were divided into five, occasionally three, four, or even seven leaflets, and they were observed to alternate along the stems or cluster on the branchlets. The papery leaves range in shape from oblong to oblong-elliptic. The leaflets have rounded to widely cuneate bases and are adaxially dark green (dark green above) and abaxially glaucous (whitish below). The tip that is farthest from the place of attachment, or the apex, is often rounded and either cuspidate (with a

point) or emarginated (notched). The terminal leaflets are 2.5 to 5 (or to 7) cm in length, while the lateral leaflets are 3 to 5 x 1.5 to 2.5 cm. The sample voucher (No. HERB/SJTU/2024/34C2A) was deposited in the herbarium of the Shanghai Jiao Tong University Affiliated Sixth People's Hospital for future reference. Fresh or mature leaves were collected from healthy plants and washed thoroughly with distilled water to remove dirt, debris, and contaminants. Leaves were air-dried in a shaded, well-ventilated area to prevent degradation of heat-sensitive compounds for 4 to 5 days. The extract was obtained after boiling the AQDE in water at 40 to 60 °C for 6 to 8 h and filtered. The filtrate was dried at room temperature for 8 to 12 days to obtain dried extract powder.

Isolation of Mouse Chondrocytes

The primary chondrocytes were extracted from the bilateral femoral head and tibial plateau cartilage of 14- to 21-day-old SD rats, which were maintained under specified pathogen-free conditions as per the previously reported procedure (Mirando *et al*. 2014; Jonason *et al*. 2015). Before being digested with 0.25% trypsin, the rat articular cartilage was sliced after 30 min, and then digested for 4 hours at 37 °C with 0.2% collagenase type II in DMEM-F12. The chondrocyte suspension was centrifuged at 1200 rpm for 5 min. After being spun in a centrifuge, the settled chondrocytes were then mixed again with 10% FBS-supplemented DMEM-F12 complete media with 1% streptomycin and penicillin. Chondrocytes that had been resuspended were divided into cultivated in incubators with 5% CO_2 at 37 °C in cell culture flasks manufactured by Corning (New York, USA) (Thermo Fisher, Waltham, MA, USA). To keep phenotypic loss to a minimum, *in vitro* studies underwent procedures employing rat chondrocytes prior to the second passage.

Effect on the Cell Viability

The viability of the extract was investigated on the mouse chondrocytes obtained from the mouse as per the previously reported procedure. The OD value was recorded at 450 nm using the microplate reader after the complete dissolution of formazan crystals (Srivastava *et al*. 2017).

qRT-PCR Analysis

Total RNA was isolated utilizing an RNA extraction kit (Tiangen, Beijing, China), in accordance with the manufacturer's guidelines. Complementary DNA (cDNA) was generated using reverse transcription utilizing Prime Script cDNA RT Master Mix (Kogen, Tokyo, Japan). Amplification was conducted using RNase DDH2O (Tiangen, Beijing, China), SYBR PreMix Ex Taq™ (Kogen, Tokyo, Japan), along with forward and reverse primers specific to the gene of interest. The complete PCR procedure was observed utilizing a LightCycler® 480 (Roche, Germany), and cycle threshold (CT) relative expression levels were determined employing the 2^-DDCT technique.

Flow Cytometry Analysis

A flow cytometer was employed to assess the apoptosis in the examined cells (10,000 events recorded). Annexin V had a strong affinity for phosphatidylserine, enabling the detection of cells at all phases of apoptosis using this protein. Propidine iodide (PI) was employed to detect late-apoptotic and necrotic cells due to its ability to stain cells with compromised cell membranes. The control consisted of cells cultivated in a drug-free medium. A positive control (cells treated with 3% formaldehyde in buffer for 30 min on ice) was employed to determine the optimal parameter settings. An analysis of the findings

was conducted with FACSDiva software.

Animals

Female Sprague-Dawley rats, aged 6 weeks, were procured from a commercial supplier and provided with standard chow and unrestricted access to water. The study adheres to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011) and was approved by the Ethics Committee for Animal Experimentation at the Shanghai Jiao Tong University Affiliated Sixth People's Hospital, China (Approval No: AE/2024/C-34/21).

Monosodium Iodoacetate Induced OA Model

Unilateral knee osteoarthritis was generated by a single intra-articular injection of monosodium iodoacetate (MIA) in the infrapatellar region of the right knee of male Wistar rats as per the previously reported procedure (Lee *et al*. 2022a; Park *et al*. 2017). A total of 30 animals were utilized, comprising six rats each group. On the first day, rats were sedated with 2% isoflurane in pure oxygen. The skin of the right leg was disinfected with 75% ethyl alcohol, and the knee was identified through palpation. A 26-gauge needle was placed vertically to breach the epidermis and then rotated distally for entry into the articular cavity, until a notable reduction of resistance was observed; thereafter, 1 mg of MIA in 50 µL saline was administered into the articular cavity. The control rats (sham) were administered 50 µL of saline solution. All animals were euthanized 21 days following the intra-articular administration of either MIA or saline solution.

Von Frey Test

The rats were subjected to testing every day after an acclimation period of one hour in a Plexiglas chamber of 28×40×35 cm with a wire mesh bottom, situated 20 cm above the bench as per the previously reported procedure (de Morais *et al*. 2016; Fusco *et al*. 2020). Following the initial adaptation phase, a servo-controlled mechanical stimulus—a pointed metallic filament with a diameter of 0.5 mm—was placed under the back paw's plantar surface. This stimulus applied a periodic pressure that increased linearly over 20 seconds, eventually reaching 50 g. The automatic recording of a distinct and unprompted withdrawal of the hind paw in response to punctuate pressure was made. The findings determined the gram-level pressure necessary to trigger a strong and rapid paw withdrawal reaction. To find the mechanical allodynic threshold, stimuli were administered alternately to the hind paws every two min for three separate measurements.

Knee Joint Thickness

Leg swelling (knee joint area) following MIA administration was measured precisely using a Vernier caliper, and the other (noninjected) leg served as a control.

Micro-CT Scanning

Bruker Micro-CT Skyscan 1276 was the system that was utilized in order to scan the specimens. Calibration of density measurements was performed using the calcium hydroxyapatite (CaHA) phantom that was provided by the manufacturer. Analysis of the data was carried out by the testing software provided by the manufacturer. NRecon (Version 1.7.4.2) was successful in completing the reconstruction. An area of interest (ROI) was selected for the purpose of conducting analyses of the bone microarchitecture. Bone morphometry was performed on the cortical and epiphyseal trabecular bone of the

tibia and femur in accordance with the procedures that were established in the past for the parameters that were investigated.

Oxidative Stress Biomarkers

The spinal cord homogenates were centrifuged at 4 °C and resulting supernatant was used for the measurement of MDA and GSH levels and SOD activity *via* commercially available kits according to the given protocols.

Enzyme-linked Immunosorbent Assay (ELISA)

The determination of TNF-α (tumor necrosis factor-α), IL (interleukin)-1β and IL-6 were performed using commercially available ELISA kits as per manufacturer's instructions.

Statistical Analysis

All data were expressed as mean ± standard deviation. Comparisons between multiple groups were performed with one-way ANOVA and post-hoc Tukey's test in the case of normal distribution or the Kruskal–Wallis and post-hoc Dunn tests for non-normal distribution. A p-value < 0.05 was considered statistically significant (GraphPad Prism version 9.4.0 for Windows, GraphPad Software, San Diego, CA, USA).

RESULTS AND DISCUSSION

Effect on the Viability of Mouse Chondrocytes: *In vitro* Activity

The effect of AQDE was evaluated on the viability of mouse chondrocytes using the MTT assay in the concentration of 0.25, 0.5, 1, 4, 8, and 12 mg. Up to the concentration of 4 mg, the viability of cells was near 100%, and on 8 mg, it was found to be 96%. However, upon increasing the concentration to 12 mg, viability was found reduced to 72%. Thus, subsequent *in vitro* experiments used 0.5, 1, and 4 mg concentrations.

Effect on the TNF-α-induced Abnormal Gene Expression

Chondrocytes, in general, have a high degree of sensitivity to TNF-α, a cytokine that inhibits the production of aggrecan and collagen type II, hence fostering the development of osteoarthritis (OA).

To evaluate the protective role of AQDE in chondrocytes that were treated with TNF-α, the Real-Time Polymerase Chain Reaction (RT-PCR) was utilized to analyze the impact of AQDE on aggrecan and collagen type II. Following that, the influence of AQDE was investigated in the production of IL-1β, IL-6, MMP13, and PTGS2 in chondrocytes, which was triggered by TNF-α activity.

As shown in Fig. 2, the cells treated with TNF-a, compared with the control, TNF-α caused significant increases in mRNA levels of IL-1β, IL-6, MMP-13, and PTGS2 and inhibited ACAN and COL2A gene expression. However, after administration of AQDE, the level of these genes was found to be restored to near to normal in a concentration dependent manner.

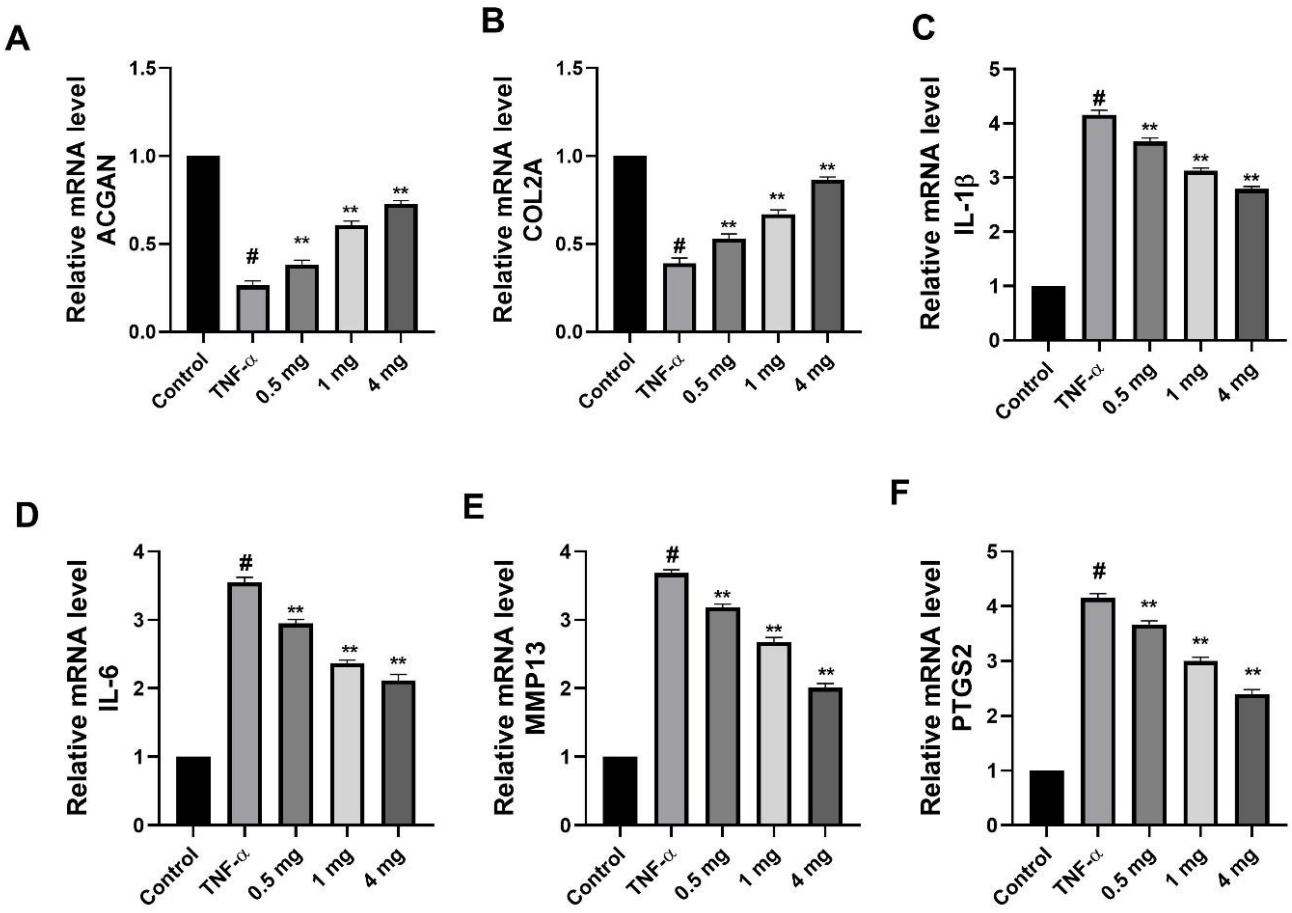

Fig. 1. Effect of AQDE on the gene expression of a) ACGAN, b) COL2A, c) IL-1β, d) IL-6, e) MMP13, and f) PTGS2 mouse chondrocytes using the qRT-PCR analysis. #P<0.05 *vs.* control and **P<0.01 *vs.* TNF-α group. Data are presented as means ± SEM

Effect on Inflammatory Mediators and Protein Synthesis Induced by TNF-α in Mouse Chondrocytes

As shown in Fig. 2 (a, b, and c), in qRT-PCR analysis, TNF-α caused significant decrease in the mRNA expression of Bcl-2, CDK1, and cyclin (CCND). However, the level of these genes was found increased and found restored near to normal in AQDE treated group. The maximum benefit was achieved in the case of 4 mg treated group of AQDE.

Effect on Apoptosis of Chondrocytes

In response of the effect of AQDE on the Bcl-2 gene, the effect of AQDE on the apoptosis of mouse chondrocytes was identified using flow cytometry analysis. The results have been presented in Fig. 2d. The rate of apoptosis was increased after the induction of TNF-α, while upon introduction of AQDE, the apoptosis was found to be significantly reduced in a concentration-dependent manner.

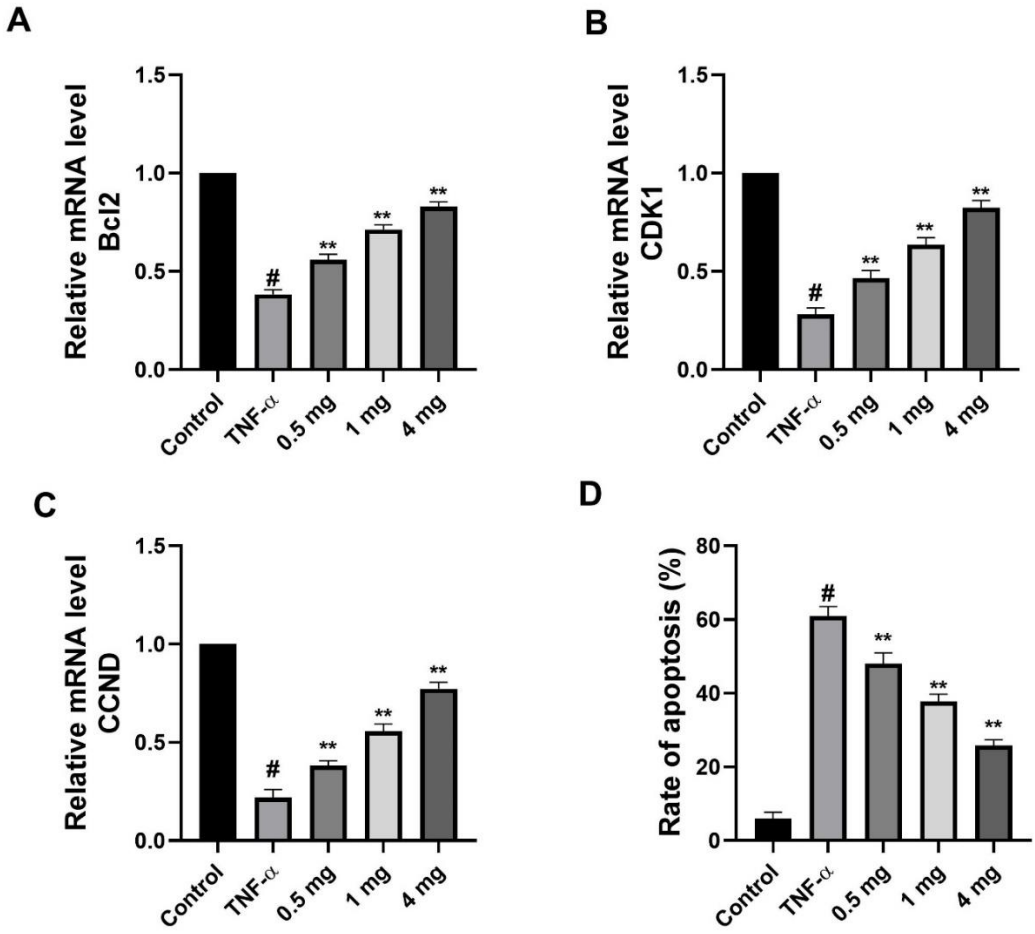

Fig. 2. Effect of AQDE on the mRNA expression of a) Bcl2, CDK1, and c) CCND using the qRT-PCR analysis, d) rate of apoptosis of mouse chondrocytes using flow cytometry analysis. #P<0.05 *vs.* control and **P<0.01 *vs.* TNF-α group. Data are presented as means ± SEM.

Effect on mRNA Level of COX-2 and NF-κB in Mouse Chondrocytes

Finally, in light of the anti-inflammatory properties of AQDE on mouse chondrocytes, its impact on the protein expression of COX-2 and NF-κB was examined. Figure 3 illustrates that the mRNA expression levels of both COX-2 and NF-κB were significantly elevated in TNF-α induced chondrocytes compared to the control group. Subsequent to the administration of AQDE, the mRNA expression of these mediators exhibited a considerable reduction in a concentration-dependent manner. The 4 mg AQDE-treated group exhibited the most significant reduction of these mediators compared to the TNF-α treated group.

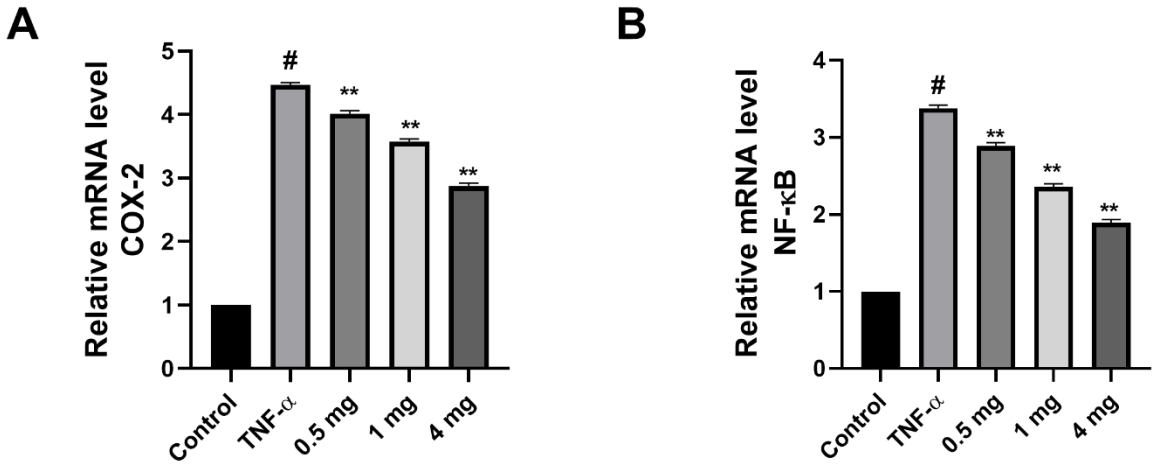

Fig. 3. Effect of AQDE on the mRNA expression of a) COX-2 and b) NF-κB using qRT-PCR analysis. #P<0.05 *vs.* control and **P<0.01 *vs.* TNF-α group. Data are presented as means ± SEM.

Effect on Pain Response and Knee Swelling *in Vivo*

Figure 4a illustrates that in the OA group, MIA elicited considerable mechanical allodynia (as measured by the Von Frey test) in the ipsilateral paw relative to the control group. The rats administered MIA exhibited a sustained reduction in withdrawal threshold in the ipsilateral paw for up to 28 days post-administration. This study assessed paw and knee withdrawal thresholds 90 min after AQDE administration. In the evaluation of mechanical allodynia, rats treated with AQDE exhibited a notable rise in the withdrawal threshold of the ipsilateral paw following acute oral treatment. Furthermore, on the 21st and 28th days, AQDE showed a substantial improvement in allodynia in rats compared to the OA group. The impact of AQDE on the knee joint thickness of rats was also studied. As illustrated in Fig. 4b, at the end of the study duration, the knee joint thickness of the animals in each group was assessed using a digital Vernier caliper. Following the administration of AQDE, a reduction in knee swelling triggered by MIA was detected in a dose-dependent manner.

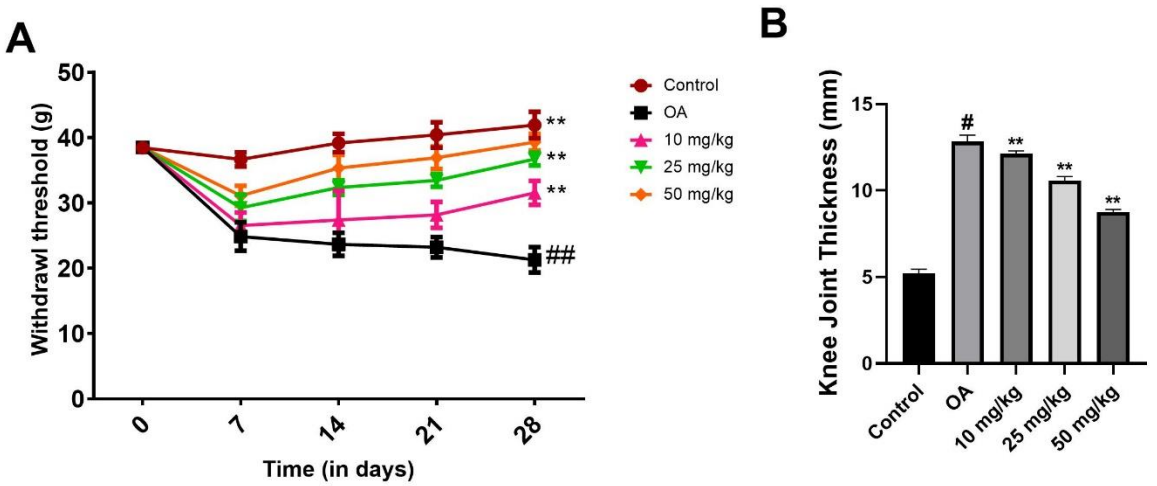

Fig. 4 Effect of AQDE on a) mechanical allodynia using von Frey test, b) knee joint thickness of MIA rats. #P<0.05 *vs.* control and **P<0.01 *vs.* OA group. Data are presented as means ± SEM.

Effect on Rat Knee Joint in Micro-CT Scanning

In the Micro-CT (μCT) investigation of rat joints, rats in the OA group exhibited a substantial reduction in BV/TV, BS/TV, Tb. N, and BMD compared to the control group. The diminished values suggest that rats exhibited considerable joint bone loss following MIA treatment. The OA model demonstrated a considerable increase in Tb. PF, Tb. Sp, and SMI in the joints of rats, with no notable alteration in Tb, indicating a transition in trabecular bone morphology from plate-like to rod-like structures. However, after administration of AQDE, these bone joints parameters were found to be significantly improved in a dose-dependent manner. The most prominent activity was achieved in the case of 50 mg/kg treated rats in comparison to OA group.

Table 1. Parameters of Rat Knee Joint Obtained after the Micro-CT Scanning

Indexes	Control	OA	10 mg/kg	25 mg/kg	50 mg/kg
BV/TV (mm^3)	47.16 ± 4.52	32.53 ± 3.61$^{\#}$	37.32 ± 3.53*	39.81 ± 4.21*	43.54 ± 4.24**
BS/TV (mm^3)	14.32 ± 2.11	10.34 ± 2.34$^{\#}$	11.26 ± 2.53*	12.78 ± 2.33*	13.23 ± 2.55*
Tb.Th (mm)	0.17 ± 0.04	0.12 ± 0.05$^{\#}$	0.13 ± 0.05*	0.15 ± 0.04*	0.16 ± 0.05*
Tb.N (mm^{-1})	3.76 ± 0.33	2.43 ± 0.26$^{\#}$	2.78 ± 0.38*	3.12 ± 0.41*	3.37± 0.52*
Tb.Sp (mm)	0.25 ± 0.02	0.32 ± 0.02$^{\#}$	0.31 ± 0.03*	0.29 ± 0.03*	0.28 ± 0.04*
Tb.PF (mm^{-1})	0.99 ± 0.14	6.12 ± 1.05$^{\#}$	4.56 ± 0.85*	3.52 ± 0.73*	2.89 ± 0.72*
SMI	0.34 ± 0.05	1.34 ± 0.44$^{\#}$	1.22 ± 0.42*	1.05 ± 0.34*	0.87 ± 0.19*
BMD ($g*mm^{-3}$)	0.83 ± 0.08	0.51± 0.06$^{\#}$	0.57 ± 0.07*	0.66 ± 0.08*	0.69 ± 0.08*

Note: Tb.SBP= Tibial subchondral bone; Fm.Cg Femoral cartilage; Tb.Cg: Tibial cartilage; $^{\#}$P<0.05 *vs.* control and **P<0.01 *vs.* OA group. Data are presented as means ± SEM.

Effect on Oxidative Stress Indices and Proinflammatory Cytokines Production in Serum

In the next part of the study, the effect of AQDE was estimated on various indices of oxidative stress and pro-inflammatory cytokines in the serum of OA rats. As shown in Fig. 5, the activity of MDA was found to be increased with subsequent reduction in GSH and SOD levels in comparison to the control. However, after administration of AQDE, the activities of this oxidative stress biomarkers were found to be restored near to normal in a dose-dependent manner. Figure 6 shows the effect of AQDE on the proinflammatory cytokines, such as, IL-1β and IL-6. The concentration of both IL-1β and IL-6 was significantly increased in OA group in comparison to the control. However, after administration of AQDE, the concentration of both the tested cytokines was significantly reduced in a dose-dependent manner. The most significant inhibitory activity was reported in the case of 50 mg/kg treated group in comparison to OA group. The results suggested that AQDE significantly diminishes oxidative stress and inflammation in MIA rats.

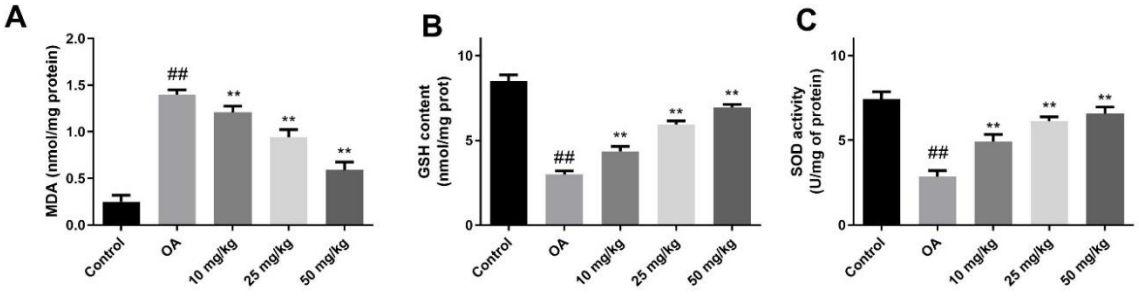

Fig. 5. Effect of AQDE on the a) MDA, b) GSH and SOD. $^{\#}$P<0.05 *vs.* control and **P<0.01 *vs.* OA group. Data are presented as means ± SEM.

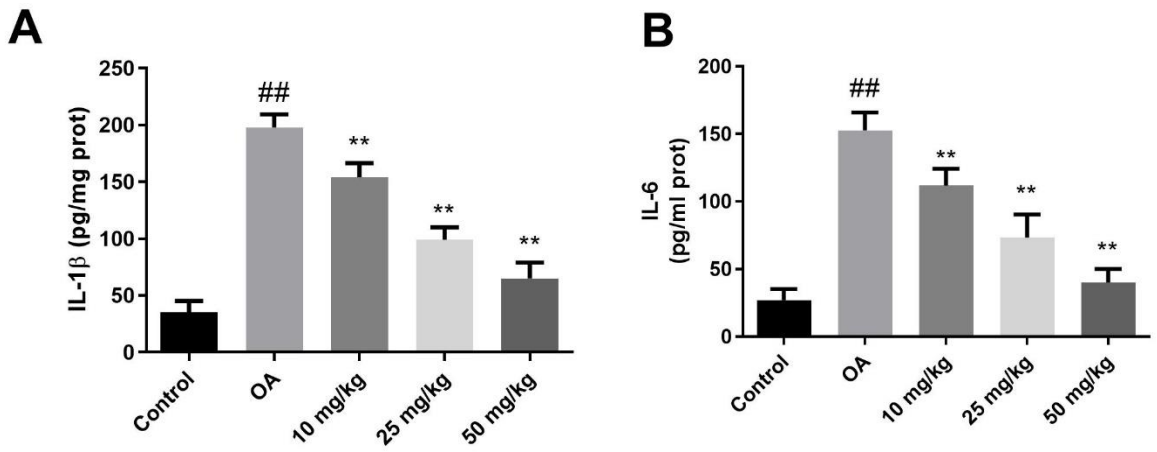

Fig. 6. Effect of AQDE on a) IL-1β, and b) IL-6. #P<0.05 *vs.* control and **P<0.01 *vs.* OA group. Data are presented as means ± SEM.

Effect on mRNA Expression of COX2, and NF-κB in Cartilage

Considering the significant anti-inflammatory effect of AQDE, in the next part of the study, its effect on the mRNA expression of COX-2 and NF-κB was examined. As shown in Fig. 7, MIA rats showed increased expression of COX-2 and NF-κB in comparison to control. Moreover, after the administration of AQDE, the expression of these biomarkers was found reduced significantly in a dose-dependent manner. Results suggest that AQDE significantly attenuated the inflammation probably by inhibiting the COX-2 and NF-κB.

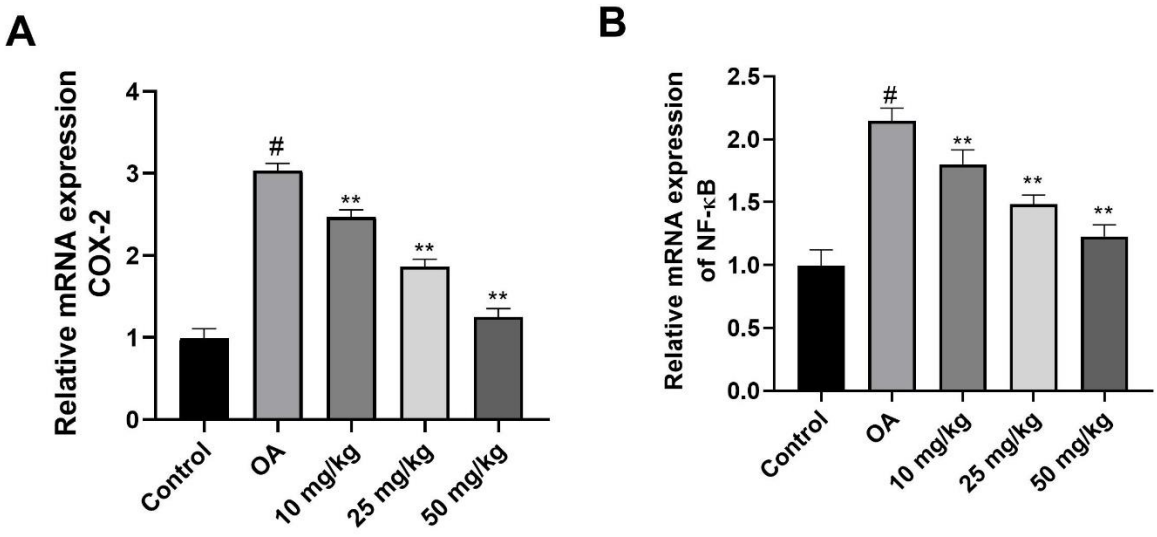

Fig. 7. Effect of AQDE on a) COX-2 and b) NF-κB using qRT-PCR analysis. #P<0.05 *vs.* control and **P<0.01 *vs.* OA group. Data are presented as means ± SEM.

Osteoarthritis (OA) is a significant public health concern, particularly among the elderly population worldwide. As life expectancy increases, the prevalence of OA has risen, placing an immense burden on healthcare systems (Steinmetz *et al*. 2023). A substantial portion of healthcare expenditures is allocated to managing OA-related

complications, which include chronic pain, reduced mobility, and diminished quality of life. This condition not only affects the physical well-being of individuals but also imposes economic and social challenges. With the elderly population projected to grow further, the demand for effective treatments and preventive strategies is becoming increasingly urgent to mitigate the healthcare costs associated with OA (Mobasheri and Batt 2016; Aubourg *et al*. 2022). Herbal extracts offer significant benefits in the prevention and management of osteoarthritis (OA) due to their natural anti-inflammatory and antioxidant properties. Key advantages include reducing inflammation, protecting cartilage, and neutralizing oxidative stress, which contribute to joint damage. Unlike conventional NSAIDs, herbal remedies such as turmeric, ginger, and *Boswellia* typically have fewer side effects, making them safer for long-term use. They also provide a cost-effective alternative, accessible to many. When used alongside lifestyle interventions like exercise and weight management, herbal extracts offer a holistic approach to managing OA and improving overall joint health (Verma 2023). The present study showed the beneficial effect of *Akebia quinata D.* aqueous extract (AQDE) against osteoarthritis. The extract showed significant ameliorative effect against various biochemical pathways known to promote OA, such as oxidative stress, inflammation, and helps to improve the cartilage density and bone erosion.

Accumulating evidence has suggested that OA entails the gradual deterioration of cartilage and persistent joint inflammation, influenced by critical molecular markers including ACGAN, COL2A, IL-1β, IL-6, MMP13, and PTGS2 (Mobasheri and Batt 2016; Sun *et al*. 2019a). Aggrecan (ACGAN), an essential cartilage matrix protein, preserves cartilage architecture and functionality. The degradation of cartilage in osteoarthritis (OA) by matrix metalloproteinases (MMPs) renders it a viable therapeutic target for the prevention of tissue loss. Collagen type II (COL2A), a major structural protein in cartilage, also degrades during osteoarthritis (OA). Decreased COL2A production or heightened degradation leads to joint impairment, and interventions that enhance collagen stability may decelerate disease advancement (Zhang *et al*. 2023). Inflammatory cytokines, including IL-1β and IL-6, are significantly increased in osteoarthritis, promoting inflammation and cartilage breakdown. IL-1β induces MMP expression, exacerbating cartilage degradation, whereas IL-6 facilitates chronic inflammation. Cytokine inhibitors, including IL-1β antagonists (*e.g.*, Anakinra) and IL-6 inhibitors (*e.g.*, Tocilizumab), have demonstrated efficacy in mitigating inflammation and cartilage degradation. MMP13 is crucial in the degradation of collagen II, expediting cartilage deterioration in osteoarthritis. It follows that inhibiting MMP13 may safeguard against more cartilage injury. Finally, PTGS2 (COX-2) is overexpressed in osteoarthritis, augmenting the synthesis of pro-inflammatory prostaglandins, which results in pain and inflammation. COX-2 inhibitors, such as celecoxib, are frequently employed to mitigate symptoms through the reduction of inflammation. Targeting these molecular pathways with anti-inflammatory medications that maintain cartilage integrity presents a viable approach for decelerating or reversing the course of osteoarthritis, as evidenced by several clinical and preclinical investigations (Fan *et al*. 2015; Lee *et al*. 2022a). In the present study, AQDE was found to cause significant restoration of these parameters to near to normal. This suggests that AQDE stimulate the formation of cartilaginous extracellular matrix (ECM) synthesis and the reduction of the expression of inflammatory genes that is caused by TNF-α.

Degeneration of cartilage and chondrocyte death are caused by apoptosis and dysregulation of cell cycle proteins in osteoarthritis (OA). These pathways involve Bcl-2, CDK1, Cyclin D (CCND), and apoptosis (Musumeci *et al*. 2015; Tan *et al*. 2019). By blocking mitochondrial-mediated apoptosis, the anti-apoptotic protein Bcl-2 aids in

controlling cell survival. Accelerated cartilage degradation is caused by downregulation of Bcl-2 in OA, which in turn increases chondrocyte death. Preventing chondrocyte mortality and slowing the course of OA may be possible by targeting Bcl-2 pathways to boost its expression. The appropriate proliferation and repair of chondrocytes are ensured by the cell cycle regulators cyclin D (CCND) and CDK1 (Saito *et al*. 2016; Kihara *et al*. 2017). Cartilage regeneration is hindered and chondrocyte proliferation is diminished in OA due to cell cycle arrest caused by abnormalities in the Cyclin D-CDK1 complex. Enhancing chondrocyte activity and supporting cartilage regeneration by restoring the function of these cell cycle regulators offers a viable therapeutic option. The planned cell death mechanism, known as apoptosis, is dramatically increased in OA, which leads to a decrease in chondrocytes (Musumeci *et al*. 2015). When cells are subjected to mechanical stress or inflammatory cytokines, they trigger apoptosis through both intrinsic (mitochondrial) and extrinsic (non-mitochondrial) routes. Potentially useful drugs for reducing chondrocyte loss and preserving cartilage include medicines that enhance anti-apoptotic proteins such as Bcl-2 or inhibitors of pro-apoptotic factors. Various preclinical and clinical investigations have demonstrated that medicines that modulate Bcl-2, CDK1, CCND, and apoptosis can protect cartilage, halt further deterioration, and improve joint health. In the present study, AQDE was found to cause significant modulation of these biomarkers near to normal and halted the process apoptosis of rat chondrocytes.

Mechanical allodynia, assessed using the Von Frey test, and knee joint swelling are critical measures in evaluating the pharmacological effects of drugs against osteoarthritis (OA) (Eitner *et al*. 2020; Rizk *et al*. 2023). The Von Frey test measures sensitivity to normally non-painful stimuli, indicating the drug's impact on OA-induced pain, a major symptom. In this, a series of filaments with increasing or decreasing forces are applied in sequence to determine the mechanical threshold, the minimum force required to elicit a response. The animal's behavioural response, such as paw withdrawal, flinching, or licking, indicates that the sensation of pain or discomfort was recorded. Based on these results, lower thresholds indicate increased sensitivity (hyperalgesia or allodynia). Higher thresholds indicate reduced sensitivity (hypoalgesia or analgesia), and reduction in mechanical allodynia reflects effective pain relief. In the present study, the significant improvement in the withdrawal threshold by the AQDE suggest that it significantly improves the sensitivity of rats against the non-pain stimuli, and thus may be effective against OA. Knee joint swelling, a marker of inflammation and joint damage, assesses the drug's anti-inflammatory properties. A decrease in swelling in AQDE in the present study indicates reduced inflammation and joint degradation. Together, improvement of these tests provides valuable insights into an efficacy of AQDE in reducing pain and inflammation in OA models.

Oxidative stress and inflammation play pivotal roles in the pathogenesis of osteoarthritis (OA). Oxidative stress arises from the overproduction of reactive oxygen species (ROS), which damage cartilage and joint tissues by degrading collagen and proteoglycans (Chatterjee 2016). This process exacerbates the inflammatory response, leading to the activation of pro-inflammatory cytokines including IL-1β and TNF-α, which further accelerate cartilage breakdown and joint degeneration (Fernández-Sánchez *et al*. 2011). Chronic inflammation perpetuates the cycle of tissue damage, resulting in pain and joint dysfunction in OA patients. Drugs used in OA treatment, such as nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids, primarily target inflammation to alleviate pain and slow disease progression. Antioxidants, such as vitamin C and E, may reduce oxidative stress, while disease-modifying osteoarthritis drugs (DMOADs) are being

explored for their potential to curb both oxidative stress and inflammation. Emerging therapies focus on modulating these pathological pathways to offer more comprehensive management of OA (Chen *et al*. 2021). In the present study, AQDE was found to cause significant ameliorative effect on the oxidative stress and inflammation, thereby exerting antioxidant and anti-inflammatory action against OA in rats.

COX-2 (cyclooxygenase-2) and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) are key molecular players in the pathogenesis of osteoarthritis (OA) (Liu *et al*. 2017). COX-2 is an inducible enzyme that becomes upregulated in response to pro-inflammatory stimuli in OA. It catalyzes the conversion of arachidonic acid to prostaglandins, which are potent mediators of inflammation and pain. In OA, excessive COX-2 activity leads to elevated prostaglandin levels in the synovial fluid (Kim *et al*. 2011), contributing to synovial inflammation, cartilage degradation, and the characteristic pain associated with OA. Moreover, NF-κB is a transcription factor that regulates the expression of various genes involved in inflammation, immune responses, and cell survival (Rahman and Fazal 2011). In OA, NF-κB is activated by inflammatory cytokines like IL-1β and TNF-α, leading to the production of additional pro-inflammatory mediators, matrix metalloproteinases (MMPs), and COX-2. This creates a vicious cycle, where inflammation perpetuates cartilage breakdown and joint damage (Khongthong *et al*. 2019). Thus, in the present study, the effect of AQDE on the both these mediators on rat chondrocytes and cartilage of MIA rats was examined. The results indicate that AQDE significantly reduces NF-κB and COX-2 levels both in vitro and in vivo, suggesting that its anti-inflammatory effects in OA rats are mediated through the inhibition of these pathways. However, future studies need to determine the possible active component of the extract to translate for future clinical usage.

CONCLUSIONS

1. **Anti-inflammatory and Antioxidant Effects**: *Akebia quinata* D. aqueous extract (AQDE) was found to exhibit significant anti-inflammatory and antioxidant properties by modulating biomarkers such as IL-1β, IL-6, MMP-13, and PTGS2, while restoring oxidative stress markers (MDA, SOD, and GSH) to near-normal levels.
2. **Cellular Protection**: AQDE enhanced the expression of protective proteins (Bcl2, CDK1, CCND) and reduced chondrocyte apoptosis, promoting cartilage health.
3. **In Vivo Efficacy**: AQDE improved mechanical allodynia, reduced joint swelling, and mitigated bone erosion in osteoarthritis (OA) rats, as confirmed by micro-CT imaging.
4. **Mechanistic Insight**: The anti-osteoarthritis effects of AQDE were mediated *via* inhibition of COX-2 and NF-κB expression, highlighting its potential as a therapeutic agent for OA.

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