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Extract from *Eucommia ulmoides* Oliv. ameliorates arthritis via regulation of inflammation, synoviocyte proliferation and osteoclastogenesis *in vitro* and *in vivo*

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ABSTRACT

Ethnopharmacological relevance: *Eucommia ulmoides* Oliv., a valuable Chinese herb, has shown a variety of health benefits. Despite the widespread clinical use of this herb to treat rheumatoid arthritis (RA) in Traditional Chinese Medicine (TCM), very few studies have described its anti-pathological effects or mechanism in RA. The present study investigated the mechanism of *Eucommia ulmoides* Oliv. in an experimental collagen-induced arthritis (CIA) rat model.

Materials and methods: The effects of four different *Eucommia ulmoides* Oliv. extracts on the proliferation of rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS) were screened in an MTT assay, and apoptotic effects were detected by flow cytometry. Among the extracts, the 70% ethanol extract (EU70) presented the best inhibition and was further investigated for its curative effect in CIA rats. Foot swelling was detected, and the arthritis index (AI) was scored. Pathological improvement was assessed by haematoxylin and eosin (H & E) staining of joint tissues. The mechanistic effects of EU70 were investigated as follows: anti-inflammatory effects in Th17-positive cells by flow cytometry; serum levels of inflammatory cytokines by ELISA; TNFα and IL-1β expression by immunohistochemistry (IHC); and anti-osteoclastogenesis by QPCR detection of RANKL and OPG mRNA.

Results: Compared with vehicle treatment in CIA model rats, EU70 significantly ameliorated foot swelling, decreased AI *in vivo* and reduced inflammatory cell infiltration and synoviocyte proliferation. EU70 decreased the number of Th17-positive cells in the spleen and the serum levels of cytokines, including IL-17, IL-1β and TNFα, and upregulated the serum levels of IL-10; these results indicated the anti-inflammatory effect of EU70. Moreover, EU70 effectively suppressed TNFα and IL-1β expression in the joint tissues and resulted in the downregulation of RANKL mRNA and the upregulation of OPG mRNA. These results revealed the possible preventive role of EU70 against bone destruction.

Conclusion: For the first time, these mechanisms and pathological improvements support the clinical use of *Eucommia ulmoides* Oliv. in treating RA. The findings indicated that the 70% ethanol extract of *Eucommia ulmoides* Oliv. could relieve RA symptoms by (1) suppressing the proliferation of synoviocytes, (2) reducing the number of Th17-positive cells and downregulating serum IL-17 expression, (3) increasing the anti-inflammatory effects of IL-10, (4) inhibiting the serum and tissue levels of key pro-inflammatory cytokines, TNFα and IL-1β, and (5) reducing the degradation of cartilage and bone.

Abbreviations: RA, rheumatoid arthritis; TCM, traditional Chinese medicine; RA-FLS, rheumatoid arthritis fibroblast-like synoviocytes; EU70, 70% ethanol extract of *Eucommia ulmoides Oliv*; EU90, 90% ethanol extract of *Eucommia ulmoides Oliv*; EUw, water extract of *Eucommia ulmoides Oliv*; EUwp, ethanol precipitant from EUw; TG, tripterygium glycosides; AI, arthritis index; NSAIDs, analgesics and non-steroidal anti-inflammatory drugs; CIA, collagen II-induced arthritis; SPF, specific pathogen-free; CII, bovine type II collagen; CFA, complete Freund's adjuvant; ELS, erythrocyte lysis solution; ELISA, enzyme-linked immunosorbent Assay; H & E, haematoxylin and eosin

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1. Introduction

Rheumatoid arthritis (RA) is a chronic, inflammatory, and systemic autoimmune disease (Arend, 2001). The basic pathological changes in RA include the proliferation of rheumatoid arthritis-fibroblast-like synoviocytes (RA-FLS), thickening of the lining layer, inflammatory cell infiltration, pannus formation, and cartilage and bone damage, which all result in irreversible joint deformity and loss of function (Kinne et al., 2000; Martínez et al., 2007). The standard treatment for RA is a combination (Nagai et al., 2012) of analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) in the early stage to prevent RA progression. Unfortunately, the use of these agents is limited by gastroenteropathy, a limited clinical remission rate, ease of suppressing immunity, secondary tumour formation, infection, and organ damage. Herbal medicines, the main source of natural medicines, have unique advantages in the treatment of RA due to their wide variety of sources, reduced side effects, and multiple activities, levels and targets (Soeken and Miller et al., 2003). The identification of effective ingredients for RA treatment from natural medicines is a topic of great interest and a focus of extensive research and development efforts (Soeken and Miller et al., 2003).

Eucommia ulmoides Oliv. is a Chinese herb with a medicinal history dating back 2000 years. The medicine-food homology of *Eucommia ulmoides* Oliv. has been established by the Ministry of Health, P.R. China, and *Eucommia ulmoides* Oliv. has been applied in the clinic, including as an RA treatment. A search of nationwide databases, including CNKI, VIP and Wanfang, reveals more than 150 Chinese publications on the use of *Eucommia ulmoides* Oliv. in RA treatment. *Eucommia ulmoides* Oliv. ranks twelfth among prescriptions used to treat rheumatic diseases at Tongxiang First People's Hospital of China (Wang et al., 2011). A statistical analysis of ten years of hospitalized cases at the First Affiliated Hospital of Guangzhou University of Traditional Chinese Medicine also supports the common use of *Eucommia ulmoides* Oliv. to treat RA (Huang, 2009). Recent studies have also revealed the effects and mechanism of *Eucommia ulmoides* Oliv. in osteoarthritis (OA) (Chen et al., 2014; Lu et al., 2013). An analysis of prescriptions for OA treatment in Taiwan in 2002 determined that *Eucommia ulmoides* Oliv. was the most commonly used single Chinese herb (Chen et al., 2014). However, few international reports have examined the mechanism of *Eucommia ulmoides* Oliv. in RA.

Eucommia ulmoides Oliv. exhibits the following pharmacological activities: blood pressure reduction, diuresis, immune regulation, anti-aging, anti-tumour, antibacterial, anti-inflammatory, and analgesic effects (Lee and Weinblatt, 2001). The effective components of *Eucommia ulmoides* Oliv. include lignans, flavonoids, iridoids, sterols, phenylpropanoids, phenolics and other molecules, such as triterpenes, that possess anti-inflammatory and immune regulatory activities. The use of terpenes to treat RA is particularly notable (Feng et al., 2015; He et al., 2014). Aucubin, an iridoid present in *Eucommia ulmoides* Oliv., exhibits anti-inflammatory effects (Bermejo et al., 2000). Kim et al. (2015) determined that *Eucommia* cortex inhibits the production of tumour necrosis factor-α (TNFα), interleukin-6 (IL-6), cyclooxygenase-2, prostaglandin E and nitric oxide (NO) in lipopolysaccharide (LPS)-treated peritoneal macrophages. Therefore, the mechanism underlying the effectiveness of *Eucommia ulmoides* Oliv. against RA may involve anti-inflammatory and immunoregulatory activities. However, relevant studies have not been reported.

Though *Eucommia ulmoides* Oliv. is widely used clinically and possesses multiple components involved in anti-inflammation and immune regulation, few studies have identified the pharmacological mechanism of this herb. The aim of this study is to use extracts of *Eucommia ulmoides* Oliv. to detect its effects in treating RA and to explore its underlying molecular mechanism of action. The inhibitory effects of *Eucommia ulmoides* Oliv. extracts on the proliferation of human synoviocytes were assessed using MTT assays and flow cytometry. A collagen II-induced arthritis (CIA) rat model was then established to investigate the *in vivo* anti-inflammatory and immune regulatory mechanisms of the pharmacological effects of *Eucommia ulmoides* Oliv. to provide experimental evidence for its clinical application in RA.

2. Materials and methods

2.1. Plant material

The cortex of *Eucommia ulmoides* Oliv. was purchased from Shanghai Kangqiao Chinese Medicine Tablet Co., Ltd., (Shanghai, China) and identified as family *Eucommiaceae* by Professor Wu Jinrong of the Shanghai University of Traditional Chinese Medicine. A voucher specimen (Wang, 9523) has been deposited in the Department of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine.

2.2. Extraction

The cortex of *Eucommia ulmoides* Oliv. (1 kg) was washed, sliced, dried and then extracted twice with 70% ethanol or 90% ethanol (1:8 and 1:6, w-v, 3 days each time). Then, the extract was evaporated to a small volume (1 g/mL) *in vacuo* to yield a 70% ethanol extract (EU70) and a 90% ethanol extract (EU90). The water extract (EUw), and ethanol precipitate from the EUw (EUwp) were purchased from Yuanmu Biotech Co., Ltd (Shanghai, China).

2.3. Cell growth inhibition using the MTT assay

Human RA fibroblast-like synoviocytes (RA-FLS) from patients were purchased from Shanghai Rochen Pharma Co., Ltd., (Shanghai, China) and cultured in 1640 medium with 10% foetal bovine serum and 1% antibiotics in an atmosphere of 5% CO_2 at 37 °C. RA-FLS at the exponential (log) phase were seeded at a density of 2,000 cells/well/200 μL in a 96-well plate. After overnight incubation, various doses of EU70, EU90, EUw, EUwp and tripterygium glycosides (TG) were added to the RA-FLS in triplicate. Medium was used as a blank. The MTT method was used to assess inhibition after 24 h, 48 h, and 72 h of growth at 37 °C. Briefly, 20 μL of 5 mg/mL MTT in PBS was added to each well. After 4 h of incubation, the OD at 490 nm was detected in DMSO after the supernatant was removed. Inhibition ratio =(1-OD/Blank OD)×100%.

2.4. Induction of CIA in rats and administration

In total, 32 female and 32 male Wistar rats (SLAC, Shanghai, China) were housed under specific pathogen-free (SPF) conditions. Animal welfare and experimental procedures were performed in accordance with the institutional guidelines for the care and use of laboratory animals and related ethical regulations of Shanghai University of Traditional Chinese Medicine (2014028).

The method for generating the CIA rat model was modified from Huang and Huang (2008). Briefly, approximately 10 mg/mL bovine type II collagen (CII) in 0.1 M acetic acid was emulsified with an equal volume of complete Freund's adjuvant (CFA) to create a stable CII/CFA emulsion (5 mg/mL). With the exception of the control group (8 male and 8 female rats), the rats were intradermally injected with the CII/CFA emulsion in the tail (0.1 mL) and right hind paw (0.1 mL). At 7 and 21 days after the primary administration, each rat received 0.1 mL of CII/CFA booster *via* intradermal injection in the tail.

On day 21, the CIA rats were randomly allocated to 3 groups containing equal numbers of males and females. The CIA group, EU70 group and TG group were intragastrically infused with PBS, 2.7 g/kg of EU70 or 5.4 mg/kg of TG once daily, respectively. The dose of EU70 was calculated from the dose for humans (China Pharmacopoeia

Committee, 2015) based on equal body surface area (Xu, 2002).

2.5. Evaluation of arthritis

The body weights and right hind paw volumes of all rats were monitored weekly. The right hind paw volumes were noted on a Digital Plethysmometer (Shandong Academy of Medical Sciences, Shandong, China). On days 7, 21, 35 and 49, the severity of arthritis was semiquantitatively scored from 0 to 4: 0, normal; 1, mild swelling confined to the ankle joint and mid-tarsals; 2, moderate swelling extending from the ankle to the metatarsal joints; 3, severe swelling encompassing the ankle, foot and digits; and 4, severe swelling causing joint swelling deformity and difficulty in walking (Leavenworth et al., 2013).

2.6. Sample collection

On the 35th and 49th days, eight rats (four male and four female) in each group were euthanized. Part of the hind limb joints from the rats were fixed in 10% formaldehyde for the subsequent experiments, whereas the other part was preserved in liquid nitrogen. Blood was obtained from the abdominal aorta, centrifuged at 4 °C to collect the serum, and stored at −80 °C. Single splenocytes suspensions were collected in an aseptic manner. Briefly, the spleens were dissected and added to PBS. The dissociated tissue was passed through a 200-mesh cell strainer with grinding using a syringe to obtain the cell suspension. After centrifugation, the cell pellet was suspended in ELS (erythrocyte lysis solution) for several minutes to remove red blood cells. After washing with PBS and centrifugation, single cells were cultured in RPMI 1640 medium containing 10% FBS.

2.7. Flow cytometry

To measure cell apoptosis, RA-FLS were cultured at a density of 5×10^5 cells/well in a 6-well plate and treated with EU70 and EU90 for 48 h. The cells were harvested, washed and incubated with Annexin V-FLUOS solution for 20 min in the dark, followed by incubation with PI (50 μg/mL) for 10 min. Apoptotic cells were then detected with a FACSCalibur (Becton Dickinson, New Jersey, USA) equipped with EXPO32 V1.2 analysis software.

IL17-positive splenocytes were detected by incubating the cells with IL-17A-FITC (eBioscience, California, USA) at room temperature for 30 min.

2.8. Enzyme-linked immunosorbent assay (ELISA)

Serum IL-10, IL-17, IL-1β and TNFα levels after treatment with EU70 were determined using ELISA kits (eBioscience, California, USA) according to the manufacturer's instructions.

2.9. Haematoxylin and eosin (H & E) staining and pathological evaluation

Right posterior knee joints were fixed in 10% formaldehyde, dehydrated in an ethanol gradient series, embedded in paraffin, serially sectioned into 5 μm slices, and stained. Changes in joint and synovial tissues were observed and evaluated pathologically on a scale from 0 to 5: 0, normal; 1, infiltration with a few inflammatory cells or mild oedema; 2, mild infiltration; 3, moderate infiltration; 4, obvious infiltration; and 5, severe infiltration (LaBranche et al., 2010).

2.10. Immunohistochemistry (IHC) for TNFα and IL-1β

Following deparaffinization, antigen retrieval was performed by heating the slices in citrate buffer, followed by treatment with 0.3% hydrogen peroxide to block endogenous peroxidase activity and incubation with goat serum to block non-specific binding. The slices were incubated with primary antibodies against TNF-α and IL-1β (Santa Cruz Biotechnology, California, USA) overnight at 4 °C. The following day, the slices were washed and incubated with a secondary antibody solution for 2 h at 37 °C. After washing, the slides were developed with DAB (3,3′-diaminobenzidine), the nucleus was counterstained with haematoxylin, and the slices were incubated with xylene (to render them transparent) and mounted. The slides were analysed using the IMS cell image analysis system, and the average optical density was calculated as the Positive area percentage (%)×optical density (OD).

2.11. Detection of RANKL and OPG mRNA levels by qRT-PCR

Fifty milligrams of joint tissue were ground in liquid nitrogen. Then, RNA was extracted with TRIzol and reverse transcribed into cDNA using an RT-PCR kit (TaKaRa, Japan). qRT-PCR was performed using β-actin as a reference and SYBR green as the fluorescent dye with the following amplification conditions: pre-denaturation at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 3 s, and annealing and extension at 60 °C for 30 s. The primers were as follows: OPG-F, tgggaatgaagatcctccag; OPG-R, cctctttctcagggtgcttg; RANKL-F, accagcatcaaaatcccaag; RANKL-R, ggacgctaatttcctcacca; β-actin-F, agccatgtacgtagccatcc; and β-actin-R, accctcatagatgggcacag.

2.12. Statistical analysis

All statistical analyses were performed with the SPSS19.0 software package (SPSS Inc., Chicago, IL, USA). Student's *t*-test was used for comparisons between independent groups. A P value of less than 0.05 indicated a statistically significant difference.

3. Results

3.1. The ethanol extract of Eucommia ulmoides Oliv. inhibits RA-FLS proliferation

The MTT assay of cell viability was performed to screen for the inhibitory effects of several extracts of *Eucommia ulmoides* Oliv. on RA-FLS. As shown in Fig. 1A, EU70, EU90 and TG inhibited RA-FLS proliferation in a dose- and time-dependent manner. Treatment with 1,000 μg/mL EU70 at 48 h and 72 h, 1000 μg/mL EU90 at 72 h, and most doses of TG resulted in greater than 50% inhibition and significant differences compared to the blank wells ($P < 0.001$), whereas 1000 μg/mL EU70 exhibited a greater anti-proliferation effect than EU90 at both 48 h (63% *vs.* 23%, respectively) and 72 h (76% *vs.* 65%, respectively). Therefore, the ethanol extracts were screened in the subsequent assay.

3.2. EU70 induces apoptosis and necrosis of RA-FLS

Apoptotic and necrotic cells were distinguished by flow cytometry with AnnexinV/PI detection. After 48 h of treatment (Fig. 1B), both EU70 and EU90 increased cell apoptosis compared with the PBS group ($P < 0.001$). Furthermore, EU70 promoted apoptosis to a greater extent than EU90. Therefore, EU70 was selected as the representative extract in the subsequent pathological and mechanistic assays *in vivo*.

3.3. EU70 ameliorates foot swelling and decreased the arthritis index (AI)

As shown in Fig. 2A, no significant weight loss was observed between the EU70 group and model group, although the CII/CFA emulsion induced arthritis and reduced weight compared with the control group. These results suggest that EU70 does not have obvious toxicity.

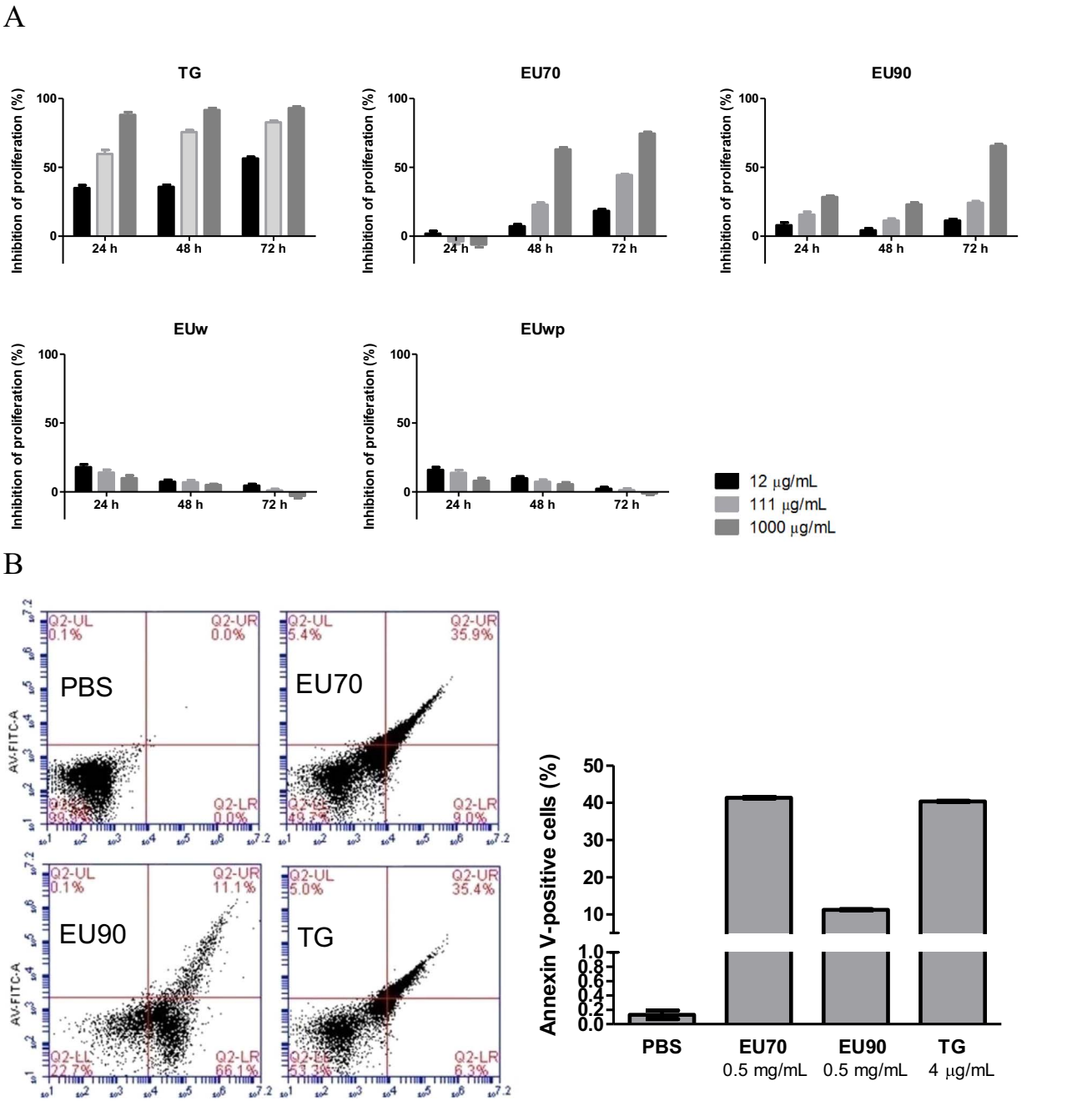

Fig. 1. Of the various extracts of *Eucommia ulmoides* Oliv., EU70 exhibited the greatest inhibitory and apoptotic effects against RA-FLS. A. MTT assay of RA-FLS cells treated with various doses of TG, EU70, EU90, EUw, and EUwp for 24 h, 48 h and 72 h. B. Apoptosis assay of RA-FLS treated with EU70 and EU90 for 48 h. TG and PBS were used as positive and negative controls, respectively.

Swelling increased obviously in the model compared with that of the control group, leading to joint deformity and difficulty walking, which were manifested as significant increases in foot volume and AI ($P < 0.05$). These results confirmed that the CIA rat model was developed successfully.

Compared to the CIA group, EU70 significantly decreased joint swelling, as indicated by reduction of both foot volume and AI, after 1 week of treatment. The maximum effect was attained in the second week (from days 35).

3.4. EU70 improves the pathology of the synovial membrane

As shown in Fig. 2B, the joint tissues of the control group were clear and smooth, without hyperplasia and inflammatory cell infiltration. After 35 days, the CIA group exhibited obvious hyperplasia of the synovial tissue, aggressive structural disorder in the articular cavity, inflammatory cell infiltration, and osteoclasts. Both the EU70 and TG group rats exhibited synovial tissue hyperplasia and significantly reduced infiltration of inflammatory cells, whereas bone tissue destruction was observed in the TG group. After 49 days, the CIA rat model exhibited serious synovial hyperplasia, inflammatory cell infiltration, and bone tissue destruction with empty cavities. However, the TG group of rats exhibited a lack of inflammation. The EU70 rats exhibited a significant decrease in inflammatory cell infiltration accompanied by synovial tissue adhesion. There were significant differences in the pathological scores of the CIA model and drug groups, but not between the EU70 and TG groups.

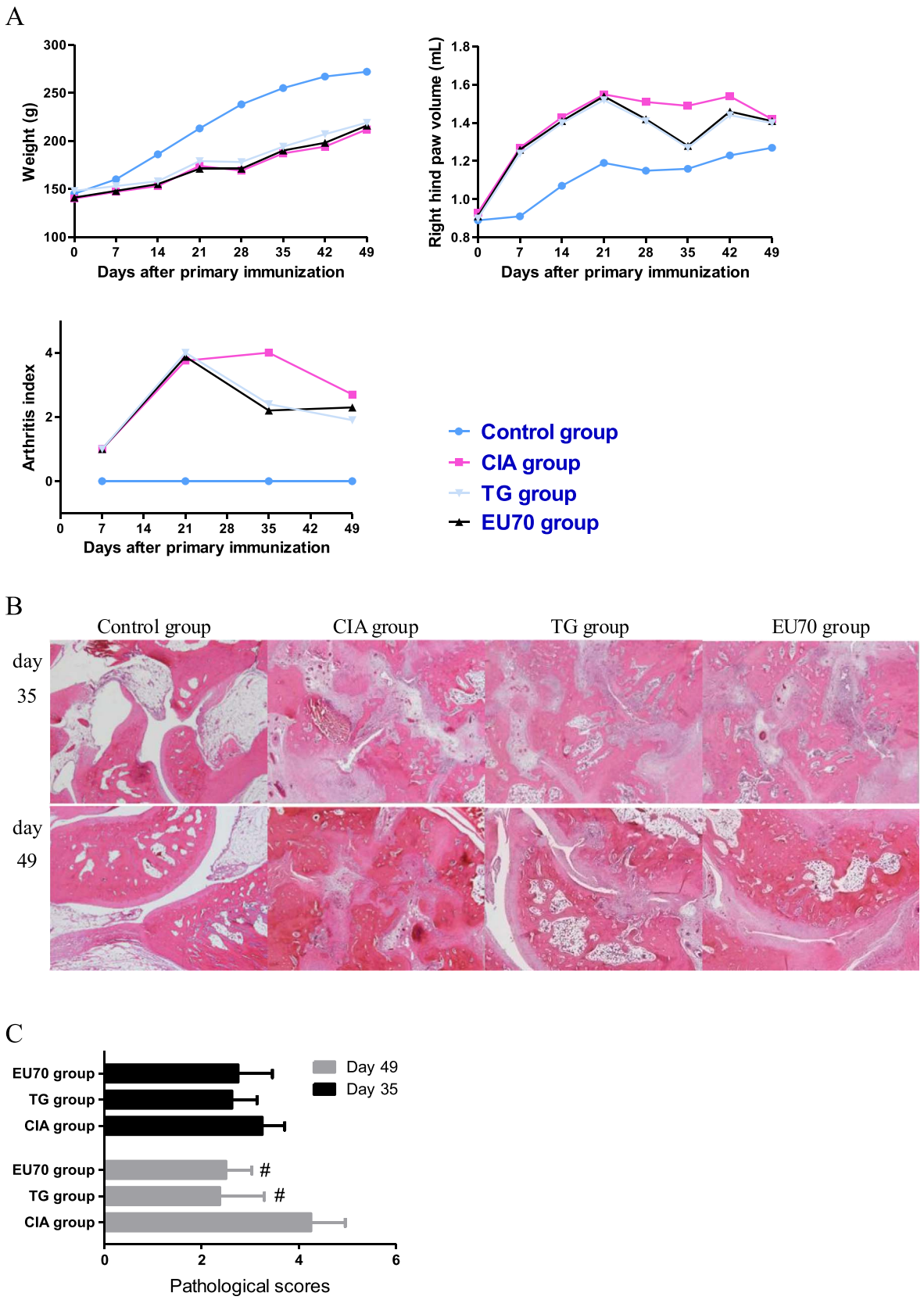

Fig. 2. EU70 alleviates the symptoms of RA *in vivo* and improves the pathological state of inflammation. A. Equal numbers of male and female rats were used to develop the CIA model with a first and booster immunization. After the first subcutaneous injection of the CII/CFA emulsion (5 mg/mL) in the tail and right hind paw, the rats' body weights, right hind paw volume, and arthritis indexes were recorded on different study days. B. Pathological sections of hind limb joints from normal (healthy), arthritic and drug-treated CIA rats (×40 magnification) were analysed by H & E staining. C. Inflammatory infiltration was assessed by scoring the H & E sections. #: $P < 0.05$ compared with the CIA group.

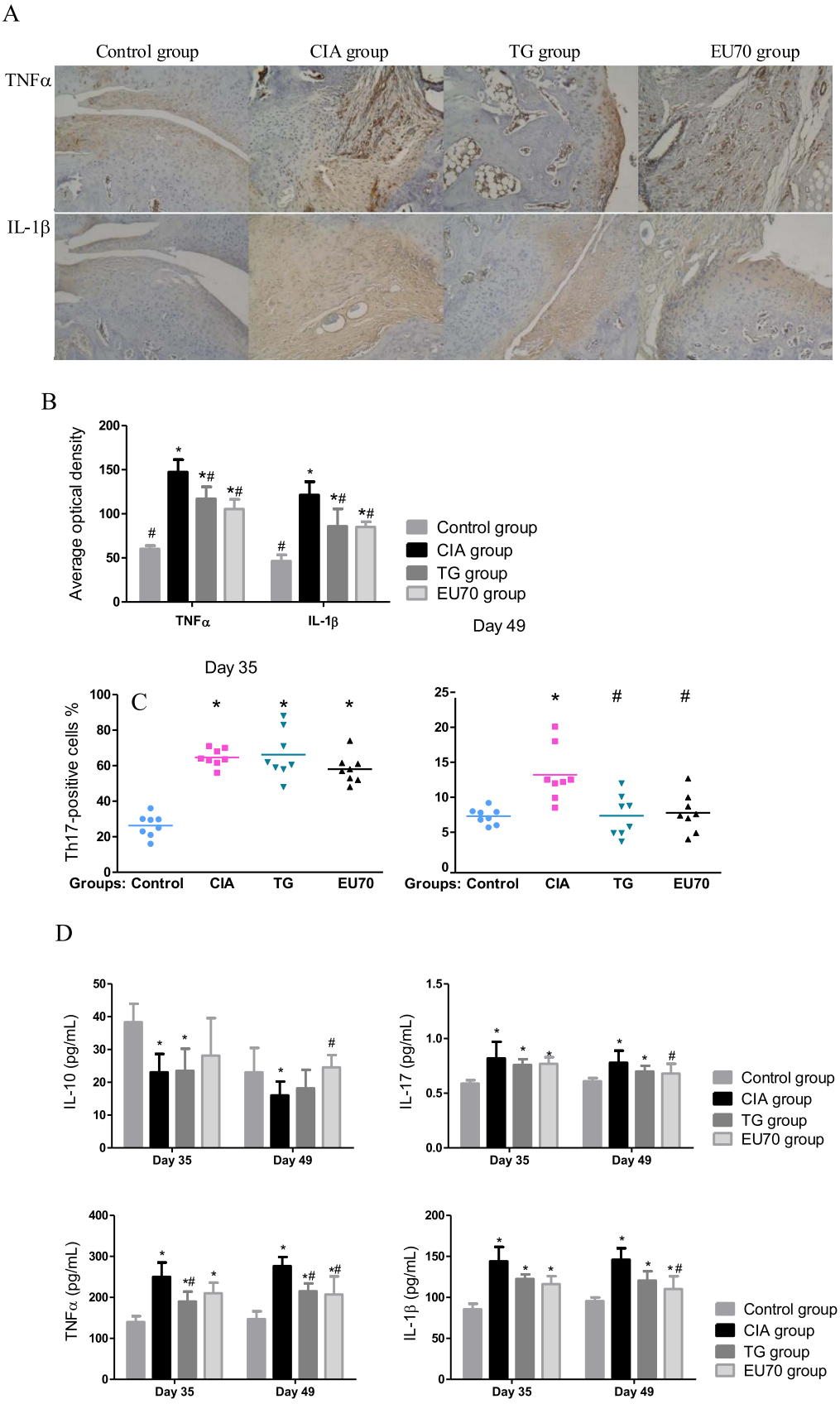

Fig. 3. EU70 decreases pro-inflammatory cytokine levels and the number of T cells (Th17-positive cells) and increases anti-inflammatory cytokine levels (IL-10). (A) IHC of TNFα and IL-1β in rat hind limb joints on day 49 (after 4 weeks of drug treatment) (×40). (B). Average optical density of IHC sections from the four groups. (C) Splenocytes were isolated from the rats on day 35 and day 49. Th17-positive cells were stained with a FITC-conjugated Th17 antibody and detected by flow cytometry. (D) Serum IL-10, IL-17, IL-1β and TNFα levels were detected by ELISA. The rats were treated with the different drugs on day 35 and day 49 after model generation. *: $P < 0.05$ compared with the normal group; #: $P < 0.05$ compared with the CIA group.

3.5. EU70 decreases the average optical density of TNFα and IL-1β staining

On day 49, the intensity of TNFα-and IL-1β-positive immunohistochemical staining in the model group was significantly increased compared with that of the control group ($P < 0.001$) (Fig. 3A and B), whereas the EU70 group exhibited a significant reduction in staining intensity compared with the CIA group ($P < 0.001$) (Fig. 3B).

3.6. EU70 decreases the number of Th17-positive lymphocytes in the rat spleen

On day 35, the percentage of Th17-positive cells in the spleen of the EU70-treated rats (58.69 ± 8.31%) exhibited a decreasing trend compared with the CIA control group (65.38 ± 6.20%). On day 49, the EU70 intervention (7.43 ± 2.98%) significantly reduced the number of Th17-positive cells compared with the model group (13.44 ± 4.54%).

3.7. EU70 induces anti-inflammatory cytokine production and reduces pro-inflammatory cytokines production

In the CIA model group, the serum levels of anti-inflammatory cytokine and pro-inflammatory cytokines (IL-17, IL-1β and TNFα) were significantly decreased and increased, respectively, compared with the control group. EU70 prevented the CIA-induced changes in IL-10 and IL-17 levels at two weeks after administration and significantly reversed the change in cytokine levels at four weeks after administration. Moreover, EU70 decreased the upregulation of IL-1β and TNFα both at 2 weeks and four weeks.

3.8. EU70 alleviates bone destruction by increasing OPG and RANKL mRNA levels

On day 35, the OPG and RANKL mRNA levels were significantly different in the CIA group compared with the levels in the control group, and EU70 significantly alleviated the changes induced by CIA. These improvements and the statistically significant differences persisted until day 49 compared with the CIA group (Fig. 4).

4. Discussion

The pathogenesis of RA involves multiple factors with complex interactions (Qian and Zhao, 2013). The main target of attack is the synovial joint, with increased inflammation leading to bone and cartilage destruction. Therefore, synoviocyte apoptosis and anti-inflammation are important targets of RA treatment (Baier et al., 2003).

Eucommia ulmoides Oliv. is a historical and valuable Traditional Chinese Medicine (TCM). The immune enhancement, anti-osteoporosis, anti-inflammation and antibacterial effects of *Eucommia ulmoides* Oliv. have been recently identified (Wang et al., 2013). However, there are few research articles describing the use of *Eucommia ulmoides* Oliv. to treat RA. We obtained four *Eucommia ulmoides* Oliv. extracts using different solvent extraction methods and screened their corresponding anti-proliferative activity in RA-FLS. EU70 exhibited the strongest inhibition. The apoptosis assay indicated that EU70 effectively promoted RA-FLS apoptosis. Additionally, H & E staining of the swelling joint revealed that EU70 inhibited the pathologic proliferation of the synovial tissue. Based on these results, EU70 was used as the agent in subsequent mechanistic assays.

For RA treatment *in vivo*, the pathological improvements of EU70-reduced AI, foot swelling, and inflammatory cell infiltration reflect the anti-inflammation mechanisms. Inflammation-related factors were explored because inflammation is the main cause and symptom of RA Th17-positive cells are a subset of T cells that produce IL-17 and promote the occurrence and development of inflammation. The upregulated production of IL-6, TNFα, IL-1 and other inflammatory cytokines by Th17 cells and IL-7 can synergistically promote the emergence and development of inflammation (Jovanovic et al., 1998; Yamaguchi et al., 2007). Th17 cells can induce synoviocyte proliferation (Lee et al., 2013), upregulate RANKL expression (Kim et al., 2015), increase osteoblast formation and lead to bone destruction (Braun and Zwerina, 2011). Flow cytometry revealed that EU70 reduced the CIA-induced increase in the number of Th17-positive lymphocytes and serum IL-17 levels, which may comprise part of the anti-inflammatory mechanism. IL-10 is a multi-functional, negative cytokine that acts to reduce the inflammatory response and the expression of inflammatory mediators (Bazzoni et al., 2010). IL-10 reduces the migration of lymphocytes to lesions by inhibiting the production of pro-inflammatory cytokines and increases the levels of endogenous anti-inflammatory substances, such as soluble TNFα receptor and IL-1 receptor antagonists, thereby suppressing TNFα and IL-1 production to inhibit disease progression (St. Clair, 1999). Our ELISA assay also demonstrated that EU70 increased serum IL-10 levels, further indicating the role for IL-10 in the anti-inflammatory mechanism of EU70.

As shown by IHC and ELISA, CIA-induced TNFα and IL-1β production in the joints and serum was significantly decreased by EU70, indicating a strong anti-inflammatory effect. TNFα is the core pro-inflammatory cytokine and plays an important role in immune regulation. TNFα upregulation induces cartilage matrix degradation and collagen decomposition, promotes platelet aggregation, and induces inflammatory cell infiltration. TNFα-targeted antagonists have been commercially developed and used in the clinic (Vinay and Kwon, 2012). Furthermore, as IL-1β may regulate numerous cytokines, immune regulators and pro-inflammatory mediators, this cytokine plays an important role in bone erosion and cartilage destruction in RA (Lee and Weinblatt, 2001). The downregulation of TNFα and IL-1β production by EU70 might by another anti-RA mechanism.

Bone destruction is the main cause of arthritis disability e regulated by the immune system; notably, RANKL-OPG is the bridge between bone and the immune system. RANKL is a member of the tumour necrosis factor super family. Several clinical studies have found

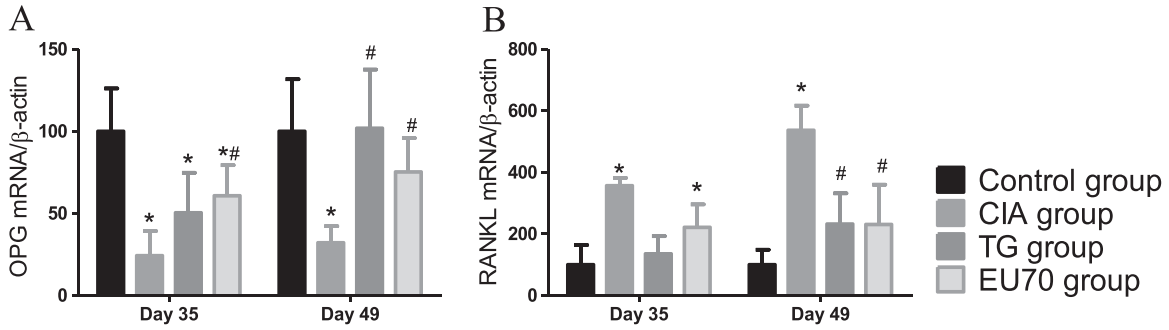

Fig. 4. EU70 decreases RANKL mRNA levels and increases OPG mRNA levels in joint tissue. *: $P < 0.05$ compared with the control group; #: $P < 0.05$ compared with the CIA group.

significantly increased RANKL levels in RA joint fluid, synovial tissue and peripheral blood mononuclear cells proportional to the degree of RA joint damage (Geusens, 2012; Miyazaki et al., 2007; Silva and Branco, 2011; Takayanagi, 2010). OPG belongs to the TNF receptor super family and is a decoy receptor of RANKL that can prevent the binding of RANKL to its receptor, thus inhibiting the intensification of osteoclast differentiation (Romas et al., 2002). The expression of RANKL in activated T lymphocytes may provide a link between the immune system and bone metabolism in RA. EU70 induced the upregulation of OPG mRNA and the downregulation of RANKL mRNA in synovial tissue, indicating resistance to the CIA-induced bone destruction.

In combination with the results of previous reports about RA cytokines, these results of this study indicate that improvements in the immune system may relieve the sensitization to RA pathology, adjust the dynamic balance of the bone tissue, and alleviate the destruction of bone tissue by CIA.

Compared with the CIA model group, body weight did not decrease significantly in the EU70 groups, suggesting that EU70 had no obvious toxicity.

This study is the first to show the effects and mechanism of *Eucommia ulmoides Oliv* on RA. EU70 acts on multiple targets of RA treatment. The possible mechanisms may include (1) inhibiting synoviocyte proliferation; (2) inhibiting Th17 cells *in vivo*; (3) increasing the downregulatory effect of IL-10 on inflammation; (4) inhibiting the expression of the pro-inflammatory cytokines IL-1β and TNFα in joint tissue and serum; and (5) upregulating the expression of the RANKL decoy receptor OPG and downregulating RANKL expression, which prevents bone tissue damage. Therefore, further research on *Eucommia ulmoides* Oliv. is warranted.

Authors' contributions

JYW and YQY formulated the original ideas and working hypothesis and designed the research study; JYW supervised and performed the detailed experiments; YY and XJC participated in generating the CIA model and pathological evaluation; SGF, LZ and YLH participated in immunohistochemistry and H & E staining; and SFY performed the ELISA. All authors analysed and interpreted the data. JYW wrote the manuscript and YQY provided revisions and submission considerations. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no competing interests.

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