International Immunopharmacology 77 (2019) 105928

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Scutellarin suppresses cartilage destruction in osteoarthritis mouse model by inhibiting the NF-κB and PI3K/AKT signaling pathways

Wenhan Wang[a,b,c], Jiayi Li[c], Feng Li[d], Jiangfan Peng[c], Mingyang Xu[c], Yangtao Shangguan[c], Yuanming Li[c], Yunpeng Zhao[b], Cheng Qiu[c], Ruize Qu[c], Weiwei Li[e], Cuijuan Zhang[a,e], Tingguo Zhang[a,*]

[a] Department of Pathology, The School of Basic Medical Sciences, Shandong University, Jinan, Shandong 250012, PR China
[b] Department of Orthopedics, Qilu Hospital, Shandong University, Jinan, Shandong 250012, PR China
[c] Cheeloo College of Medicine, Shandong University, Jinan, Shandong 250012, PR China
[d] Department of Medical Imaging, First People's Hospital of Jinan, Jinan, Shandong 250012, PR China
[e] Department of Pathology, Qilu Hospital, Shandong University, Jinan, Shandong 250012, PR China

ARTICLE INFO

Keywords:
Osteoarthritis
Scutellarin
NF-κB and PI3K/AKT signaling pathway

ABSTRACT

Osteoarthritis (OA), a common and severe disease, is predominantly characterized by cartilage destruction, which results in the degeneration of joint surfaces. Nowadays, it is accepted that TNFα plays a critical role in OA. Scutellarin, the main bioactive flavonoid glycoside extracted form *Erigeron breviscapus*, has been reported to exert positive effects on anti-inflammatory reactions. However, the effect of scutellarin in OA is still unknown. In this study, we isolated and cultured primary murine chondrocytes, stimulating TNF-α, in the presence or absence of scutellarin treatment. We found that the inflammatory response stimulated by TNF-α was significantly inhibited by the addition of scutellarin. Moreover, we established OA mouse models induced by surgery. In this mouse model, both inflammatory reaction and cartilage degeneration were markedly inhibited by oral administration of scutellarin. Furthermore, the cellular mechanism underlying the protective effect of scutellarin in OA was clearly associated with the NF-κB and PI3K/AKT signaling pathways. Collectively, this study proposes scutellarin as a potential therapeutic to treat joint degenerative diseases, including OA.

1. Introduction

Osteoarthritis (OA), a biomechanical disease, which is estimated to be the fourth leading cause of disability by 2020 [1,2]. Osteoarthritis (OA) is one of the main types of arthritis, and millions of people suffer from this disease worldwide. OA is characterized by progressive degeneration of chondrocytes [3,4]. OA presents a progressive prevalence as people over 65-year-old suffered diverse degree of OA. OA-associated cartilage destruction, synovitis, and the formation of osteophyte have become the main reasons underlying adult disability, especially in the elderly [1,2]. As a progressive degenerative joint disease, progressive degradation of the articular cartilage lead to progressive degradation of the articular cartilage so as to bone-to-bone abrasive articulation, which causes severe pain, stiffness, and disability. It is reported that inflammatory and catabolic alterations were strongly associated with the onset and development of OA. However, the pathogenesis was still unknown [5]. Thus, nowadays, there is limited therapeutic approaches for OA and no therapy to successfully prevent OA progression. Recently, as the development of biological agent, it is reported that etanercept, a TNFα inhibitor, is a potent strategy in medicine that can render a biological solution for tissue regeneration and repair [5,6]. However, Limited efficacy and expensive cost of etanercept in the treatment of OA present in clinic. Total joint arthroplasty is still the main treatment for mid and late stage's patient. Thus, to find a new effective and low-cost medicine is necessary.

Scutellarin, extracted from the traditional Chinese medicine plant *Erigeron breviscapus*, performs multiple functions in both physiological and pathological processes [7]. Scutellarin is a flavone glucuronide which is widely used clinically to treat cardiovascular and cerebrovascular diseases [8]. Reportedly, scutellarin exerts both anti-oxidant and anti-inflammatory effects on ischemic cerebrovascular disease [9]. Recently, its anti-inflammatory effect was confirmed in a number of diseases, including dermatitis, atherosclerosis, and myocardial infarction [10]. However, whether scutellarin affects the degeneration of

* Corresponding author at: Department of Pathology, The School of Basic Medical Sciences, Shandong University, 107 Wenhuaxi Road, Jinan 250012, PR China (T. Zhang).
E-mail address: qlyyzhangtingguo@126.com (T. Zhang).

https://doi.org/10.1016/j.intimp.2019.105928
Received 18 July 2019; Received in revised form 20 September 2019; Accepted 20 September 2019
Available online 08 November 2019

Table 1
Primer sequences in this study.

Gene Symbol	Primer sequences
IL-1β	5′-AATCTCACAGCAGCACATCA-3′; 5′-AAGGTGCTCATGTCCTCATC-3′
IL-6	5′-CCTTCCTACCCCAATTTCCAAT-3′; 5′-GCCACTCCTTCTGTGACTCCAG-3′
iNOS	5′-ACA GGAGGGGTTAAAGCTGC-3′; 5′-TTGTCTCCAAGGGACCAGG-3′
NF-κB1	5′ TACAAGCTGGCTGGTGGGGA-3′; 5′-GTCGCGGGTCTCAGGACCTT-3′
GAPDH	5′-AGAACATCATCCCTGCATCC-3′; 5′-AGTTGCTGTTGAAGTCGC-3′
MMP-13	5′-ACTTTGTTGCCAATTCCAGG-3′; 5′-TTTGAGAACACGGGGAAGAC-3′
ADAMTS-5	5′-GCATTGACGCATCCAAACCC-3′; 5′-CGTGGTAGGTCCAGCAAACAGTTAC-3′
COX-2	5′-AACATCTCAGACGCTCAGGAAATAG-3′; 5′-GCCGTAGTCGGTGTACTCGTAG-3′
Collgen2	5′-ACTAGTCATCCAGCAAACAGCCAGG-3′; 5′-TTGGCTTTGGGAAGAGAC-3
Aggrecan TNFα	5′-AATGCTGGTACTCCAAACCC-3′; 5′-CTGGATCGTTATCCAGCAAACAGC-3′
	5′-AGGGTCTGGGCCATAGAACT-3′; 5′-CCACCACGCTCTTCTGTCTAC-3′

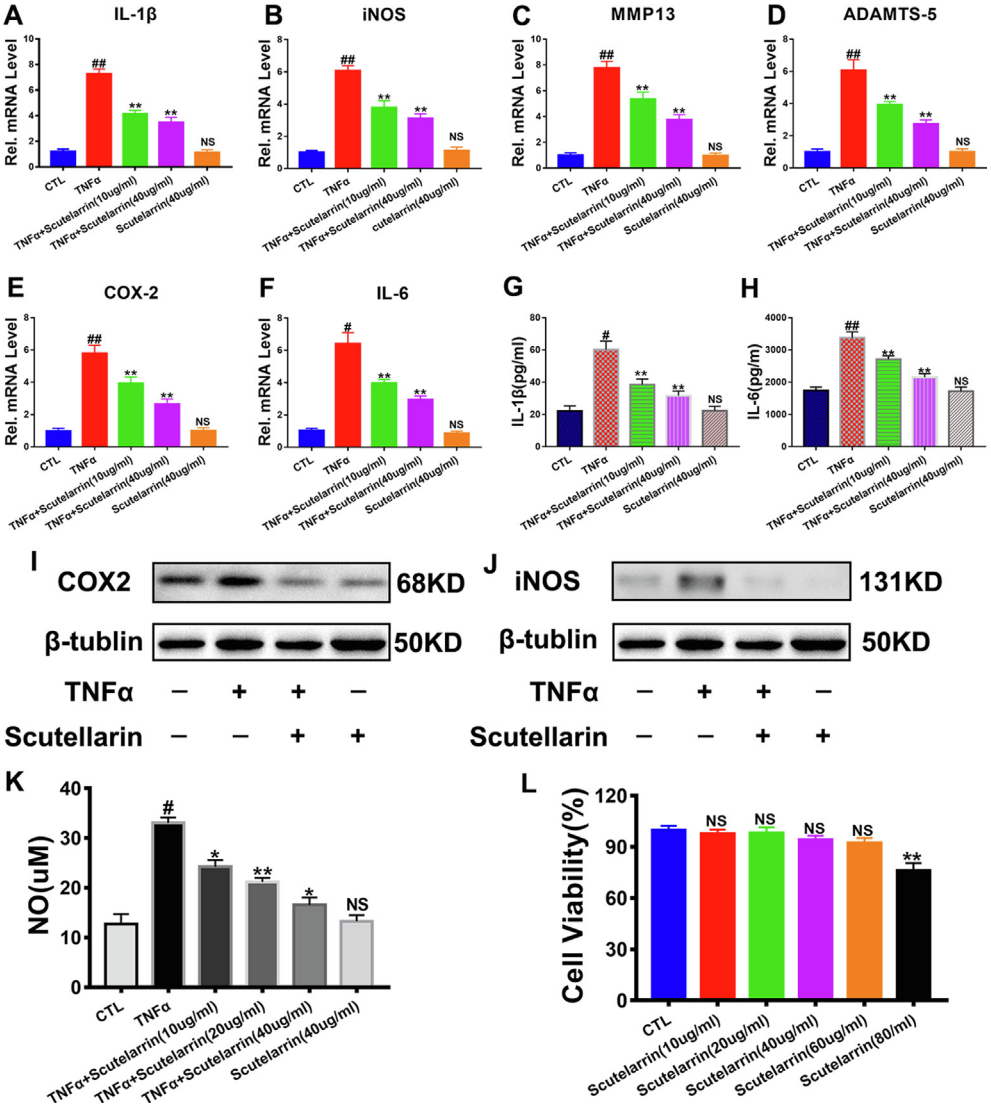

Fig. 1. Scutellarin antagonized cartilage degeneration and inflammation induced by TNF-α in vitro. (A–F) mRNA levels of inflammatory biomarkers including IL-1β, iNOS, MMP13, ADAMTS-5, COX-2, and IL-6 were assayed in each group by real-time PCR. (G–H) The levels of IL-1β and IL-6 in culture medium of all groups were tested by ELISA. (I–J) The protein levels of COX2 and iNOS in each group were assayed by western blot. (K) Analysis of NO production in culture medium was performed by Griess experiments. (L) Cell viability assay performed by CCK8 test. The values are mean ± S.E.M of at least three independent experiments. #P < 0.05; ##P < 0.01 versus CTL group; *P < 0.05; **P < 0.01 versus TNFα stimulated group; NS: not significant versus CTL group.

cartilage requires further investigation.

It is generally accepted that the NF-κB and PI3K/AKT signaling pathways play critical roles in the degeneration of cartilage [11]. Moreover, TNF-α has been identified as a key cytokine in the genesis and development of cartilage destruction [12]. Thus, the suppression of TNF-α function is a well-accepted clinical treatment of OA [13]. Interestingly, the anti-TNF-α, anti-NF-κB, and PI3K/AKT signaling pathway effects of scutellarin were proven recently [14], and this prompted us to investigate whether scutellarin might play a key role in cartilage degradation and OA progression. The purpose of this study is to determine: (1) whether scutellarin could antagonize inflammatory procedure in osteoarthritis and (2) the potential involvement of scutellarin in the pathogenesis of osteoarthritis.

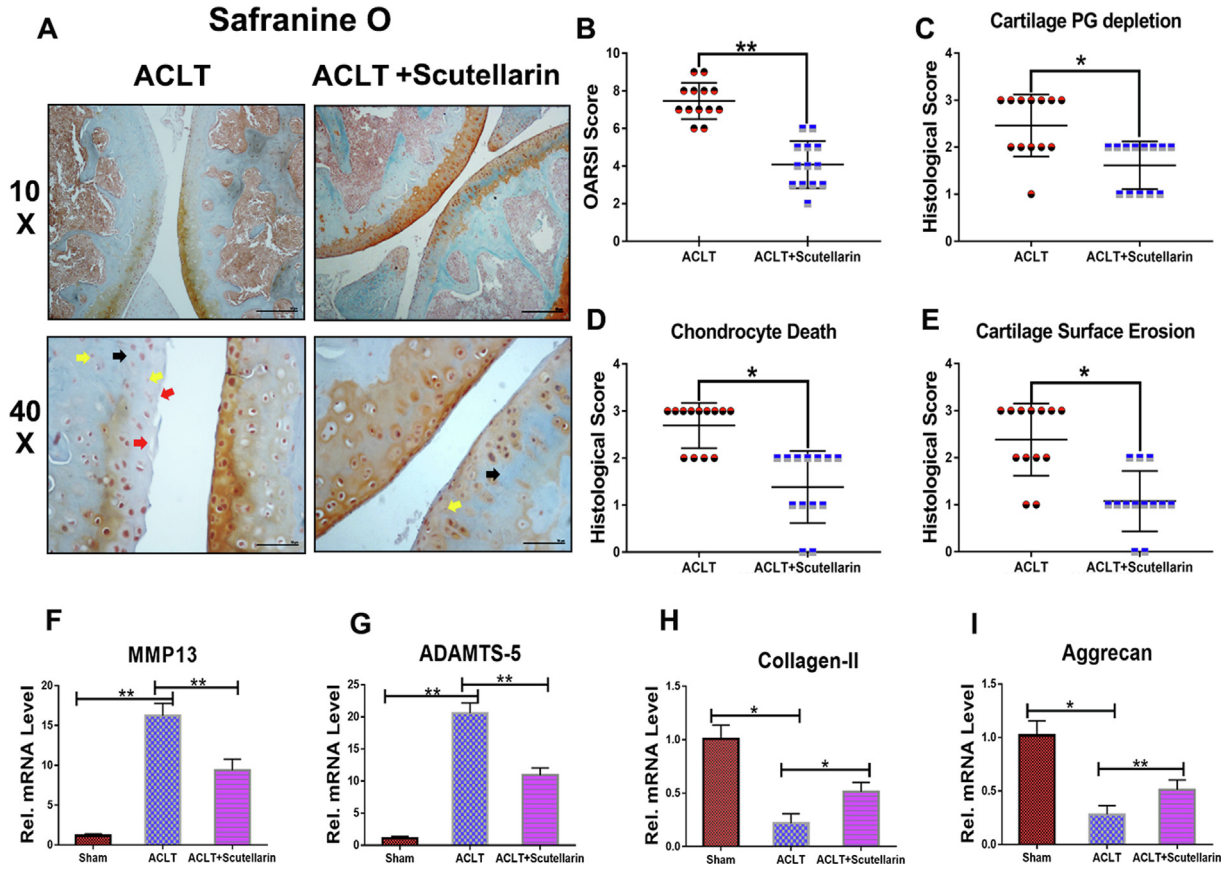

Fig. 2. Scutellarin protected against cartilage degeneration in ACLT mice models in vivo. (A) Cartilage degeneration present in the ACLT and scutellarin treatment groups, assayed by Safranin O staining (Black Arrow: cartilage PG depletion; Red Arrow: Cartilage Surface Erosion; Yellow Arrow: Chondrocyte Death). (B) The OARSI score of osteoarthritis in the two groups were analysis based on the results of Safranin O staining. (C–E). Treatment of naringin protected cartilage stability in ACLT models, assayed by histological score analysis, according the score of cartilage PG depletion, cartilage surface erosion and chondrocyte death. (F–I) The mRNA levels of MMP13, ADAMTS-5, Collagen II and aggrecan were test by real time PCR. The values are mean ± S.E.M of at least three independent experiments. *P < 0.05; **P < 0.01. Scale bar at 50 μm. N = 6 for each group.

2. Materials and methods

2.1. Animals

Wild type BL6/C57 male mice (10-week old) were purchased from Shandong University Laboratory Animal Center (Jinan, Shandong, China). Mice were housed in Shandong University Laboratory Animal Center under specific pathogen-free (SPF) conditions conforming to the Institutional Animal Care and Use Committee of Shandong University, with standard rodent water and food. Experiments performed in this study were all approved by the Animal Experiment Committee of Shandong University.

2.2. Isolation and culture of primary murine chondrocytes

As previously reported, Primary Murine Chondrocyte cells were isolated from articular cartilage in wild type mice (10-weeks old). Briefly, after the mice were euthanized by administering pentobarbital sodium, the articular cartilage of the knee was collected and minced into 1 mm^3-thick slices. After washing in 1% cold PBS three times, cartilage slices were treated with 0.25% Trypsin at 37 °C for 30 min. Cartilage slices were again washed in 1% cold PBS thrice, and digested in a mixture of 0.1% collagenase II in DMEM (Gibco, USA) at 37 °C overnight in an incubator with 5% CO2. Next, primary murine chondrocyte cells were collected and cultured in DMEM (Gibco, USA) containing fetal bovine serum (10%) (Gibco, USA).

2.3. LAL test and cell viability

Scutellarin, provided by Shanghai Macklin Biochemical Co., Ltd, was dissolved in bioclean PBS and filtered through a filter membrane (0.22 μm). To assess the endotoxin contamination of the scutellarin solutions (2 mg/ml in vivo; 10 μg/ml, 20 μg/ml and 40 μg/ml in vitro), LAL testing was performed according the protocol of the ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript; limit of detection < 0.01 EU/ml). A negative result was presented, which indicated that the endotoxin contamination of scutellarin solution is at least < 0.01EU/ml. Mo [6] reover, to further investigate the effect of scutellarin on cell viability, the Cell Counting Kit-8 (CCK-8) kit (Vazyme Corporation, Nanjing, P.R. China) was used. Briefly, chondrocyte cells (1×10^5 cells/ml) were cultured in a 96-well plate with varying concentrations of scutellarin for 24 h. Next, 10 μL of CCK8 reagent was added into each well and incubated at 37 °C for 4 h.

2.4. The effect of scutellarin in vitro

In this study, Greiss assay, western blot as well as real-time PCR were performed to examine whether scutellarin inhibits TNF-α-mediated inflammation in Primary Murine Chondrocyte cells. According previously reported, the concentrations of scutellarin accepted in this study were 0 μg/ml, 10 μg/ml, 20 μg/ml and 40 μg/ml. And the preparation method was described above. Chondrocyte cells were cultured in 12-well flat-bottom plates (5×10^5 cells/well) and divided into 5 groups: Chondrocyte cells in group 1 were cultured in culture medium

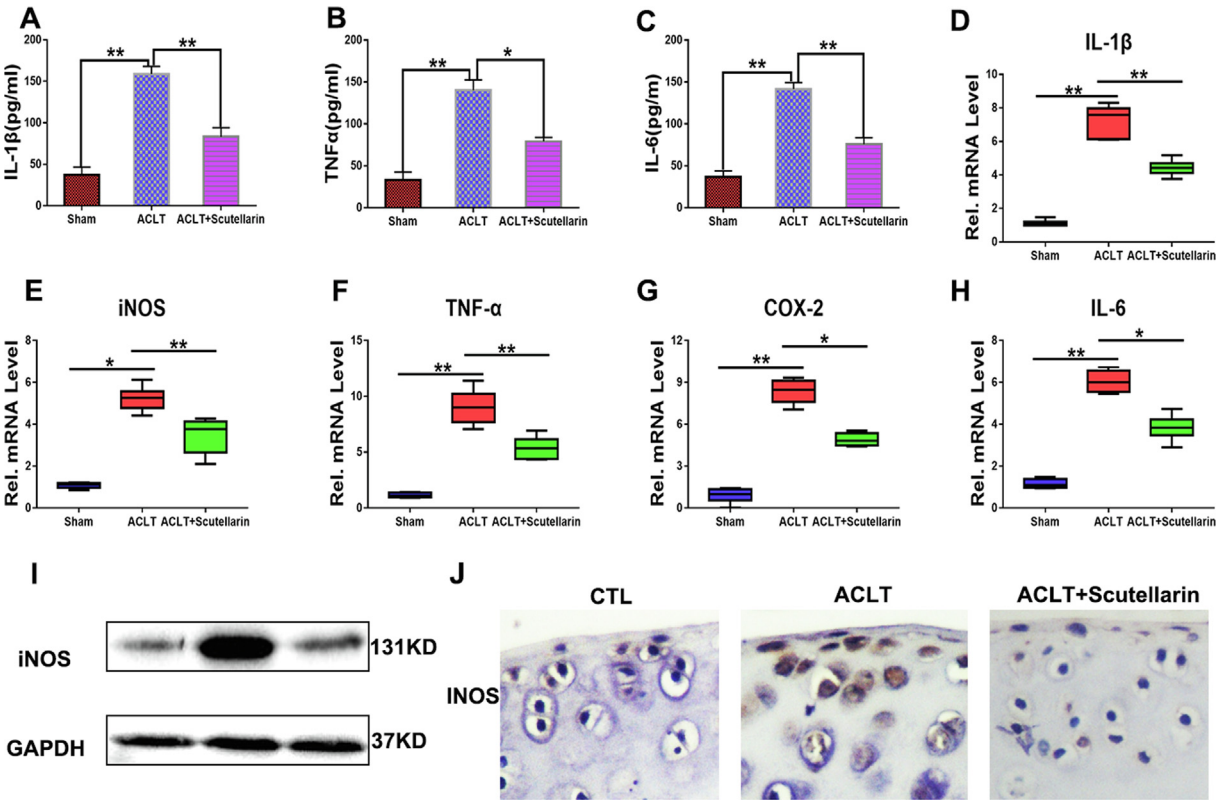

Fig. 3. Scutellarin protected against the inflammatory response in ACLT mice models in vivo. (A–C) The circulating levels of IL-1β, TNF-α, and IL-6 in serum in each group were tested by ELISA. (D–H) The mRNA levels of IL-1β, iNOS, TNF-α, COX-2, and IL-6 in the knee joint were tested by real time PCR. The expression protein levels of iNOS in the cartilage of the knee in all groups was tested by western blot (I) and Immunostaining (J). The values are mean ± S.E.M of at least three independent experiments. *P < 0.05; **P < 0.01. Scale bar at 50 μm. N = 6 for each group.

without any addition for 24 h. Chondrocyte cells in group 2 were cultured in culture medium with 10 ng/ml TNF-α (R&D systems) for 24 h, while group 3 with TNF-α (10 ng/ml) and scutellarin (low dose:10 μg/ml) addition. And in group 4, cells were cultured with TNF-α (10 ng/ml) and scutellarin (high dose: 40 μg/ml) for 24 h. Group 5 were cultured with scutellarin (high dose: 40 μg/ml) for 24 h. Culture supernatants in these three groups were collected for NO test via the Griess reaction through a commercial kit (Beyotine Biotechnology Corporation, Shanghai, P.R. China), and total protein and mRNA were extracted for next test.

2.5. OA surgical models

To further determine the effect of scutellarin in osteoarthritis, anterior cruciate ligament transection (ACLT) surgery was performed in 12-week-old WT mice as previously reported [15]. At first, mice were divided into the following three groups (n = 6): the sham-operation, ACLT, and treatment. In the sham-operation group, mice underwent sham-operation, in which the joint capsule of knee was exposed without ligament destruction, and PBS (0.2 ml) was administered intraperitoneally once a day for 4 weeks. In the ACLT group, the anterior cruciate ligament (ACL) was transected, and the mice received intraperitoneal injection containing 0.2 ml of PBS once a day for 4 weeks. In the scutellarin treatment group, ACLT was also performed by the same method, and the mice were treated with scutellarin (20 mg/kg/day) once a day for 4 weeks. Four weeks following surgery, mice were euthanized with pentobarbital sodium, and the knee joint tissue and serum of each mouse was collected for further testing.

2.6. Histological and immunostaining analysis

Knee joint samples collected from each mouse were fixed in 4% PFA for at least 4 days. After decalcification in 10% EDTA at room temperature for two weeks, Knee joint samples were embedded in paraffin. Sections (5 μm) were stained with Safranin-O/fast green. For performing immunohistochemistry analysis, after being dewaxed with xylene and eluted with 100%, 90%, 80%, and 75% ethanol, serial sections were pretreated with compound digestive juice (Boster Biological Technology, China) at 37 °C for 30 min. To block nonspecific proteins, 10% goat serum (Boster Biological Technology, China) was added to the sections at room temperature for at least 45 min. Sections were then incubated with anti-p-IκBα (1:150 dilution, Boster Biological Technology, China) and anti-iNOS antibodies (diluted 1:150, Boster Biological Technology, China) at 4 °C overnight. Next, detection was performed using an enhanced polymer detection system kit (Boster Biological Technology, China) according to the protocol, following counterstaining with 1% hematoxylin (Boster Biological Technology, China).

2.7. Quantificational evaluation in histology

To grade the proteoglycan (PG) content of cartilage, the OARSI scoring system was performed in this study based on the Safranin-O–stained sections. PG depletion was scored as 0–3 according to previous reports (4); complete depletion of PGs (3), distained cartilage (2), fully stained cartilage (1), and normal (0). Moreover, we scored chondrocyte death on a scale of 0–3; from no empty lacunae (0) to complete loss of chondrocytes (3) in the cartilage layer, and we also scored cartilage surface erosion as 0–3; from no cartilage loss (0) to complete loss of articular cartilage (3). Image J was used to analyze the articular cartilage thickness. Each group contained at least 3 mice, and each mouse was determined and averaged in all sections in 3 parameters.

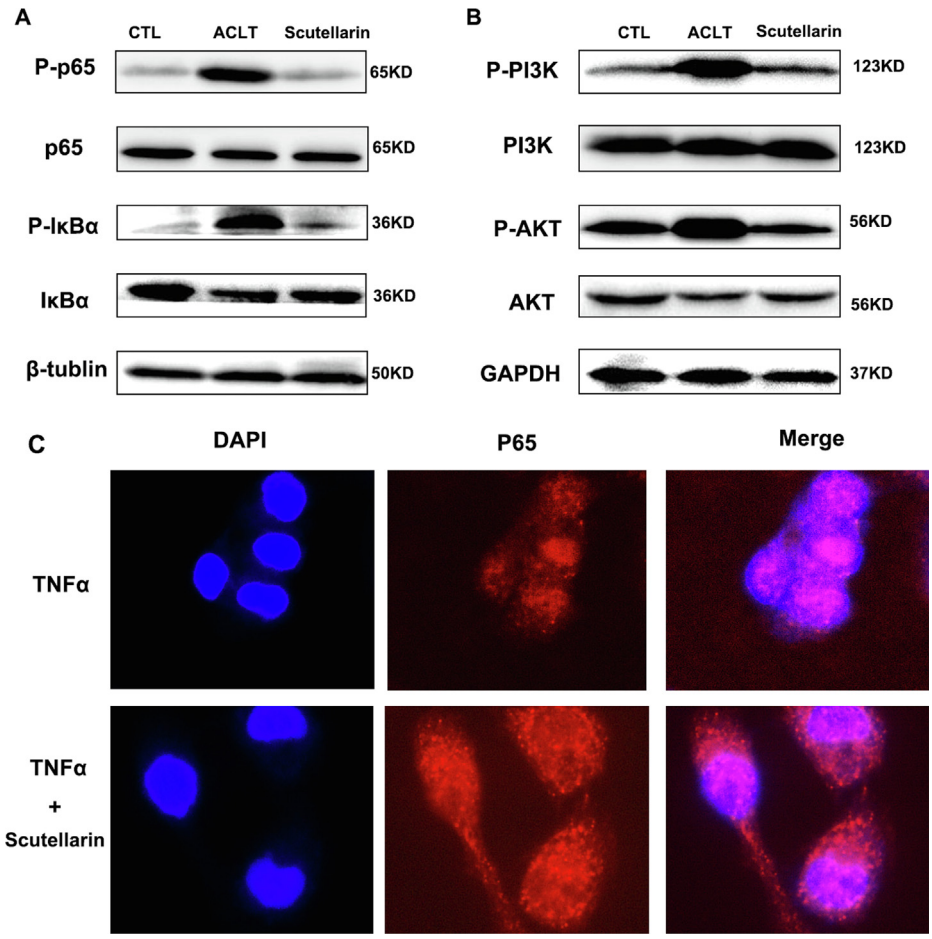

Fig. 4. Scutellarin antagonizes activation of NF-κB and PI3K/AKT signaling Pathways both in vivo and in vitro. (A) The phosphorylation of IκBα and P65 in the control group, ACLT and scutellarin treatment group were tested by western blotting. (B) The phosphorylation of PI3K and AKT in the three groups was also assayed by western blot. (C) In chondrocytes, immunofluorescent staining was performed to further investigate the nuclear translocation of NF-κB-P65.

2.8. Western blotting

Proteins of primary chondrocytes and knee joints from all groups were extracted and collected using RIPA Lysis Buffer (Boster Biological Technology, China), mixed with 1% PMSF (Boster Biological Technology, China) and 1% protease and phosphatase inhibitor (Boster Biological Technology, China). After heating in loading buffer (Boster Biological Technology, China) at 100 °C for 10 min, proteins were analyzed by electrophoresis in 10% agarose gel (Boster Biological Technology, China) and electroblotted onto a nitrocellulose membrane (Boster Biological Technology, China). To block non-specific proteins, the protein blots were treated with 5% non-fat milk. Protein blots were then incubated with the following antibodies at 4 °C overnight: p-IκBα (diluted 1:1000, Boster Biological Technology, China), COX-2 (diluted 1:1000, Boster Biological Technology, China), iNOS (diluted 1:1000, Boster Biological Technology, China). Next, the protein blots were treated with horseradish peroxidase-conjugated secondary antibody (1:2000 dilutions, Boster Biological Technology), and detected using an enhanced chemiluminescence system (Thermo Scientific, Shanghai, China).

2.9. Real-time PCR

We extracted total RNA from mice and chondrocytes in each group by using a RNeasy kit (Vazyme Corporation, Nanjing, P.R. China). According the manufacturer's instructions, all mRNA was reverse transcribed into cDNA by RT- PCR kit (Vazyme Corporation, Nanjing, P.R. China). Next, we performed real-time PCR using the Thermal Cycler Dice Real-Time System with SYBR Green I dye. In this study, we designed the sequence-specific primers to generate products between 100 bp and 200 bp in length and are listed in Table 1. Mean Ct values of genes under investigation were normalized to GAPDH and the results were quantified using the ΔΔCt method. All experiments were repeated at least three times.

2.10. ELISA assays for IL-1β and IL-6 in vivo and in vitro

Serum from each mouse were test for IL-1β, TNFα and IL-6 by using ELISA kits (Boster Biological Technology, Wuhan, China) after harvest. Moreover, culture supernatant from chondrocytes in each group were collected for ELISA test for IL-1β and IL-6. According the manufacturer's instructions, sandwich ELISA assay performed as follows: 96-well Plates were incubated with samples (100 μl) and standards (100 μl) for 90 mins at 37 °C without any wash. Then Plates incubated with detection Abs (100 μL per well) at 37 °C for 60 mins with 4 washes by 0.01 M TBS. Next, 100 μL ABC complex (per well) were added into plates and incubated at 37 °C for 30 mins and then washed by 0.01 M TBS for 5 times. Finally, plates were incubated with TMB Substrate solution and stopped by the addition of 100 μL of 1 N HCl. All results were test by Thermo Scientific Microplate Reader (Thermo Scientific, Shanghai, China), and all the samples were assayed in triplicate and repeated at least three times.

2.11. Statistical analysis

Results are shown as average values ± SEM. One-way ANOVA and nonparametric comparison (Bonferroni's multiple comparison test) were performed using Prism software version 4.0b. P values less than 0.05 were considered significant.

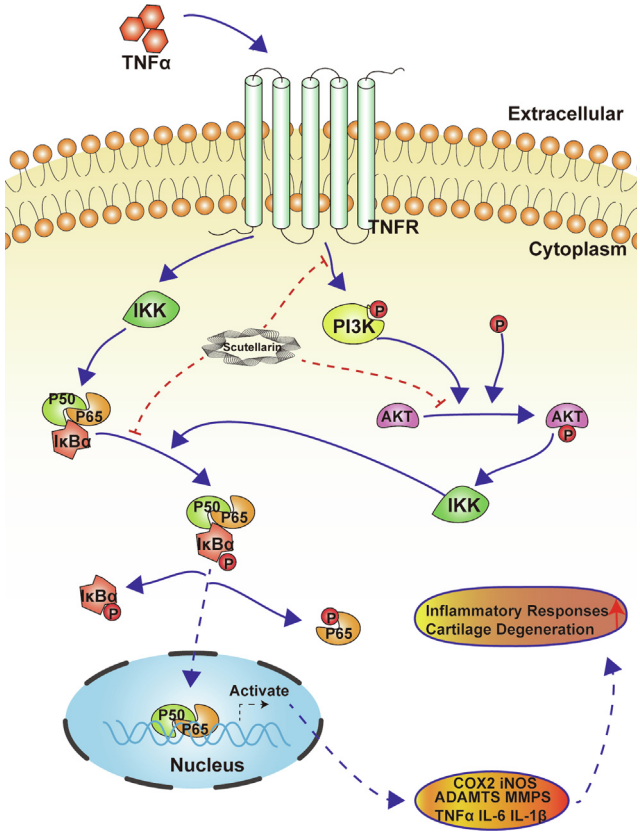

Fig. 5. Pattern diagrams for the effect of scutellarin in osteoarthritis.

3. Results

3.1. Scutellarin antagonizes cartilage degeneration and inflammation induced by TNF-α in vitro

It has been reported that cartilage degeneration and inflammation are key to cartilage degeneration in osteoarthritis [16]. Moreover, inflammatory cytokines, especially TNF-α, play a critical role in cartilage degeneration and inflammation [17]. Interestingly, according to a recent study, TNF-α function was antagonized by scutellarin [18]; this prompted us to investigate the effect of scutellarin in osteoarthritis by targeting TNF-α function. Inflammatory cytokines play important roles in the whole inflammatory reactions, such as IL-1β, iNOS, MMP13, ADAMTS-5, COX-2, and IL-6, which is also the biomarkers for inflammation. As described above, primary murine chondrocytes were isolated and treated in four groups. Total mRNA was extracted and reverse transcribed into cDNA. As Fig. 1A–F (Each P value < 0.05) indicate, the mRNA levels of inflammatory biomarkers including IL-1β, iNOS, MMP13, ADAMTS-5, COX-2, and IL-6 were highly upregulated following stimulation by TNF-α (10 ng/ml), while this tendency was abrogated by addition of scutellarin in both low (10 μg/ml) and high (40 μg/ml) doses. Meanwhile, culture supernatant was collected and ELISA was performed. As shown in Fig. 1G–H (Each P value < 0.05), the release of IL-1β and IL-6 was significantly inhibited by scutellarin in both low and high dose groups when chondrocytes were stimulated with TNF-α (10 ng/ml). Western blot analyses of the total protein collected was also performed. The protein levels of COX2 and iNOS were consistent with the mRNA levels, which indicated that the increased expression of COX2 and iNOS stimulated by TNF-α were abrogated by the addition of scutellarin (Fig. 1I–J). NO production promoted the activation of inflammatory reactions, and inflammatory reactions further increased the level of NO. In addition, analysis of NO production was performed by Griess experiment. Results showed that chondrocytes produced less nitrite when stimulated by TNF-α in the presence of scutellarin (low and high doses) (Fig. 1K) (Each P value < 0.05). Collectively, these results demonstrate that scutellarin suppressed TNF-α-induced cartilage degeneration and inflammation in chondrocytes.

3.2. Scutellarin protects against cartilage degeneration and inflammation in ACLT mouse models

To further investigate the protective effects of scutellarin on cartilage degeneration and inflammation in mice, ACLT mouse models were established in 12-week-old WT mice as described above, followed by treatment with PBS or 20 mg/kg/day scutellarin. As indicated in Fig. 2A, after surgery, the cartilage of the knee was significantly degenerated and destroyed. However, in the scutellarin treated group, the degeneration of cartilage was remarkably inhibited. As described above, OARSI score was used in this study for histological grading analysis. As for the OARSI score of osteoarthritis, the scutellarin-treated group showed more improvement than the PBS treatment group (Fig. 2B) (P value < 0.05). Furthermore, the score of cartilage PG depletion, cartilage surface erosion, and chondrocyte death was in line with the OARSI score (Fig. 2C–E) (Each P value < 0.05), which indicates that scutellarin exerts marked protective effect on OA. Matrix metalloproteinase-13 (MMP13), also called collagenase 3, plays a critical role in the degradation of collagen II to destroy the cartilage. Moreover, ADAMTS-5, a well-known extracellular matrix (ECM) degrading enzyme, shows proteolytic activity toward the hyalectan group of chondroitin sulfate proteoglycans, including aggrecan. Thus, MMP13 and ADAMTS-5 play important roles in assessing the degeneration of cartilage. As shown in Fig. 2F–G (Each P value < 0.05), the mRNA levels of MMP13 and ADAMTS-5 in PBS group were markedly increased. However, after treatment with scutellarin, the mRNA levels of MMP13 and ADAMTS-5 were lower than those of the PBS treatment group, which indicates that scutellarin plays a protective role in degeneration of cartilage by inhibiting the degradation of Collagen II and aggrecan (Fig. 2H–I) (Each P value < 0.05). This is consistent with the result presented in Fig. 2F–G.

To assess the levels of circulating inflammatory cytokines, serum was extracted from each mouse and ELISA was performed for IL-1β, TNF-α, and IL-6. As shown in Fig. 3A–C (Each P value < 0.05), the circulating levels of IL-1β, TNF-α, and IL-6 in the PBS treatment group were higher than those of the sham-operation group, while the levels of circulating inflammatory cytokine levels in the scutellarin treatment group were lower than those of the PBS treatment group. After harvesting, total RNA was collected from the knee of each mouse, and real time PCR was performed. As Fig. 3D-H (Each P value < 0.05) shows, the mRNA levels of IL-1β, iNOS, TNF-α, COX-2, and IL-6 were significantly decreased following treatment with scutellarin, which indicates that the expression of inflammatory cytokines was inhibited by scutellarin in OA in mice. Moreover, after WB was performed, the protein levels of iNOS in the knee cartilage were consistent with the mRNA levels (Fig. 3I). This was further confirmed by histological analysis through immunostaining for iNOS (Fig. 3J). Collectively, osteoarthritis mice models, ACLT mice models, were established in this study with or without scutellarin treatment to assess the therapeutic effects of scutellarin in OA. As the results described above, not only the degree of cartilage degeneration but also the inflammation in arthritis was deeply improved after scutellarin treatment.

3.3. Scutellarin antagonizes activation of NF-κB and PI3K/AKT signaling pathways both in vivo and in vitro

As previously reported, scutellarin is an effective inhibitor of the NF-κB signaling pathway [19]. Moreover, a recent study reported that scutellarin plays a protective role in many inflammatory reactions through action on the NF-κB and PI3K/AKT signaling pathways [14]. Furthermore, it was also reported that the NF-κB and PI3K/AKT

signaling pathways play a significant role in the degeneration of cartilage and inflammatory responses [20].This prompted us to investigate the effect of scutellarin on OA and the associated underlying mechanism. In the present study, to investigate the activation of the NF-κB and PI3K/AKT pathways, levels of related proteins were assayed in each group; the control, ACLT. and scutellarin treatment groups. As Fig. 4A indicates, the phosphorylation of IκBα and P65 was markedly upregulated in the ACLT group compared to the control group, but phosphorylation of IκBα and P65 was downregulated by scutellarin treatment in the ACLT group. Similarly, the phosphorylation of PI3K and AKT was significantly upregulated in the ACLT group compared with the control, while scutellarin downregulated both PI3K and AKT in the scutellarin treatment group compared with the ACLT group (Fig. 4B). In chondrocytes, immunofluorescent staining was performed to further investigate the nuclear translocation of NF-κB-P65. As Fig. 4C indicates, NF-κB-P65 was translocated into the nucleus when chondrocytes were stimulated by THFα. However, this nuclear translocation was blocked with the addition of scutellarin. Collectively, our data suggest that scutellarin may affect the inflammatory response and cartilage degeneration via the NF-κB and PI3K/AKT signaling pathways, which are described in pattern diagrams (Fig. 5).

4. Discussion

OA, a common and severe disease, is predominantly characterized by cartilage destruction, which results in the degeneration of joint surfaces [21]. However, further investigation is needed to understand its pathogenesis and to develop a curative treatment [22]. As previously reported, TNFα plays a critical role in the process of cartilage destruction. The expression of MMPs and ADAMTS was markedly increased by TNFα, this increase lead to significant degradation of the cartilage matrix, including cartilage oligomeric matrix protein, Col II, and aggrecan [23]. Recently, TNFα has become a new target for OA therapy [23]. Moreover, the NF-κB and PI3K/AKT signaling pathways have been considered to play key roles in the pathogenesis of osteoarthritis [24]. Furthermore, NF-κB activation and PI3K/AKT phosphorylation induced by TNFα has been confirmed to occur in the process of cartilage degeneration [25].

Scutellarin, the main bioactive flavonoid glycoside extracted form *Erigeron breviscapus*, has been reported to possess positive effects in anti-inflammatory reactions [7]. Moreover, the therapeutic effect of scutellarin in rheumatoid arthritis in rats has been reported in recent years [26]. Furthermore, as recently reported, a combination of *scutellarin baicalensis and acacia catechu extracts* have exhibited marked therapeutic effects in patients suffering with arthritis in clinic [27]. However, the unique effect of scutellarin on OA and its associated underlying mechanism still need to be investigated. In this study, we demonstrated the protective effect of scutellarin in osteoarthritis and clarified the underlying mechanism behind this.

NO plays a critical role as a main cytotoxic mediator in the inflammatory reaction, regulated by iNOS [28]. iNOS is expressed in low levels under normal conditions but is increased in activated microphages. Expression of iNOS is activated by inflammatory cytokines, such as IL-1β, THFα and IL-6. In the present study, the production of NO in chondrocytes was increased when stimulated with TNFα, but this tendency was abrogated by the addition of scutellarin in a dose dependent manner [19]. Moreover, the increased expression of detrimental inflammatory cytokines such as IL-1β, iNOS, ADAMTS-5, COX-2, and IL-6, in chondrocytes were significantly antagonized by the addition of scutellarin both in low and high dose, when assayed by real time PCR, ELISA and Western Blot. It is a widely held view that the production and activation of MMPs is promoted by NO and that the synthesis of collagen II and proteoglycan are suppressed by NO, causing ECM degradation in osteoarthritis [29–31]. In this study, as described above, the increased expression levels of MMP13 and NO in chondrocytes were significantly inhibited by scutellarin.

The anterior cruciate ligament transection (ACLT)-induced OA model is a well-established model used to investigate the pathogenesis and treatment of osteoarthritis [32]. As previously reported, the ACLT mouse model demonstrates great similarities with human osteoarthritis, including subchondral change, articular cartilage damage and synovitis [33]. To determine the effect of scutellarin, ACLT mouse model was established and scutellarin was treated by ig administration in this study. The inflammatory response in ACLT mice model was remarkably inhibited following scutellarin treatment. Moreover, the degeneration and destruction of articular cartilage damage and subchondral change progressed slowly in scutellarin treatment group after the ACLT mouse model was established, as tested by Safranin O staining and assayed by OARSI.

As previously reported, the protective effect of a combination of scutellarin baicalensis and acacia catechu extracts have been proven clinically [27]. However, the effect of scutellarin and the associated mechanism of scutellarin action remain unknown. In this study, the protective effect of scutellarin was confirmed in mice and the associated mechanism was elucidated. It has previously been reported that the NF-κB and PI3K/AKT signaling pathways are two key signaling pathways in OA [34], which could downregulate the transcription of cellular inflammatory response-associated genes. Moreover, as recently reported, the activation of NF-κB and PI3K/AKT pathways was inhibited by scutellarin in the inflammatory response [35]. In this study, the activation of NF-κB and PI3K/AKT was assayed both in vivo and in vitro. In chondrocytes, with the lower expression of inflammatory cytokines and lower synthesis of pro-inflammatory mediators, the activation of NF-κB and PI3K/AKT signaling pathway was significantly abrogated. Furthermore, in the ACLT mouse model, the increased expression of NF-κB and PI3K/AKT were remarkably inhibited by treatment with scutellarin. In conclusion, scutellarin plays a protective role in osteoarthritis through modulation of the NF-κB and PI3K/AKT signaling pathways.

In this study, the positive effect of scutellarin in cartilage degeneration and inflammation in OA was verified both in vivo and in vitro. However, the effect of scutellarin in patient is still unknown. Till now, it is just reported that a compound Chinese herbal preparation contains scutellarin was effective in clinic [27]. No one performed elaborate effect of scutellarin treatment alone in patient, including us. Because of the limitation of ethics, we did not perform experiment in patient in this study. But the effective mechanism of scutellarin treatment in OA in mice was explored, which provided evidence to investigate the effect of scutellarin in patient in the future. At present, widely used medicine is still non-steroidal anti-in-flammatory drugs (NSADS), which is effective in inflammation but with various side effect, such as peptic ulcer and cardiovascular events. Moreover, it has little benefit in cartilage degeneration. Thus, scutellarin, which benefit inflammation and cartilage degeneration was a new and suitable target for medicinal development in OA.

Author contributions

Conceived and designed the experiments: Weiwei Li, Yunpeng Zhao, Cuijuan Zhang, Tingguo Zhang.

Performed the experiments: Wenhan Wang, Jiayi Li, Jiangfan Peng, Mingyang Xu, Yangtao Shangguan, Yuanming Li, Cheng Qiu, Ruize Qu,

Analyzed the data: Weiwei Li

Contributed reagents/materials/analysis tools: Tingguo Zhang, Yunpeng Zhao, Weiwei Li

Declaration of Competing Interest

None

Acknowledgments

This work was supported by the Natural Science Foundation of Shandong Province (Grant No. ZR2019MH05, ZR2019MH075 and BS2015SW028), Key Research and Development Projects of Shandong Province (No. 2015GSF118115), the Cross-disciplinary Fund of Shandong University (Grant No. 2018JC007) and the National Natural Science Foundation of China (Grant No. 81572191 to Lei Cheng, Grant No. 81501880 to Yunpeng Zhao and 81602761 to Weiwei Li)

References

[1] N.G.M. Thielen, P.M. van der Kraan, A.P.M. van Caam, TGFbeta/BMP signaling pathway in cartilage homeostasis, Cells 8 (9) (2019).
[2] C.H. Teirlinck, et al., Prognostic factors for progression of osteoarthritis of the hip: a systematic review, Arthritis Res. Ther. 21 (1) (2019) 192.
[3] T. Neogi, Structural correlates of pain in osteoarthritis, Clin. Exp. Rheumatol. 35 (Suppl 105(5)) (2017) 75–78.
[4] Y. Zhao, et al., Naringin protects against cartilage destruction in osteoarthritis through repression of NF-kappaB signaling pathway, Inflammation 39 (1) (2016) 385–392.
[5] H. Ahn, et al., The relationship between beta-endorphin and experimental pain sensitivity in older adults with knee osteoarthritis, Biol. Res. Nurs. 21 (4) (2019) 400–406.
[6] E.E. Mehana, A.F. Khafaga, S.S. El-Blehi, The role of matrix metalloproteinases in osteoarthritis pathogenesis: an updated review, Life Sci. 234 (2019) 116786.
[7] L. Wang, Q. Ma, Clinical benefits and pharmacology of scutellarin: a comprehensive review, Pharmacol. Ther. 190 (2018) 105–127.
[8] J. Gao, et al., Therapeutic effects of breviscapine in cardiovascular diseases: a review, Front. Pharmacol. 8 (2017) 289.
[9] J.B. Sun, et al., Scutellarin protects oxygen/glucose-deprived astrocytes and reduces focal cerebral ischemic injury, Neural. Regen. Res. 13 (8) (2018) 1396–1407.
[10] J. Mo, et al., Scutellarin protects against vascular endothelial dysfunction and prevents atherosclerosis via antioxidation, Phytomedicine 42 (2018) 66–74.
[11] X. Huang, et al., Vanillic acid attenuates cartilage degeneration by regulating the MAPK and PI3K/AKT/NF-kappaB pathways, Eur. J. Pharmacol. 859 (2019) 172481.
[12] G. Hu, et al., MicroRNA-145 attenuates TNF-alpha-driven cartilage matrix degradation in osteoarthritis via direct suppression of MKK4, Cell Death Dis. 8 (10) (2017) e3140.
[13] K.S. Zhang, et al., Effects of tumor necrosis factor alpha on the expression of programmed cell death factor 5 in arthritis, Orthop. Surg. (2019).
[14] P. You, et al., Scutellarin suppresses neuroinflammation via the inhibition of the AKT/NF-kappaB and p38/JNK pathway in LPS-induced BV-2 microglial cells, Naunyn Schmiedebergs Arch. Pharmacol. 391 (7) (2018) 743–751.
[15] C. Zhao, Q. Liu, K. Wang, Artesunate attenuates ACLT-induced osteoarthritis by suppressing osteoclastogenesis and aberrant angiogenesis, Biomed. Pharmacother. 96 (2017) 410–416.
[16] S. Zhang, et al., MSC exosomes alleviate temporomandibular joint osteoarthritis by attenuating inflammation and restoring matrix homeostasis, Biomaterials 200 (2019) 35–47.
[17] Y.P. Zhao, et al., Progranulin protects against osteoarthritis through interacting with TNF-alpha and beta-catenin signalling, Ann. Rheum. Dis. 74 (12) (2015) 2244–2253.
[18] Y. Yuan, H. Zha, P. Rangarajan, E.A. Ling, C. Wu, Anti-inflammatory effects of Edaravone and Scutellarin in activated microglia in experimentally induced ischemia injury in rats and in BV-2 microglia, BMC Neurosci. 15 (2014) 125.
[19] T. Baluchnejadmojarad, H. Zeinali, M. Roghani, Scutellarin alleviates lipopolysaccharide-induced cognitive deficits in the rat: Insights into underlying mechanisms, Int. Immunopharmacol. 54 (2018) 311–319.
[20] Y.Q. Qian, et al., Downregulating PI3K/Akt/NF-kappaB signaling with allicin for ameliorating the progression of osteoarthritis: in vitro and vivo studies, Food Funct. 9 (9) (2018) 4865–4875.
[21] K. Nagai, et al., Anterior cruciate ligament reconstruction affects tibiofemoral joint congruency during dynamic functional movement, Am. J. Sports Med. 46 (7) (2018) 1566–1574.
[22] A.E. Nelson, Osteoarthritis year in review 2017: clinical, Osteoarthritis Cartilage 26 (3) (2018) 319–325.
[23] A. Bottini, et al., PTPN14 phosphatase and YAP promote TGFbeta signalling in rheumatoid synoviocytes, Ann. Rheum. Dis. 78 (5) (2019) 600–609.
[24] Z.C. Hu, et al., The protective effect of magnolol in osteoarthritis: in vitro and in vivo studies, Front. Pharmacol. 10 (2019) 393.
[25] W. Li, et al., GDF11 antagonizes TNF-alpha-induced inflammation and protects against the development of inflammatory arthritis in mice, FASEB J. 33 (3) (2019) 3317–3329.
[26] L. Zhang, et al., Effect of Scutellarin inhibits collagen induced arthritis through TLR4/NF kappa B mediated inflammation, Mol. Med. Rep. 16 (4) (2017) 5555–5560.
[27] B.H. Arjmandi, et al., A combination of Scutellaria baicalensis and Acacia catechu extracts for short-term symptomatic relief of joint discomfort associated with osteoarthritis of the knee, J. Med. Food 17 (6) (2014) 707–713.
[28] X.F. He, W. Li, L.M. Zhu, J.W. Zhang, Investigation for effects of iNOS on biological function of chondrocytes in rats with post-traumatic osteoarthritis, Eur. Rev. Med. Pharmacol. Sci. 22 (21) (2018) 7140–7147.
[29] B. Ji, Y. Ma, H. Wang, X. Fang, P. Shi, Activation of the P38/CREB/MMP13 axis is associated with osteoarthritis, Drug Des. Devel. Ther. 13 (2019) 2195–2204.
[30] F. Fei, et al., Paeoniflorin inhibits Th1 and Th17 cells in gut-associated lymphoid tissues to produce anti-arthritis activities, Inflammopharmacology (2019).
[31] J. Chen, H.Y. Chen, W. Dai, Q.J. Lv, C.Y. Chen, Artificial intelligence approach to find lead compounds for treating tumors, J. Phys. Chem. Lett. (2019).
[32] K. Feng, et al., Curcumin Inhibits the PERK-eIF2alpha-CHOP pathway through promoting SIRT1 expression in oxidative stress-induced rat chondrocytes and ameliorates osteoarthritis progression in a rat model, Oxid. Med. Cell Longev. 2019 (2019) 8574386.
[33] L. Guo, et al., Ipriflavone attenuates the degeneration of cartilage by blocking the Indian hedgehog pathway, Arthritis Res. Ther. 21 (1) (2019) 109.
[34] X. Zhang, et al., Increased LGALS3BP promotes proliferation and migration of oral squamous cell carcinoma via PI3K/AKT pathway, Cell Signal (2019) 109359.
[35] Y. Fu, et al., Scutellarin exerts protective effects against atherosclerosis in rats by regulating the Hippo-FOXO3A and PI3K/AKT signaling pathways, J. Cell Physiol. 234 (10) (2019) 18131–18145.