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# Anti-inflammatory and joint protective effects of extra-virgin olive-oil polyphenol extract in experimental arthritis☆

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**Abstract**

The consumption of extra virgin olive oil (EVOO) in Mediterranean countries has shown beneficial effects. A wide range of evidence indicates that phenolic compounds present in EVOO are endowed with anti-inflammatory properties. In this work, we evaluated the effects of EVOO-polyphenol extract (PE) in a model of rheumatoid arthritis, the collagen-induced arthritis model in mice. On day 0, DBA-1/J mice were immunized with bovine type II collagen. On day 21, mice received a booster injection. PE (100 and 200 mg/kg) was orally administered once a day from days 29 to 41 to arthritic mice. We have demonstrated that PE decreases joint edema, cell migration, cartilage degradation and bone erosion. PE significantly reduced the levels of proinflammatory cytokines and prostaglandin $E_2$ in the joint as well as the expression of cyclooxygenase-2 and microsomal prostaglandin E synthase-1. Our data indicate that PE inhibits c-Jun N-terminal kinase, p38 and signal transducer and activator of transcription-3. In addition, PE decreases nuclear factor κB translocation leading to the down-regulation of the arthritic process. These results support the interest of natural diet components in the development of therapeutic products for arthritic conditions.


*Keywords:* Rheumatoid arthritis; CIA; EVOO; Polyphenols; Inflammatory response

## 1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by synovial inflammation and pannus leading to cartilage and bone damage. The earliest event is activation of the innate immune by exogenous and autologous antigens and thus auto-antibodies and later high levels of cytokines can be detected years before clinical symptoms [1]. At a joint level, there are synovial hyperplasia and massive infiltration of immune cells, including $CD4^+$ T cells, B cells, natural killer cells, macrophages, dendritic cells, neutrophils and mast cells [2,3]. A wide range of evidence has demonstrated the contribution of cytokines to the chronicity of inflammation and tissue damage in RA. Therefore, the importance of proinflammatory cytokines has been confirmed by the success of cytokine-directed therapies in RA [4]. Proinflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor α (TNFα) activate many signal transduction pathways involved in inflammation. The mitogen-activated protein kinase (MAPK) pathway includes extracellular signal-regulated kinases (ERK1/2 or p42/p44), c-Jun N-terminal kinases (JNK)1/2/3 and p38, which are activated in the synovium of patients with RA [5]. The janus kinase-signal transducer and activator of transcription (JAK-STAT) is another relevant inflammatory pathway activated in response to cytokines. In particular, overexpression of STAT-3 has been reported in synovial membranes from RA patients [6].

Proinflammatory cytokines activate different transcription factors to induce the expression of inflammatory and catabolic mediators [7]. Nuclear factor κB (NF-κB) and activating protein-1 (AP-1) are activated in RA synovium and play an important role in metalloproteinase induction [8]. NF-κB regulates a wide range of genes that contribute to inflammation, such as IL-1β, TNFα, IL-6, chemokines and microsomal prostaglandin E synthase-1 (mPGES-1), an efficient downstream enzyme functionally coupled with cyclooxygenase-2 (COX-2).

Biological therapies have improved the treatment of chronic inflammatory diseases such as RA. Nevertheless, these drugs are effective in a fraction of patients only and have other limitations including a high cost, the requirement for parenteral administration and important side effects. Therefore, new therapeutic strategies are under investigation. Epidemiological studies about consumption of functional foods, particularly extra virgin olive oil (EVOO) in Mediterranean countries, have showed important beneficial effects.

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EVOO, obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to oil alteration, has shown antioxidant, anti-inflammatory, immunomodulatory, antiproliferative and anti-apoptotic effects [9–15]. Although these health-protective effects have been traditionally ascribed to its high monounsaturated fatty acid content [15,16] a wide range of evidence indicates that many of the beneficial effects of EVOO intake are due to its minor highly bioactive components. Among them, phenolic compounds such as hydroxytyrosol, tyrosol and oleuropein have shown anti-inflammatory and antioxidant effects [17]. Olive oil polyphenols have also been associated with neuroprotective, antiaging and antiatherogenic effects and the regulation of important cellular signaling pathways. On the other hand, because of their ability to modulate cell death, olive polyphenols have been proposed as chemopreventive and therapeutic anti-cancer agents [18–21]. Our research group has previously demonstrated that a diet made with EVOO enriched with hydroxytyrosol, could improve inflammatory processes in a chronic colitis model [22]. In addition, we evaluated the protective effect of dietary EVOO-polyphenol extract (PE) supplementation in the inflammatory response associated to a chronic colitis model [23].

Collagen-induced arthritis (CIA) in DBA/1 mice is an animal model of RA widely used to test potential therapeutic agents [24]. The pathogenesis of CIA is dependent on the host's response to type II collagen challenge and the subsequent generation of antibodies that recognize collagen-rich joint tissue [25]. Joint inflammation in this model is characterized by significant cellular exudate and infiltrate, synovitis, and articular degradation. High levels of proinflammatory cytokines are detected at the onset of the disease, leading to the production of a wide range of mediators relevant in CIA pathogenesis [26,27]. Taken into account the anti-inflammatory properties of olive oil constituents, we hypothesized that PE may control the development of inflammation and articular lesion. In fact, intraperitoneal administration of oleuropein aglycone, a major constituent of the leaves and unprocessed olive drupes, has shown beneficial effects in CIA [28], and a recent study has reported that EVOO diet in conjunction with physical activity is able to preserve the articular cartilage in an osteoarthritis model [29].

The present study was designed to evaluate the effects of the oral administration of PE in the arthritis model of CIA in mice. In addition to macroscopic and histological analyses, we have determined the effects of PE on the production of inflammatory mediators. In order to gain a better insight into its mechanisms of action, signaling pathways were also explored.

## 2. Materials and methods

### 2.1. Extraction of PE

PE was obtained by the method described by Vazquez Roncero et al. [30] with some modifications [31]. Fifty grams of EVOO (Oleoestepa, Seville, Spain) was extracted with methanol/water (80:20, vol/vol, 125 ml). The mixture was mixed with a vortex at 5000 g for 1 min and sonicated for 15 min. After decantation, the methanolic extract was concentrated in vacuum under a stream of nitrogen at <35°C until it reached a syrup consistency; finally, it was lyophilized and stored at −80°C.

### 2.2. Characterization of phenolic compounds in PE

PE (70–75 mg) extracted from EVOO (75 g) was dissolved in $CDCl_3$ (750 μl) or in DMSO-d6 (750 μl) and an precisely measured volume of the solution (550 μl) was placed in a 5 mm NMR tube for the detection and quantification of phenolic compounds. The phenolic mixture to be analyzed in DMSO-d6 was previously dissolved in a MeOD-$D_2O$ 1:1 (1 ml) and concentrated to dryness at reduced pressure in order to exchange hydroxyl protons with deuterium nuclei. The NMR solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). NMR experiments were conducted on a Bruker Avance III 700 spectrometer, operating at 700.25 M Hz. The probe temperature was 24.8±1°C. All chemical shifts were given in ppm, and the J couplings in Hz. The solvent itself was used as a chemical shift reference (7.26 ppm for $CHCl_3$, and 2.50 for DMSO-d6). $^1$H NMR spectra were acquired with the following acquisition parameters: acquisition time 2.3s, relaxation delay 5s, spectral width of 0–20 ppm, data points 32k, number of scans 32, and line broadening of 0.3 Hz. Postacquisition processing included zero-filling to 64k. The spectra were phase corrected automatically using TOPSPIN and integration was performed manually.

We have determined the levels of oleocanthal and oleacein following a modification of the method developed by Karkoula et al. [32] for direct measurement of both dialdehydes in olive oil by quantitative high resolution $^1$H NMR in $CDCl_3$. The levels of other phenolic compounds have been determined by the procedure described by Christophoridou and Dais [33].

### 2.3. Induction of CIA

Arthritis was induced in male DBA-1/J mice (Janvier, Le Genest St Isle, France) of 11 weeks of age. All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals. The protocols were approved by the institutional Animal Care and Use Committee (University of Valencia, Spain). Bovine type II collagen (CII; 2 mg/ml in dilute acetic acid; MD Biosciences, Zürich, Switzerland) was emulsified in equal volumes of Freund's complete adjuvant (2 mg/ml *Mycobacterium tuberculosis*, strain H37Ra; Difco, Detroit, MI, USA). On day 0, DBA-1/J mice were immunized at the base of the tail with 100 μg of bovine CII. On day 21, mice received an intraperitoneal booster injection of 100 μg of CII dissolved in phosphate-buffered saline. Mice were considered to have arthritis when significant changes in redness and/or swelling were noted in the digits or in other parts of the paws. Joint inflammation was scored visually in each paw, using a scale of 0–2 where 0=uninflamed, 1=mild, 1.5=marked and 2=severe. Scoring was performed by two independent observers without knowledge of the experimental groups. In addition, photographs and X-ray (Carestream MS FX, Gainesville, FL, USA) images of hind paws were obtained at the end of the experiment (day 42).

### 2.4. Treatment groups

On day 29, animals were randomized into control and treatment groups. The study was performed in four groups of mice ($n$=10): naive group (NA), CIA group (CIA) and two treatment groups: CIA mice with PE treatment (PE 100 and 200 mg/kg, orally, once a day from days 29 to 41). On day 42 after immunization mice were anesthetized, blood samples were taken by intracardiac puncture and animals were killed by cervical dislocation. Paws were amputated and processed for either histological analysis or homogeneization and assessment of inflammatory mediators and mechanisms.

### 2.5. Histological and immunohistochemical analyses

Knee joints were fixed in 4% formalin. After decalcification in 10% EDTA, specimens were processed for paraffin embedding. Tissue sections (7 μm) were stained with hematoxylin and eosin to perform histological analyses. For immunohistochemistry, the endogenous peroxidase activity was inhibited with hydrogen peroxide and then the sections were incubated in normal horse serum (Vectastain Kit; Vector Laboratories, Burlingame, CA, USA) for 20 min to reduce nonspecific staining and successively incubated with rabbit anti-COX-2 (Cayman Chemical, Ann Arbor, MI, USA) at dilution 1:250 overnight at 4°C. Later on, slides were treated with antirabbit IgG antibody (Vectastain Kit, Vector Laboratories, Burlingame, CA, USA) for 30 min and incubated with the streptavidin–peroxidase complex of the kit for 30 min, at room temperature. After incubation with the peroxidase substrate 3,3′-diaminobenzidine and washing with water, the sections were counterstained with hematoxylin. Negative control sections were treated in the same way but in the absence of primary antibody. Positive cells and total cells were counted in five random high-power fields by two independent observers.

### 2.6. Enzyme-linked immunosorbent assay

Hind paws (knees) were homogenized in liquid $N_2$ with 1 ml of A buffer pH 7.46 (10 mM HEPES, pH 8, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM dithiothreitol (DTT), 5 mM NaF, 1 mM $Na_3VO_4$, 1 μg/ml leupeptin, 0.1 μg/ml aprotinine, and 0.5 mM phenylmethyl sulfonyl fluoride (PMSF)). The tissue homogenates were sonicated (10 s three times at 20% with a 10-s incubation on ice between bursts) in an ultrasonic processor (VC130PB, Sonics & Materials Inc., Newtown, CT, USA) and centrifuged at 600×$g$, 15 min at 4°C. Supernatants were removed and used for determination of inflammatory mediators by enzyme-linked immunosorbent assay (ELISA): $PGE_2$ (Cayman Chemical, Michigan, USA), TNFα and IL-1β (R&D Systems Inc., Minneapolis, MN, USA), and IL-6 (Diaclone, Besançon Cedex, France).

### 2.7. Western blot

Hind paws were homogenized in liquid $N_2$ with 1 ml of ice-cold phosphate buffer (0.01 mM $K_2HPO_4$, $KH_2PO_4$ and 0.15 M NaCl) and centrifuged (800×$g$, 10 min at room temperature). Pellets were resuspended in hypotonic buffer [1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 1 μg/ml leupeptin, 20 mM NaF, 0.5 mM DTT, 1 mM EDTA and 10 mM HEPES, pH 7.9], incubated for 10 min on ice and centrifuged (14,000×$g$, 30 s at room temperature). Supernatants (cytoplasmic proteins) and pellets (nuclear proteins) were collected. Pellets were resuspended in ice-cold low-salt buffer and nuclear proteins were released by adding a