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# Fisetin Attenuates Cartilage Destruction in Adjuvant-Induced Arthritis by Modulating Cartilage Cytokine Expression Correlated with Oxidative Status in the Early Phase in Experimental Animals

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Original article

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The present study was designed to evaluate the effect of Fisetin on the progression of adjuvant-induced arthritis in rats and explore the mechanisms underlying fisetin mediated immunomodulation. Adjuvant-induced arthritis (AIA) was induced by a single subcutaneous injection of Freund's complete adjuvant (FCA). AIA rats were treated with Fisetin daily via oral gavage, for a period of 28 days. Paw swelling changes were assessed and histopathological and radiographic analysis was conducted to evaluate the antiarthritic effect. Lipid peroxidation and antioxidant enzyme activities in the joint tissue homogenate were performed to observe the modulation of the antioxidant status along the expression of different pro-inflammatory cartilage cytokines, such as TNF-α and IL-6. Fisetin promotes both the antiarthritic and the antioxidant effect, as well as the suppression of lipid peroxidation. Fisetin significantly inhibited the development phase of arthritis, as supported by histopathological and radiographical observations, and reduced overexpression of cartilage cytokines. Fisetin not only suppressed the arthritic progression and tissue destruction, but it also demonstrated a pronounced anti-inflammatory and immunomodulatory action against the immunosuppressive properties of AIA and provided a superior effect against inflammation. Furthermore, fisetin therapy restored the BMD loss and acts as a potent antioxidant and immunomodulator, suggesting that oral administration can suppress arthritic progression in rats.

Key words: Flavanol, fisetin, adjuvant-induced arthritis, antioxidant, cytokines.

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Rheumatoid arthritis (RA) is characterized by systemic chronic and autoimmune diseases in which the immune system erroneously assaults the body's own tissues, which leads to irreversible joint damage and motor disability (VALESINI *et al.* 2015). Synovial hyperplasia, synovitis, joint swelling, pannus formation, and stiffness are the traits of RA which have also been associated with bone erosion and cartilage destruction during the late stages of the disease. Arthritis affects between about 3.3% and 3.6% of the population globally. It causes moderate to severe disability in 43 million people, making it the 11th most debilitating disease around the world (BORTOLUZZI *et al.* 2018). The prevalence of RA was reported to amount to 1.02% in China, affecting primarily laborers with an economic burden of $3826 per capita per year (XU *et al.* 2014). The etiology of arthritis is unclear, but reports suggest that its pathogenesis is strongly associated with oxidative stress, as the overproduction of reactive oxygen species (ROS) from activated neutrophils and macrophages can mediate tissue

injury (DATTA *et al*. 2014). Furthermore, these elevated levels of ROS via the modulation of signaling pathways promote the generation of proinflammatory cytokines, including the tumor necrosis factor (TNF-α), interleukin IL-1β, IL-6, interferon (IFN-γ), and IL-10, solely responsible for initiation and progression of arthritis (SHAH *et al.* 2011).

Accordingly, the pharmacological attenuation of oxidative stress and chemical agents that can mediate the downregulation of these inflammatory components appears to be a promising approach for the treatment of arthritis. Although nonsteroidal anti-inflammatory drugs (NSAIDs), such as indomethacin, disease-modifying antirheumatic drugs (DMARDs), such as methotrexate and corticosteroids, suppress the symptoms of rheumatoid arthritis, their clinical use is limited due to their side effects coupled with a lack of robust efficacy, which discourages long-term compliance or use by the patients (FEI *et al.* 2019). In an attempt to address this problem, plant-based natural products with various anti-inflammatory effects and low toxicity with the potential to treat arthritis have attracted an increasing interest.

Fisetin (3,3',4',7-tetrahydroxyflavone) is a bioactive flavonol molecule found in many fruits and vegetables, such as apple, strawberry, grape, persimmon, cucumber, and onion. This compound has gained an enormous interest in the scientific community due to its antioxidant and ROS-scavenging activity (PJØRKLUND *et al.* 2013). Previous preclinical reports suggested that it has strong anti-inflammatory, antidiabetic, antioxidant, cardioprotective, and neuroprotective effects (BJØRKLUND *et al.* 2017). Fisetin protected against cisplatin-induced renal injury by modulating the activation of the nuclear factor-kappaB (NF-κB), inhibited apoptosis, and restored antioxidant defenses (SAHU *et al.* 2014). In addition, fisetin attenuated the ischemia/reperfusion (I/R)-induced cardiac injury by decreasing oxidative stress, induced apoptosis, and restored the structure and function of the mitochondria (SHANMUGAM *et al.* 2018). In addition, fisetin was shown to decrease ROS levels and upregulate the expression of antioxidant genes in cardiomyocyte hypertrophy induced by phenylephrine (PE) *in vitro* (DONG *et al.* 2018). In a recent report, fisetin protected against doxorubicin- (DOX) induced cardiac injury through the inhibition of multiple processes, including oxidative stress and inflammation (MA *et al.* 2019). Despite the multiple pharmacological effects, the ameliorative effect of fisetin on arthritis has not been reported yet. We further explore the anti-arthritic activity of fisetin using the adjuvant-induced rat arthritis model. Adjuvant-induced arthritis (AIA) rats are a widely accepted experimental animal model of arthritis that shows histopathological and clinical features similar to human arthritis (KANNAN *et al.* 2005). The hallmark of this model is a reliable onset, progression of a robust, easily measurable polyarticular inflammation, marked bone resorption, periosteal bone proliferation, and cartilage destruction. In addition, this model has the ability to assess the role of several pathogenic factors, including cytokines and chemokines at various phases of disease progression (SZEKANECZ *et al.* 2000). Furthermore, the use of Freund's complete adjuvant (FCA) remains scientifically justified in many systems, especially in the induction of autoimmune disease models for which no comparable alternative exists (BILLIAU & MATTHYS 2001). Prompted by the evidence, the present study was designed to unveil the effect of fisetin on the progression of AIA in rats by examining the hind paw volume, the hematological, radiographic, and histopathological analysis correlates with the modulation of antioxidant status, and the expression of cartilage cytokines.

## Materials and Methods

### Materials

All experimental procedures involving animals were performed strictly according to the recommendations by "The Traditional Chinese Medicine and Western Medicine Hospital of Cangzhou of Hebei" Committee (Ethical committee No. HBZX201908102) for the care and use of laboratory animals.

All reagents used for the experiment were of analytical grade. Fisetin, Freund's complete adjuvant (FCA), biotinylated horseradish peroxidase, and 3,3'-diaminobenzidine (DAB) were purchased from Sigma Chemical, Co. (St. Louis, MO, USA). Rabbit anti-rat TNF-α and IL-6 polyclonal antibodies and biotinylated goat anti-rat IgG were purchased from ANASPEC, Inc. (San Jose, CA). The Target retrieval solution was acquired from Dako Cytomation (Carpinteria, CA). Avidin-biotin blocking kit was purchased from Vector Laboratories (Burlingam, CA). Other reagents used for the experiment were obtained in their purest forms from local firms.

### Animals and diet

Inbred male Wistar rats (150-180 g) were purchased from the Department of Laboratory Animal Science, Tianjin Medical University, China, and were housed separately in animal cages (Tarsons) in a room and acclimated for 1 week under

standard laboratory conditions at a temperature of 25±1°C and humidity of 50-60%, and were exposed to a 12:12 h light and dark cycle and allowed free access to both drinking water and commercial standard rat chow diet throughout the experiment.

### Induction of adjuvant arthritis

Adjuvant arthritis was induced by subcutaneously injecting 0.1 ml of FCA suspension of heat-killed *Mycobacterium butyricum* in paraffin oil (10 mg/ml) into the plantar surface of the right hind paw and two booster FCA doses (0.1 ml) into the tail root (ARAB *et al.* 2017).

### Dose selection and treatment

The dose of fisetin was selected based on the previously reported therapeutic and toxicological properties. Reports suggests that a small dose of fisetin inhibits inflammation-related cytokines in arthritic fibroblasts, such as the synovial cells in rats (LEE *et al.* 2009).

Fifty rats were randomly allocated to five groups (10 rats/group):

1) Control group: normal rats, administered the oral vehicle only (0.5% carboxymethyl cellulose) for the entire study and served as the negative control group.

2) Adjuvant arthritis group: arthritic rats, administered the oral vehicle for 28 days. Arthritis was induced by a subcutaneous inoculation of FCA (0.1 ml) into the plantar surface of right hind paw and two booster FCA doses (0.1 ml) *via* the tail root.

3) Fisetin (25 mg/kg) treated arthritic group: arthritic rats, administered fisetin by oral gavage (25 mg/kg/day) for 28 days daily.

4) Fisetin- (50 mg/kg) treated arthritic group: arthritic rats administered fisetin by oral gavage (50 mg/kg/day) for 28 days daily.

5) Fisetin (100 mg/kg) treated arthritic group: arthritic rats administered fisetin by oral gavage (100 mg/kg/day) for 28 days daily.

### Paw volume assessment

As the disease progressed, the swelling of the adjuvant-injected hind paw was established plethysmographically, and paw volumes were assessed once per week.

### Serum albumin level

Serum albumin was assessed on days 14, 21 and 28 in the rat plasma with spectrophotometric methods using a commercially available albumin estimation kit (Quanti ChromTM BCG Albumin Assay Kit) at 620 nm.

### Bone mineral density

Dual-energy X-ray absorptiometry (DXA) using a Hologic QDRs-4500 device (Waldham, MA, USA) for measuring small laboratory animals was used to analyze bone mineral density. Bone mineral density ($g/cm^2$) of the tibiotarsal region of the rats was determined on day 28.

### Radiography

On day 28, the rats were anesthetized with diethyl ether, and an intact animal was kept in a radiographic box at a distance of 90 cm from the X-ray source. A radiographic analysis of normal and arthritic hind paws was performed using an X-ray machine (Philips X12, Germany) with a 45 kW exposition, 5 mA, and 30 seconds of exposure time.

### Determination of *in vivo* antioxidant activity

#### Assessment of lipid peroxidation

Joint cartilage tissue samples were homogenized in a solution of 5% trichloroacetic acid and 5 mM EDTA and centrifuged for 10 min at 15,000×g in 4°C. Lipid peroxidation from the tissue homogenate can be determined by the formation of malondialdehyde (MDA) and measured using the thiobarbituric acid reactive substance method (ZHANG *et al.* 2015). The level of lipid peroxides was expressed as millimoles of TBA reactants/100 g of wet tissue.

#### Measurement of reduced glutathione

Reduced glutathione (GSH) was estimated using the Smyth method; 0.1 ml of tissue homogenate was mixed well with 5% TCA for a complete precipitation of proteins followed by centrifugation. To an aliquot of clear supernatant, 2.0 ml of 0.6 mM DTNB reagent and 0.2 M of phosphate buffer (pH 8.6) were added to obtain a final volume of 4.0 ml. Absorbance was read at 412 nm (SMYTH *et al*. 2006). The amount of glutathione was expressed as mg/100 g of wet tissue.

#### Analysis of SOD Activity

Tissue homogenate was centrifuged at 1600 rpm for 15 min. The supernatant was collected and mixed with reaction mixtures, containing 0.2 ml of EDTA (6 mM) with 0.1 ml of sodium cyanide (NaCN, 3 mM), 0.05 ml of riboflavin (2 mM), 0.1 ml of nitro blue tetrazolium (NBT, 50 mM), and phosphate buffer in a final volume of 3 ml. The absorbance was measured at 530 nm. The specific activity of SOD was expressed as a unit/mg protein in the supernatant (BORA *et al.* 2011).

### Measurement of Catalase Activity

Catalase activity was assessed according to the method suggested by NARENDHIRAKANNAN; 0.2 ml of the tissue homogenate was mixed thoroughly with 1.2 ml of 50 mM phosphate buffer (pH 7.0), and the enzyme reaction was started by the addition of 1.0 ml of 30 mM $H_2O_2$ solution. The decrease in absorbance was measured at 240 nm at 30 s intervals for 3 min. The enzyme activity was expressed as micromoles of $H_2O_2$ decomposed/min/mg of protein (NARENDHIRAKANNAN *et al.* 2005).

### Histopathological Evaluation

The hind paws were dissected and processed for histology. Ankles were fixed into 10% neutral buffered formalin. After 48 h in the fixative, the specimens were placed into 5% formic acid for decalcification for at least 7 days. After the ankles were sufficiently decalcified, they were transected in approximately equal halves processed for paraffin embedding. The ankles were cut into 5-µm-thick sections using microtome and stained with hematoxylin and eosin (H & E) for histological evaluation using light microscopy.

### Immunohistochemical analysis of TNF-α and IL-6

The formalin fixed, paraffin embedded tissues were cut into 5-µm-thick sections to mount in the glass slides and deparaffinized, followed by rehydration with ethanol. Endogenous peroxidase activity was blocked for 1 h in the dark with 1% $H_2O_2$ and 5% normal goat serum for 30 min to reduce background staining due to nonspecific binding sites. Subsequently, endogenous biotin was blocked with avidin for 30 min and biotin for additional 15 min. Thereafter, the sections were incubated overnight at 4°C with 50 µl of rat cytokine specific antibodies: polyclonal ligand affinity-purified rabbit anti-rat IL-6 and polyclonal antigen affinity-purified rabbit anti-rat TNF-α. After washing, the sections were incubated with biotinylated goat anti-rat IgG for 30 min at room temperature. Biotinylated horseradish peroxidase was added and incubated for 1 h at room temperature, and immunoperoxidase labeling was detected using DAB as a substrate. As soon as the sections turned brown, the slides were submerged in $ddH_2O$ two times for 5 min each. All slides were counterstained with hematoxylin, rinsed, dehydrated, and mounted with cover slips. Scoring was performed by two investigators using coded slides. The labeling index was calculated as a percentage of IL-6 and TNF-α positive cells per total number of cells counted.

### Statistical analysis

Data were expressed as means ± standard error of the mean (SEM), which were calculated from quantified data obtained from at least three replicative experimental conditions. Statistical analysis was performed using the *t* test and one-way analysis of variance (ANOVA), followed by the *post hoc* Dunnett's test as appropriate using the Graph Pad Prism. Statistical significance was achieved at $p<0.05$, $p<0.01$ and $p<0.001$.

## Results

No signs of general toxicity were observed with the fisetin therapy, and all the animals survived the 28-day study period. The inoculation of FCA subcutaneously in the plantar region of the right hind paw resulted in inflammatory and arthritic lesions that were discernable by edema and erythema within 7 days (Fig. 1. A-E). The effect of fisetin therapy on body weight gain (Table 1) indicated that all groups receiving the fisetin treatment recovered from the diseased state.

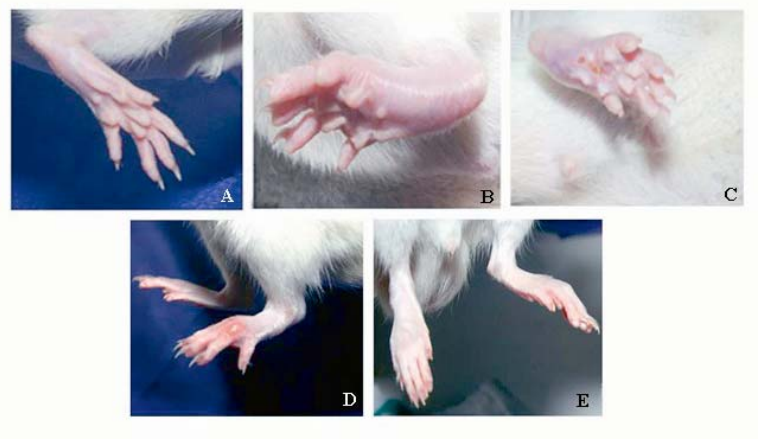


Fig. 1. Representative photographs of the right hind paw of normal control (A), arthritis control (B), Fisetin 25mg/kg (C), Fisetin 50 mg/kg (D), and Fisetin 100 mg/kg (E) adjuvant-induced arthritic rats, taken on day 28 after immunization with FCA. Diffuse soft tissue swelling and redness with joint thickening was detectable in the arthritis control, whereas there were no signs of joint thickening, redness, or swelling in fisetin treatment.

Table 1

Effect of fisetin on body weight of AIA rats

| Treatment | Dose (mg/kg) | Body weight (g) | | % Increase in body weight |
|---|---|---|---|---|
| | | Initial | Final | |
| Normal control | Normal saline (0.1 ml) | 152.3 ± 1.3 | 181.5 ± 1.09 | 8.74* |
| Arthritic control (AIA) | CFA (0.1 ml) | 164.4 ± 1.02 | 169.6 ± 1.6 | 1.56 |
| Fisetin | 25 mg/kg | 158.7 ± 1.31 | 166.5 ± 0.92 | 2.40 |
| Fisetin | 50 mg/kg | 163.6 ± 0.94 | 172.1 ± 0.73 | 5.35 |
| Fisetin | 100 mg/kg | 164.2 ± 0.98 | 190.5 ± 1.03 | 7.41* |

Values are mean ± SEM ($n$ = 10), significantly different from arthritic control rats: *p<0.05

## Effect of fisetin in paw swelling changes

The swelling of the hind paw suggests both inflammatory and arthritic changes taking place in rats with AIA. Changes in the paw volume of all groups of rats were assessed for inflammation once a week. Figure 2 indicates a time-dependent increase in hind paw volume of the rats injected with FCA. Maximal paw volume was attained by day 28 in the adjuvant-induced rats. Fisetin treatment significantly suppressed hind paw swelling on days 21 and 28 following adjuvant arthritis induction. Fisetin therapy at 100 mg/kg displayed a constant suppression of significant hind paw swelling from days 14 to 28 following adjuvant arthritis immunization, and the maximal reduction in paw swelling was observed on day 28 (p<0.001), as compared to the arthritic control.

## Serum albumin level and bone mineral density

Serum albumin is considered one of the primary inflammatory markers. Both fisetin 50 mg/kg (p<0.01) and fisetin 100 mg/kg significantly increased (p<0.001) the serum albumin level throughout the treatment period (Table 2). Fisetin at 25 mg/kg did not significantly impact the inflammatory marker throughout the study.

Another indicator of arthritis-induced osteoporosis in adjuvant arthritic rats is the bone mineral density. On day 28 of post immunization, non-treated arthritic rats and arthritic rats treated with fisetin 25 mg/kg had a prominently lower bone mineral density values compared to the other treated groups (Table 2). Among the fisetin-treated groups, only a dose of 100 mg/kg of fisetin presented a significantly higher bone mineral density (p<0.01), as compared to non-treated arthritic rats.

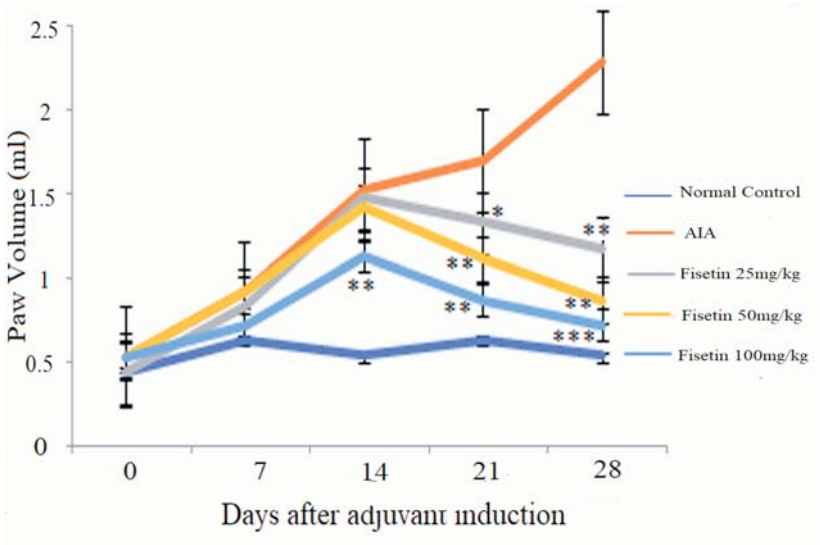


Fig. 2. Effect of fisetin on paw swelling of AIA rats, taken on every 7th day after the immunization with FCA. Data represents mean values ± SEM ($n$ = 10). *p<0.05; **p<0.01; ***p<0.001. Paw volumes of different treated groups were compared with non-treated arthritis control rats. The severity of paw swelling was significantly reduced in the fisetin 50 mg/kg treated group (p<0.01) and fisetin 100 mg/kg treated group (p<0.001).

Table 2

Effect of fisetin on serum albumin concentration (g/dl) and bone mineral density (BMD) in AIA rats in the consecutive weeks

| Group | 14 day | 21 day | 28 day | BMD (Day 28) |
|---|---|---|---|---|
| Normal control | 4.51 ± 0.14*** | 4.44 ± 0.09*** | 4.58 ± 0.09*** | 0.196 ± 0.01*** |
| Arthritic control (AIA) | 2.65 ± 0.11 | 3.22 ± 0.07 | 3.38 ± 0.08 | 0.127 ± 0.01 |
| Fisetin 25 mg/kg | 2.92 ± 0.05 | 3.18 ± 0.1 | 3.45 ± 0.12 | 0.131 ± 0.002 |
| Fisetin 50 mg/kg | 3.26 ± 0.02** | 3.47 ± 0.04** | 3.60 ± 0.02** | 0.149 ± 0.002** |
| Fisetin 100 mg/kg | 3.29 ± 0.01*** | 3.63 ± 0.02*** | 3.83 ± 0.02*** | 0.165 ± 0.003** |

Values are mean ± SEM (n = 10). Results were analyzed using one-way ANOVA followed by the Dunnett's test and compared with the arthritic control (AIA) group: **p<0.01, ***p<0.001.

## Radiographic analysis of the tibiotarsal joints

The clinical examination of RA permits the therapeutic observation of cartilage tissues and continues to be the standard technique for assessing the progression of the disease. Radiographic irregularities, such as loss of articular cartilage, may contribute to the weakening of joint space, which may be caused by an assortment of pathological mechanisms. Changes in the bone structure in the adjuvant-induced group were designated by bone matrix resorption, osteophyte development, and an increased joint space with critical bone erosion and soft tissue swelling (Fig. 3B). However, no such changes were observed in the control group (Fig. 3A). Animals treated with 25 mg/kg of fisetin displayed a modest inflammation with less diffused joint space (Fig. 3C), whereas animals treated with 50 mg/kg of fisetin showed less cartilage destruction and diminished swelling with a partial diffusion of the joint space (Fig. 3D). A diminished joint space with no joint swelling or bone erosion was observed in the 100 mg/kg fisetin-treated groups (Fig. 3E).

## Lipid peroxidation and *in vivo* antioxidant status

Treatment of AIA rats with various doses of fisetin and its effect on lipid peroxidation and *in vivo* antioxidant status are shown in Table 3.

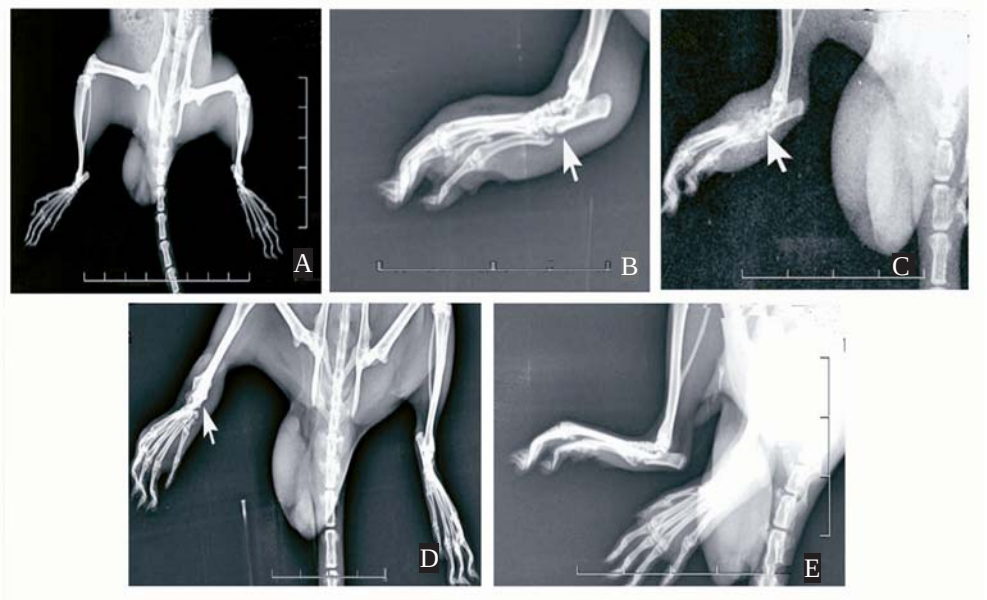


Fig. 3. Radiographic changes in joints of AIA rats and AIA rats treated with fisetin, taken on day 28 after the immunization with FCA. No evidence of pathological changes was observed in normal control animals (A). AIA control group showing severe inflammation with diffused joint space and cartilage destruction along with bone erosion (B). Fisetin 25 mg/kg (C) and fisetin 50 mg/kg (D) shows mild to moderate inflammation with less diffusion in joint space. Fisetin 100 mg/kg therapy shows a clear joint space with no evidence of bone erosion or inflammation (E).

Table 3

Effect of fisetin on lipid peroxidation and *in vivo* antioxidant status in joint tissue homogenate of AIA rats

| Parameters | MDA content (mM/100 g tissue) | SOD activity (U/mg tissue) | GSH content (mg/100 g tissue) | Catalase activity (μM $H_2O_2$decomposed/min/mg protein) |
|---|---|---|---|---|
| Normal control | 1.24 ± 0.006*** | 271.2 ± 1.3*** | 36.1 ± 1.19*** | 4.37 ± 0.3*** |
| Arthritic control (AIA) | 2.64 ± 0.01 | 95.1 ± 0.7 | 23.2 ± 0.79 | 2.74 ± 0.2 |
| Fisetin 25 mg/kg | 2.37 ± 0.013 | 124.1 ± 1.14* | 24.2 ± 0.94 | 3.28 ± 0.1 |
| Fisetin 50 mg/kg | 1.79 ± 0.008*** | 221.7 ± 1.55*** | 28.5 ± 0.8*** | 4.05 ± 0.02*** |
| Fisetin 100 mg/kg | 1.57 ± 0.018*** | 236.3 ± 1.87*** | 34.4 ± 0.96*** | 4.22 ± 0.01*** |

Results were expressed as mean ± SEM (n = 10). Statistical analysis was performed using one-way ANOVA followed by the *post hoc* Dunnett's test. All data were compared with the arthritic control group: *p<0.05, ***p<0.001.

Overall MDA content was higher ($p<0.001$) in the arthritic control group, in contrast to the normal control animals. Fisetin in the 100 and 50 mg/kg doses provided a significant reduction ($p<0.001$) in the MDA content of the cartilage tissue, while the reduction of MDA content was found to be insignificant ($p>0.05$) following treatment with 25 mg/kg of fisetin. Reduced glutathione, SOD, and catalase activity decreased significantly ($p<0.001$) in the arthritic control group, as compared to the normal control. Treatment with the 100 and 50 mg/kg doses considerably increased the *in vivo* antioxidant markers, as compared to the arthritic control groups of animals. Antioxidant activities were not significantly increased on treatment with 25 mg/kg of fisetin.

## Histopathological evaluation

The histological architecture of normal rats denoted a joint structure comprising of tibio-tarsal bone articulation with bone, cartilage, marrow cavity and synovial cartilage junction. Inflammation or tissue destruction of any kind was not observed in the H & E sections of normal control rats (Fig. 4A). In comparison, the tissue slides of the right tibiotarsal joints of adjuvant-induced arthritic animals showed prominent synovial proliferation along with a constriction of the joint space of the synovial cartilage junction, subchondral bone damage, cartilage impairment, articular bone tissue attrition, pannus formation, and invasion of the bone matrix (Fig. 4B, C, D). These symptoms were significantly amended on treatment with 100 mg/kg (Fig. 4E), 50 mg/kg (Fig. 4F), and 25mg/kg of fisetin. The proliferation and expansion of the synovial tissue were also significantly mitigated by the fisetin treatment compared to AIA animals. Reduced cartilage and subchondral bone destruction, which evidenced the reversal of inflammation symptoms, was considerably more intense following the 100 mg/kg fisetin treatment. Additionally, the fisetin treatment demonstrated a prominent constructive effect on articular bone and tissue erosion along with pannus formation (Fig. 4G).

## Immunohistochemical analysis

In the current study, we detected the expressions of pro-inflammatory cytokines, such as TNF-α and IL-6. They were subjectively observed under light microscopy with the immunohistochemical technique against antigen expressions by using TNF-α and IL-6 antibodies.

### Tumour Necrosis Factor-α (TNF-α)

The cells showing TNF-α expression are located in the synovial lining and, to a lesser extent, in the cartilage and bone tissues. Figure 5A denotes the expressions of TNF-α in AIA (Group II) rats. Figure 5B, 5C, and 5D represents the histological sections of the 25, 50, and 100 mg/kg fisetin treated rats. A large number of TNF-α immunopositive cells were identified in the tissue joint of the arthritic control rats; however, a significant decrease in TNF-α immunopositive cells was noted upon fisetin supplementation compared to the arthritic control group. The highest suppression of the TNF-α expression ($p<0.01$) was noted in the 100 mg/kg fisetin treated animals (Table 4).

### Interleukin-6 (IL-6)

The expression of IL-6 in the ankle joints of different groups of animals is denoted in Figure 6. IL-6, recognized as the most widely distributed cytokine, was observed throughout the synovial and

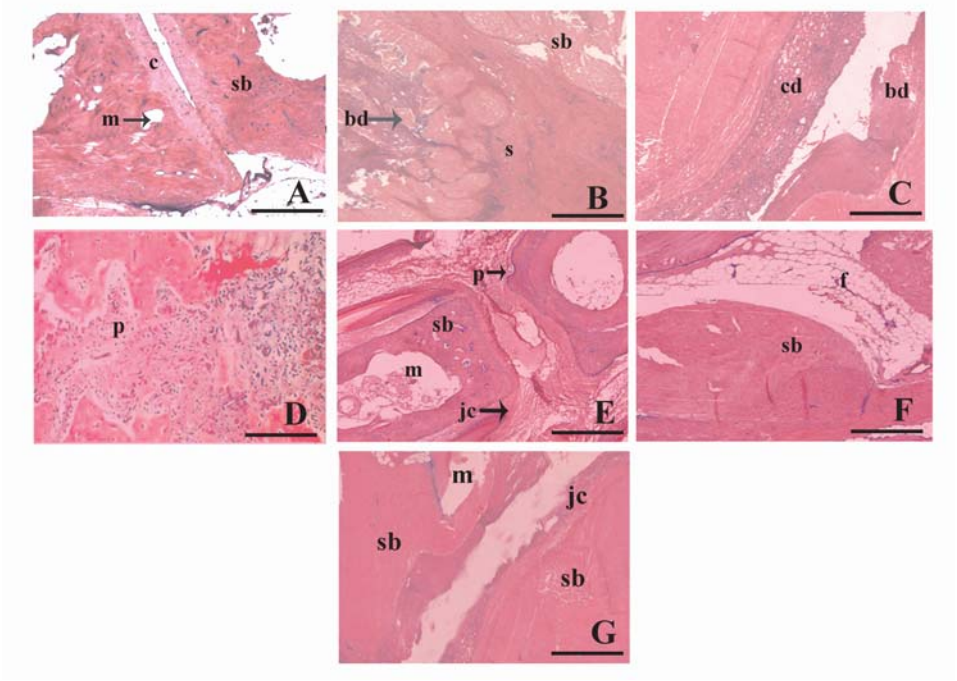


Fig. 4(A-G). Effect of fisetin on tibiotarsal joint histology in AIA rats, taken on day 28 after immunization with FCA. A – indicates a normal joint structure with subchondral bone destruction (sb) and an increase in marrow cavity (m) and cartilage (c). B, C, and D – denotes tissues from AIA rats. B – shows discernable synovial proliferation (s), subchondral bone destruction (sb), and severe bone destruction (bd). C – denotes severe bone (bd) and cartilage destruction (cd). D – signifies the pannus formation (p) and invasion of the bone matrix, which indicates bone erosion. E, F, and G represents fisetin 25 mg/kg, fisetin 50 mg/kg and fisetin 100 mg/kg treatment in adjuvant arthritis rats, respectively. E – portrays moderate changes involving the pannus penetration (p) into the articular cartilage initiating focal erosion and junctional cartilage loss (jc). However, the subchondral bone (sb) and bone marrow cavity (m) destruction still persists. F – represents progress towards the restoration of subchondral bone (sb) and mild inflammatory reaction with fibrin exudation (f). G – denotes an almost normal architecture of joints with intact subchondral bone (sb), marrow cavity (m), and junctional cartilage (jc). Original magnification 10x with a scale bar of 50 μm.

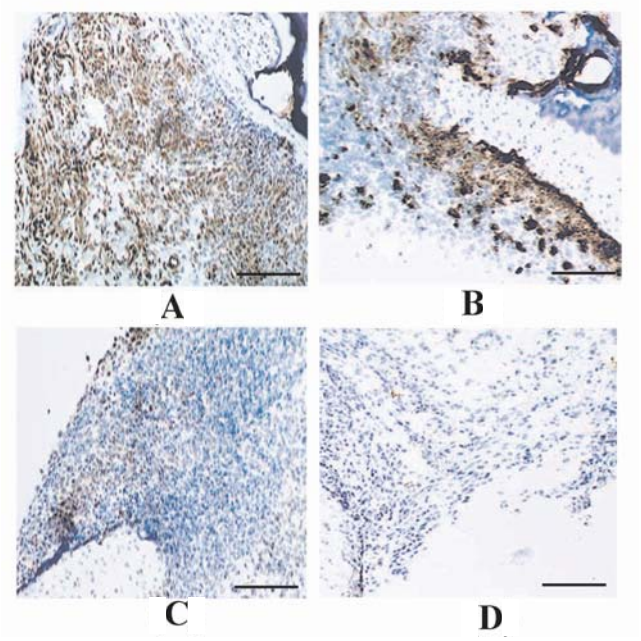


Fig. 5(A-D). Representative photomicrographs illustrating brown (diaminobenzidine) staining of the TNF-α of tibiotarsal joint tissue from arthritic animals and the treated groups. A – represents adjuvant arthritic animals at the time point of maximal arthritis (day 28 after immunization) with sections stained for the expression of TNF-α. B-D – represents various groups with fisetin therapy, namely, 25 mg/kg, 50 mg/kg and 100 mg/kg respectively. Articular cartilage and bone (bluish staining) were evident in all figures. Original magnification 40x with a scale bar of 200 μm.

Table 4

Expression of TNF-α and IL-6 immunopositive cells of FCA-induced cartilage after 28 days of treatment with fisetin in AIA rats

| Group | TNF-α | IL-6 |
|---|---|---|
| Arthritic control (AIA) | 29.5 ± 0.25 | 19.6 ± 0.26 |
| Fisetin 25 mg/kg | 24.7 ± 0.33 | 18.1 ± 0.16 |
| Fisetin 50 mg/kg | 20.6 ± 0.34* | 16.5 ± 0.20* |
| Fisetin 100 mg/kg | 15.2 ± 0.17** | 9.9 ± 0.14** |

Approximately 200 cells were counted per field, five fields were examined per slide, and five slides were examined per group. Values are presented as mean ± SEM. Results were analyzed using one-way ANOVA, confirmed by Dunnett's test, all data were compared with the AIA group: *p<0.05, **p<0.01.

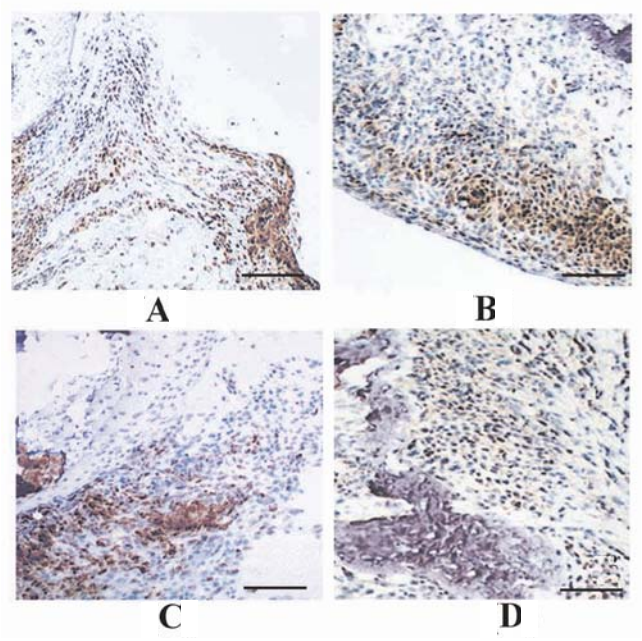


Fig. 6. Representative photomicrographs illustrating brown (diaminobenzidine) staining of IL-6 of the tibiotarsal joint tissue from arthritic animals and the treated groups. A – represents adjuvant arthritic animals at the time point of maximal arthritis (day 28 after immunization) with sections stained for the expression of IL-6. B-D – represents various groups with fisetin therapy, namely, 25 mg/kg, 50 mg/kg, and 100 mg/kg, respectively. Articular cartilage and bone (bluish staining) were evident in all figures. Original magnification 40x with a scale bar of 200 μm.

bone tissue in the AIA rats. The expression of IL-6 has been found to be associated with the osteoblasts present within and on the surface of bone tissues. A large number of IL-6 immunopositive cells was found in the AIA control animals (Fig. 6A); however, the expressions of IL-6 in the tissues of animals treated with fisetin displayed a substantial decrease in IL-6 immunopositivity (Fig. 6B, 6C, and 6D). The reduction of IL-6 expressions in the rat ankle joint was found to be significant in the 50 mg/kg fisetin-treated ($p<0.05$) and 100 mg/kg fisetin-treated ($p<0.01$) animals, but no significant decrease was observed in the 25 mg/kg fisetin-treated animals (Table 4).

## Discussion

Several murine models of immune-mediated arthritis are considered the standard methods for the evaluation of novel antiarthritic drug molecules and their mechanisms of actions during pre-clinical research (BEVAART *et al*. 2010). Even though animal models may be insufficient to replicate all the manifestations of human rheumatoid arthritis, they certainly assist us in recognizing the typical inflammatory and immune responses presented during the pathogenesis of rheumatoid arthritis (QUINONES *et al.* 2004). Arthritis induced by FCA is one of the fundamental animal models for

screening anti-inflammatory drugs owing to its histological and immunological resemblance to RA in human patients (GÓMEZ-SANMIGUEL *et al.* 2016). The bacterial peptidoglycan and muramyl dipeptide present in FCA are responsible for the initiation of adjuvant arthritis in rats (PETCHI *et al.* 2015) and are regarded as the standard for adjuvants by many immunologists.

Rheumatoid arthritis is a chronic inflammatory and autoimmune disorder that can be identified through synovial hyperplasia, inflammation, and angiogenesis in synovial tissues, which leads to pannus formation and extensive cartilage and bone destruction (MCINNES & SCHETT 2011). To date, there is no cure for RA. However, clinical studies indicate that remission of symptoms is more likely when treatment begins early with medications known as disease-modifying antirheumatic drugs (DMARDs) (GOUGH & FOO 2011). Nevertheless, once remission is reached with DMARDs, there are no distinct guidelines for maintenance therapy, giving rise to several situations requiring drug withdrawal/dose modification due to such events as adverse drug reactions (ADRs), lack of efficacy, and development of resistance (MITTAL *et al.* 2012). An enhanced understanding of the prognosis of RA has directed to the development of various alternative modes of treating RA. Plants used in traditional medicine currently provide a rich source of candidate drugs for the treatment of chronic inflammatory diseases. It has been reported that dietary flavonoids can regulate the symptoms of joint inflammation and improve arthritis manifestations in both human RA and animal models; however, no scientific evidence about their mechanism of action in RA is currently available (HUGHES *et al.* 2017). Additionally, a single mediator has not been able to demonstrate a consistent level of acceptability and a continued degree of efficacy in a large patient population. Our study was designed to evaluate the anti-arthritic efficacy of fisetin, a bioactive flavonol molecule in adjuvant arthritic rats.

Rheumatoid arthritis was assessed by observing the architectural modifications of the synovial tissue and the serological conditions of the disease. In addition, changes in body weight and paw volume were assessed, and an immunohistochemical examination of the expression of pro-inflammatory cytokines, such as TNF-α and IL-6, was performed. Treatment with fisetin not only suppressed the arthritic progression and tissue destruction, but it also demonstrated pronounced anti-inflammatory and immunomodulatory action against the immunosuppressive properties of AIA and provided a superior effect against inflammation. It was further noted that fisetin therapy restored the BMD loss that is prominent in the arthritic animals.

Paw swelling assessed weekly showed a significant reduction of swelling in adjuvant-injected rat paws and joints throughout the experiment following treatment with different doses of fisetin. The improvement of paw volume indicated that fisetin treatment can produce both an anti-inflammatory and an immune response attenuation in all the treatment groups. Results demonstrate that fisetin at a dose of 100 mg/kg significantly inhibited the development period of chronic joint swelling induced by FCA when compared to the AIA control rats. Body weight of rats in all treatment groups increased by the end of day 28.

The concentrations of serum albumin served as a negative acute phase indicator of RA and were low in patients and arthritic rats. The reduction in serum albumin values denoted alterations in the synthesis of the protein by the liver owing to the stimulation of hepatic cells by inflammatory cytokines (GUICCIARDI *et al.* 2013). In this experiment, fisetin at a dose of 50 and 100 mg/kg reduced serum albumin levels at all time points, whereas a dose of 25 mg/kg had no effect on this inflammatory marker.

A radiographic analysis of the tibiotarsal joint in arthritis further potentiated the anti-arthritic effect of fisetin on the AIA rats. A histopathological estimation of the AIA rats demonstrated pronounced inflammatory reactions with granulomas and pannus formation, which caused the progressive destruction of articular cartilage and bone in the tibiotarsal regions. Fisetin treatment provided effective support against inflammatory changes involving the synovium and articular cartilage. Fisetin treatment further diminished the rate of disease progression, and there were no inflammatory cell infiltrations. Furthermore, fisetin treatment caused the bone and cartilage structures to return to the normal state. Even though severe synovitis in the tibiotarsal joint region of the hind paw was noted in rats induced with the adjuvant, no noticeable synovitis was found in the joint region of animals treated with fisetin.

For the purposes of evaluating the antiarthritic properties of a drug, the detection of MDA levels of MDA provides an excellent yet simple tool (ZHAO *et al.* 2016). An increase in the lipid peroxide level in the arthritic tissue of AIA rats designates increased oxidative stress. Treatment with fisetin effectively decreased the MDA content by interrupting the formation of or by scavenging RSO (EKAMBARAM *et al*. 2011). Recent studies indicate that RA and oxidative stress are closely associated with both humans and animals (ARULMOZHI *et al.* 2011). The most essential enzymes associated with the antioxidant defense sys-

tem are SOD, catalase, and reduced glutathione. The production of oxygen-free radicals follows the development of arthritis, leading to diminished SOD, catalase, and reduced glutathione levels as a result of their depletion in the course of oxidative stress and cellular lysis (SINDHU *et al.* 2012). Treatment with fisetin upregulated catalase activity, facilitating the scavenging of the free radicals produced during the progression of the disease. The triggering of superoxide radicals under oxidative stress generates the dismutation of the radical into $H_2O_2$, which cannot be completely detoxified due to the declining levels of catalase, ultimately leading to increased lipid peroxidation (BAUEROVÁ *et al.* 2011). Reduced glutathione (GSH) is an intracellular thiol-rich tripeptide, which safeguards the structure of cells and tissues. Activity of GSH was significantly reduced in the AIA rats and was restored to normal levels on treatment with fisetin. These findings substantiate the observation of a modified response in arthritic animals treated with fisetin owing to the consequent induction of the anti-oxidant enzymes.

One of the primary intermediaries of joint inflammation in RA is the tumor necrosis factor (TNF). Various experimental studies have confirmed that it plays a major role in local joint damage and systemic bone loss by increasing osteoclast- (OC) mediated bone resorption (MANARA & SINIGAGLIA 2015). Another pro-inflammatory cytokine, IL-6, is known to activate local synovial leukocyte and antibody production. Earlier studies indicate that TNF-α and IL-6 play dominant roles in facilitating the advancement of several inflammatory joint diseases (HASHIZUME & MIHARA 2011). The levels of both cytokines in local and systemic circulation may reflect the severity of the disease progression. TNF-α is produced by various cell types in RA, primarily by macrophages and dendritic cells in response to the pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) and pattern-recognition receptors (PRRs) or to the cytokine environment (SEMAAN *et al.* 2011). Furthermore, other biological effects, such as the modulation of inflammation, immune response, and hematopoiesis in RA, are brought about by another multifunctional cytokine, IL-6 (HASHIZUME & MIHARA 2011). Macrophages bring about various changes in RA through the release of several cytokines, including TNF-α and IL-1, -6, -12, -15, -18, and -23; among these, TNF-α and IL-6 are considered the most dominant mediators that eventually break down the extracellular matrix of the bones and cartilage (NI *et al.* 2013).

Recent studies list senescent cells as one of the detrimental factors facilitating inflammation and damage in human and animal models of arthritis (JOUSEFZADEH *et al.* 2018). Furthermore, YOUSEFZADEH and his team demonstrated that fisetin was capable of expressing sonotherapeutic activity in murine and human tissues (YOUSEFZADEH *et al.* 2018). Similar studies confirm that fisetin therapy inhibited the activity of several pro-inflammatory cytokines, including TNFα and IL-6 (GUPTA *et al*. 2014). Thus, in this study, the expression of the pro-inflammatory cytokines TNF-α and IL-6 was observed using immunohistochemical methods involving specific antibodies against TNF-α and IL-6. Fisetin at a dose of 25 mg/kg did not significantly diminish the TNF-α and IL-6 expression; however, a considerable decrease in cytokine level was detected in animals treated with 50 and 100 mg/kg of fisetin. Consequently, our findings suggest that fisetin therapy improves various parameters of RA in AIA rats, such as paw edema, and provides a decrease in the serum albumin level along with a reduced expression of cartilage cytokines. Additionally, fisetin therapy significantly improves the antioxidant status compared to the AIA rats.

There is an emerging interest in using natural substances as an alternative therapy to prevent the development of arthritis and related complications. Consequently, it is imperative to recognize the role of natural supplements, such as bioflavonoids, in alleviating bone damage and related symptoms during the development of arthritis. Fisetin, or 3,7,3',4'-tetrahydroxyflavone, is a bioactive flavanol that has been observed to reduce the progression of various diseases with multiple bioactivities, including the decline of oxidative stress and inflammatory responses (XU & LI 2018). Our results provide concrete evidence that fisetin is capable of providing a beneficial antioxidant effect in arthritic tissue.

The capability to reduce the production of TNF-α and IL-6, along with a modulation of the antioxidant status, suggest that the protective effect of fisetin on adjuvant arthritis in rats may be arbitrated by changes in the immune system and an adjustment of cytokine imbalance. Therefore, fisetin has the potential to be used as an anti-arthritic agent and requires further studies to establish its anti-inflammatory activity in clinical settings.

## Conclusion

This study successfully demonstrates that fisetin provides a protective effect on adjuvant-induced arthritic rats by inhibiting inflammation and preventing bone and cartilage destruction. The inhibi-

tory effect of fisetin is primarily brought about by the suppression of inflammatory modulators, such as TNF-α and IL-6, along with the modification of antioxidant stress. This study supports the observation that fisetin can mediate the symptoms of RA in adjuvant-induced arthritic rats.

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## Author Contributions

Research concept and design: X.M.; Collection and/or assembly of data: J.Z.; Data analysis and interpretation: X.W., H.W., Y.Z.; Writing the article: X.L., D.G.; Critical revision of the article: S.M.; Final approval of article: X.M.

## Conflict of Interest

The authors declare no conflict of interest.

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