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ORIGINAL RESEARCH

The Effect of Aqueous Extract of *Trachyspermum ammi* Seeds and Ibuprofen on Inflammatory Gene Expression in the Cartilage Tissue of Rats with Collagen-Induced Arthritis

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Background and Objectives: Rheumatoid arthritis (RA) is an inflammatory disease treated with nonsteroidal anti–inflammatory drugs that have different side effects. One of the plants used for this purpose in the traditional medicine is *Trachyspermum ammi*. The present study aimed at investigating the anti–inflammatory effect of this plant on type II collagen-induced arthritis (CIA) in Wistar rats.

Materials and Methods: The study was performed on 35 male Wistar rats. Seven rats were considered as the healthy control group (normal group), and CIA was stablished in the rest. The rats with a model of inflammatory arthritis were divided into four groups. One group did not receive any treatment and three groups were treated orally with ibuprofen (15 mg/kg), aqueous extract of the *T. ammi* seeds (100 mg/kg), or their combination for 30 days. The effect of different treatments was investigated on the paw thickness, arthritis score, and mRNA level of *COX2* and *iNOS* genes.

Results: CIA increased paw thickness, arthritis score, and *COX2* and *iNOS* mRNA levels compared to those of the normal group. Treatment with ibuprofen and aqueous extract alone or in combination reduced the studied variables. Reduction in the paw thickness, arthritis score, and *iNOS* mRNA level was more in the ibuprofen-treated group than the *T. ammi* extract-treated group, but treatment with *T. ammi* extract reduced *COX2* mRNA level more than ibuprofen.

Conclusion: It seems that the aqueous extract of *T. ammi* can be used alone or in combination with ibuprofen to treat RA.

Keywords: rheumatoid arthritis, *Trachyspermum ammi*, ibuprofen, cyclooxygenase-2, nitric oxide synthase type II

Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory disorder that affects about 1% of the world's population and more than 2 million adults in the United States.[1,2] RA is clinically diagnosed with joints involvement, immune activation, and increased inflammatory markers such as rheumatic factor (RF), anti-cyclic citrullinated peptide (anti-CCP), and antinuclear antibodies (ANAs).[3] Even after decades of research, the understanding of pathogenesis and mechanisms involved in it are primitive and primordial. This disease is affected by environmental and genetic factors.[1]

Prostaglandins and nitric oxide (NO) are two important inflammatory mediators involved in RA and other inflammatory disorders. NO is produced by NO synthases

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(NOS) from the amino acid L-arginine. Two forms of NOS are reported: cNOS (constitutive NOS) that contains eNOS (endothelial NOS) and nNOS (neuronal NOS), which are calcium-dependent regulators of homeostasis, and inducible NOS (iNOS) that is independent of calcium. Various inflammatory agents such as interleukin-1 (IL-1), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), and lipopolysaccharide (LPS) increase the expression of iNOS. NOS are produced mainly by synoviocytes, chondrocytes and endothelial cells in inflamed joints. NO stimulates inflammation, promotes tumor growth, and enhances the invasion of cancer cells.[4,5] Prostaglandins are lipids with extensive biological activity involved in inflammatory responses, pain, and cancer. The main form of that is prostaglandin E2; the key enzyme of the prostaglandin production pathway is cyclooxygenase (COX) that has two isoforms, COX1 and COX2. COX1 is continuously produced in all tissue and plays a role in many physiological processes. COX2 is inducible and is associated with inflammation and tumorigenicity.[5]

Inhibition of prostaglandin production is a promising and practical way to treat inflammatory diseases, including RA. For this purpose, nonsteroidal anti–inflammatory drugs (NSAIDs) are used. These drugs selectively only inhibit COX2 – eg, celecoxib – or in a non-specific way inhibit both isoenzymes – eg, ibuprofen. Several studies suggest that ibuprofen in spite of directly inhibiting the activity of the COX2 enzyme can also change its mRNA levels.[6–8] Ibuprofen can play a role in suppressing inflammation by acting on mRNA levels of the iNOS enzyme.[9] Ibuprofen side effects are digestive and cardiovascular problems that occur more often in high doses and for long periods of time. For this reason, one of the important goals of the researchers is to achieve medicines with fewer side effects. The use of medicinal herbs to treat human and animal diseases is not a new idea. The use of such plants in many developing countries is rising, since three-quarters of the world's population does not have access to new drugs.[10]

Interest in the use of herbal products is steadily increasing since many herbal medicines do not have known side effects. Of the traditional herbs, *Trachyspermum ammi* is widely used to treat various diseases.[11] *Trachyspermum ammi* seeds have stimulant, antiseptic, anti-spasm, and anti-diarrheal properties and are used as anti-corrosive, anti–inflammatory, and laxative, as well as for abdominal pain and hemorrhoids. One of its important components is thymol, which is a polyphenol compound with antiseptic, antiflatulent, antifungal, and antibacterial activities.[11,12] Thymol also has antioxidant and anti–inflammatory properties, and reduces CRP (C-reactive protein), IL-1β, IL-6, TNF-α, TNF-β, and MMP9 (matrix metalloproteinase 9) levels.[13] This plant contains isomerism of thymol, called carvacrol, which has the same anti–inflammatory properties as Thymol.[14] Aqueous extract of the *T. ammi* seeds has anti–inflammatory activity in mice with edema and granuloma.[15] Treatment of mice arthritis with the aqueous extract of the *T. ammi* seeds can increase the antioxidant markers and reduce the inflammatory marker.[16] Animal models of RA are valuable tools to study the mechanisms associated with various stages of RA.Collagen-induced arthritis (CIA) is the most commonly used animal model of RA, since it has immunologic and pathologic features of human RA.[1,17]

To the best of authors' knowledge, since no study thus far investigated the effect of the aqueous extract of *T. ammi* seeds on the expression of *COX2* and *iNOS* genes in the cartilage of patients with RA, the current study aimed at investigating the effect of the aqueous extract of *T. ammi* seeds on RA compared with ibuprofen, a commonly used drug in the treatment of RA, using a rat model of RA.

Materials and Methods

Totally, 35 adult male Wistar rats (200–250 g) were purchased from the Animal House Facility of Baqiyatallah University of Medical sciences. The protocols of the study, which followed the NIH guidelines for animal use and care, were approved by the Ethics Committee of Baqiyatallah University of Medical Sciences (code No. IR.BMSU.REC.1396.564). Rats were kept in special cages under controlled conditions (temperature 25°C ± 2 and 12:12 hrs light/dark cycle). Water and food were provided ad libitum. After 1 week, when the rats were accustomed to the new conditions, seven rats were selected as the healthy control group (normal group), and in others, CIA was induced by bovine type II collagen and incomplete Freund's adjuvant (IFA), according to the method described by Trentham et al[18]. In brief, an equal volume of bovine type II collagen (2 mg/mL, Sigma, dissolved in 0.05 M acetic acid) and IFA (Razi Vaccine and Serum Research Institute, Karaj, Iran) was mixed. The rats were immunized intradermally at the base of the tail with 0.2 mL of this emulsion; 10 days after the first immunization, 0.1 mL of the emulsified liquid was intradermally injected as a booster shot. When the symptoms of arthritis appeared, a macroscopic semi-quantitative scoring system was used to evaluate the arthritis severity as follows: 0=normal joint; 1= swelling and redness in 1 joint; 2= swelling

in >1 joint; 3= swelling in the entire paw, and 4= joint deformity and/or ankylosis. The cumulative score for all four paws of each rat was used as arthritis score (maximum of 16 per rat) to represent overall disease severity and progression.[19] Swelling was quantified by measuring the thickness of the first arthritic hind paw with a caliper in all groups. CIA rats were divided into four groups based on their arthritis score. The same arthritis score was considered for rats in each group. The first group was considered as a CIA control group that did not receive any treatment; the second group received ibuprofen (IBU); the third group received the aqueous extract of *T. ammi* seeds (T.A) and the fourth group received both the aqueous extract and ibuprofen (IBU+T.A).

To prepare the aqueous extract of *T. ammi* seeds, 500 g of seed was added to 750 mL of distilled water and incubated at 55°C for 6 hrs; then the extract was filtered. After evaporation of water, the extract was transferred to the glass vial and stored at −10°C.[15] Two weeks after immunization, the treated groups received ibuprofen (15 mg/kg), aqueous extract (100 mg/kg), or a combination of them daily as a single dose by gavage for 30 days. During this time, all rats had a uniform diet. The CIA control group received water and food only after immunization.

After the treatment period, rats were first anesthetized with ether; then the volume of blood was removed from the heart that caused death in the rats. Blood samples were used to measure the white blood cells (WBCs) number and erythrocyte sedimentation rate (ESR). In order to investigate gene expression, the cartilage was surgically removed from the rat's foot and frozen quickly with the liquid nitrogen; then it was kept for RNA extraction at −70°C. To investigate the expression level of *COX2* and *iNOS* genes, total RNA were extracted from cartilage samples. RNA extraction was performed by TRIZOL (Invitrogen) according to the manufacturer's instructions. Then, using the Qiagen QuantiTect Rev Transcriptase kit, the cDNA was synthetized. After the preparation of cDNA, the *COX2*, *iNOS*, and *β-actin* genes were amplified using a quantitative fast SYBR Green PCR kit (Qiagen) and specific primers by real-time PCR technique. The real-time PCR profile of genes are shown in Table 1. The primers were designed with Primer 3 plus and GenscriptTaq Man software; NCBI Blast software was also employed to align primer sequences (Table 2).

In the current study, the *β-actin* gene was used as an internal control gene and the $2^{-\Delta\Delta ct}$ method was employed to examine the relative expression of genes in different groups. Statistical analyses were performed using SPSS 18 software. Quantitative data were expressed as mean ± standard deviation (SD). The normal distribution for quantitative variables was evaluated by the Kolmogorov–Smirnov test. To compare the measured data between the groups, one-way ANOVA and Tukey multiple comparison test were utilized; the significance level was considered $P < 0.05$.

Table 1 Real-Time PCR Profile of Genes

Step	Temp (°C)	Time (sec)	Cycle, N
Initial denaturation	95	300	1
Denaturation	95	20	35
Annealing	64	20	35
Extension	72	60	35
Final extension	72	240	1

Results

ESR and WBCs values of the various groups are shown in Figures 1 and 2, respectively; these values in the CIA groups had a significant increase compared to those of the normal group ($P < 0.05$), which confirmed the inflammation in the CIA group. Treatment with ibuprofen and the aqueous extract, both individually and in combination, decreased ESR and WBCs values in comparison with the CIA control group ($P < 0.05$); however, the changes were not significant in different treatment groups ($P > 0.05$)

Thickness of the paw and arthritis score of different groups are shown in Figures 3 and 4, respectively. The study results indicated that after different treatments, paw swelling and arthritis score decreased compared to those of the CIA control group ($P < 0.001$). The combination of ibuprofen and the aqueous extract reduced the arthritis score and paw swelling more than each one separately.

The results of the relative expression of *COX2* gene are shown in Figure 5. As shown, the relative expression of

Table 2 Real-Time PCR Primer Sequences

Gene	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	Size (bp)
iNOS	TCCCAGCACAAAGGGCTCAA	TGCGGACCATCTCCTGCATT	106
COX2	AGCTTCACTTGCCACCAACG	TCGGAAGAGCATCGCAGAGG	70
β-actin	AGCCATGTACGTAGCCATCC	CTCTCAGCTGTGGTGGTGAA	141

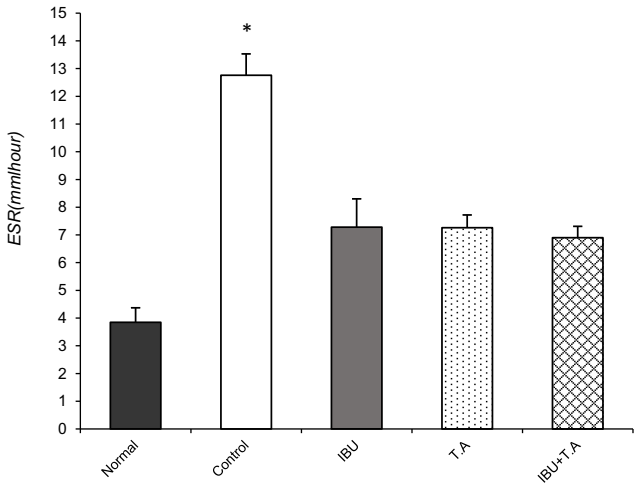

Figure 1 ESR values in different groups.
Note: *Compared to all groups (P <0.001).
Abbreviations: Control, CIA control; IBU, Ibuprofen; T.A, *Trachyspermum ammi.*

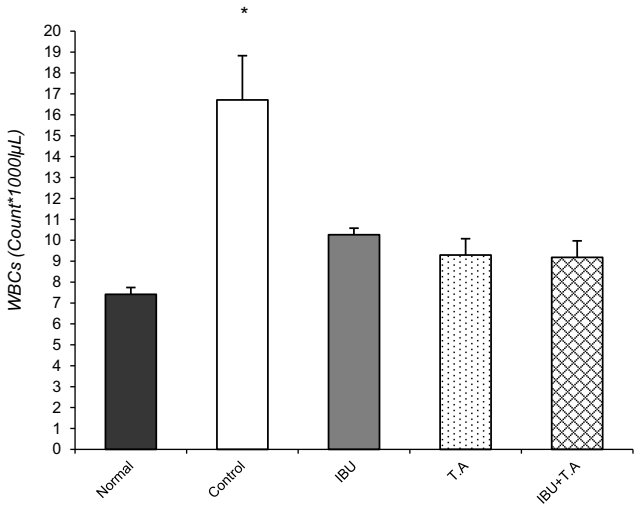

Figure 2 WBCs count in different groups.
Note: *Compared to all groups (P <0.001).
Abbreviations: Control, CIA control; IBU, Ibuprofen; T.A, *Trachyspermum ammi.*

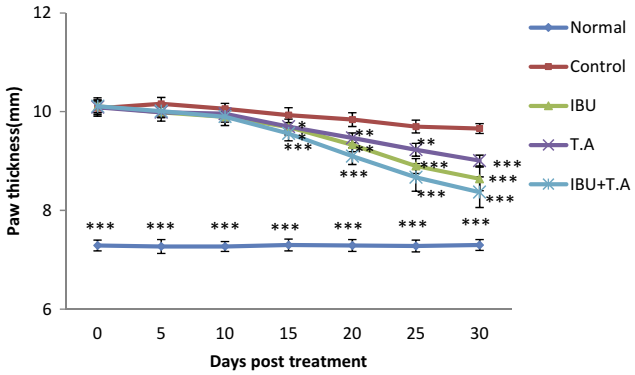

Figure 3 The effects of different treatments on paw thickness.
Notes: *(P <0.05), **(P <0.001), ***(P <0.001) compared to the control group.
Abbreviations: Control, CIA control; IBU, Ibuprofen; T.A, *Trachyspermum ammi.*

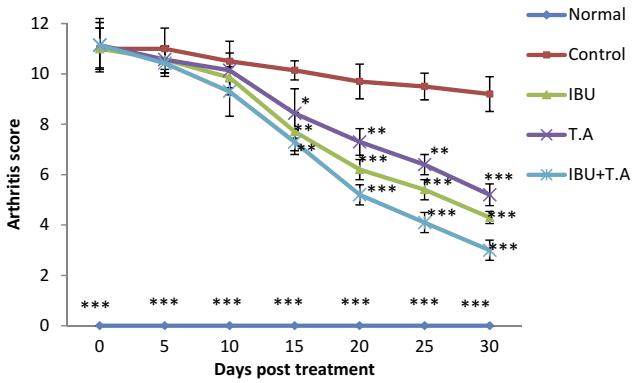

Figure 4 The effects of different treatments on arthritis scores.
Notes: *(P <0.05), **P (<0.01), ***(P <0.001) compared to the control group.
Abbreviations: Control, CIA control; IBU, Ibuprofen; T.A, *Trachyspermum ammi.*

COX2 gene in the CIA control group was 15.15 ± 1.23 times more than that of the normal group (P <0.001), which meant that the expression of this gene was well induced by CIA. Treatment with ibuprofen and the aqueous extract of *T. ammi* seeds significantly reduced the expression of this gene in comparison with that of the CIA control group; the gene expression in the ibuprofen group was 10.02 ± 0.78, in the aqueous extract of the *T. ammi* group was 5.82 ± 0.59, and in the combination group (ibuprofen + aqueous extract) was 4.29 ± 0.80 times higher than that of the normal group (P <0.001 for all groups).This reduction was higher in the *T. ammi* group than in the ibuprofen group (P <0.001). The combination of ibuprofen and the aqueous extract compared to ibuprofen (P <0.001) and the aqueous extract (P <0.01) alone resulted in a higher reduction in the *COX2* gene expression.

As shown in Figure 6, the relative expression of *iNOS* gene in the CIA control group was 13.65 ± 1.37 times higher than that of the normal group (P <0.001), which indicated that the gene played a role in the development of inflammatory arthritis. The expression of this gene in the ibuprofen group was 6.34 ± 0.49, in the aqueous extract group was 5.67 ± 0.45, and in the combination group was 3.45 ± 0.32 times higher than that of the normal group. Treatment with ibuprofen and the aqueous extract of *T. ammi* seeds individually and in combination reduced the expression level of *iNOS* gene compared to that of the CIA control group (for all groups, P <0.001). There was no statistically significant difference in the expression

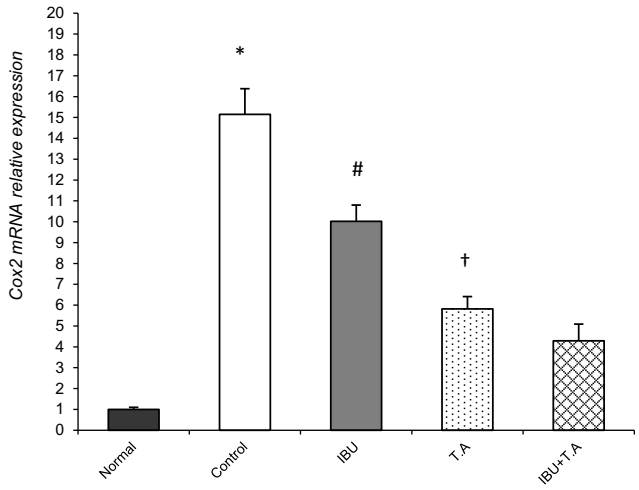

Figure 5 Relative expression of *COX2* gene in different groups (data are expressed as mean± standard deviation.).
Notes: *Compared to all groups (P <0.001); #Compared to all groups (P <0.001); †Compared to IBU+T.A group (P <0.01).
Abbreviations: Control, CIA control; IBU, Ibuprofen; T.A, *Trachyspermum ammi.*

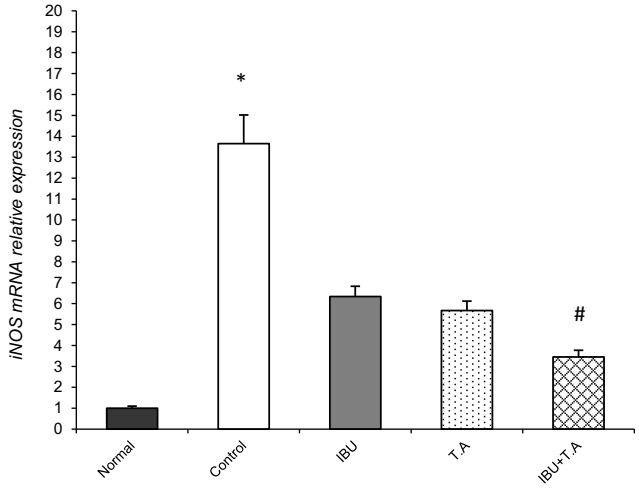

Figure 6 Relative expression of *iNOS* gene in different groups (Data are expressed as mean± standard deviation.).
Notes: *Compared to all groups (P <0.001); #Compared to IBU and T.A groups (P <0.001).
Abbreviations: Control, CIA control; IBU, Ibuprofen; T.A, *Trachyspermum ammi.*

level of *iNOS* between the ibuprofen and the aqueous extract groups (P >0.05). The combination of drug and aqueous extract more reduced the *iNOS* expression level compared to each of the treatments alone (P <0.001).

Discussion

RA is an autoimmune chronic inflammatory disease with significant effects on the quality of life of affected people. The common treatment for this disease is the use of non-steroidal anti–inflammatory drugs (NSAIDs). Long-term use of such drugs causes multiple digestive and renal complications. Therefore, it is evident that researchers are seeking alternative therapies to treat RA. One of the treatments that are especially appreciated in the developing countries is the use of medicinal herbs. These plants contain substances that are biologically active and can have therapeutic applications. Many of the drugs currently used in modern medicine are derived from plants. One of the plants used in traditional medicine is *T. ammi* that is native to Iran. The seeds of this plant have stimulant, antiseptic, anti-spasm, and anti-diarrheal properties and are also used as an anti–inflammatory and anti-fungal agent. In the current study, the effect of the aqueous extract of *T. ammi* seeds on the expression of *COX2* and *iNOS* genes, two genes expressed in inflammatory diseases such as RA, was investigated and compared with the effect of ibuprofen, which is commonly used in the treatment of RA.

The results of the current study showed that ESR, WBCs, paw thickness, arthritis score, and the expression level of *COX2* and *iNOS* genes increased in CIA. Treatment with aqueous extract of *T. ammi* seeds and ibuprofen alone or in combination reduced these values. These reductions were significantly higher in the combination group, which indicates that the simultaneous use of these compounds synergistically increases the effect of each other on inflammation reduction. Reduction in paw thickness and arthritis score was higher in the ibuprofen group than the aqueous extract group, but the aqueous extract reduced the expression level of *COX2* gene more than that of ibuprofen.

There are limited studies on the role of the aqueous extract or effective components of *T. ammi* seeds on the inflammation that are comparable to the present study, although none of these studies investigated the effect of the aqueous extract on the expression level of genes studied in the current study.

Umar et al[16] reported that treatment with the aqueous extract of *T. ammi* decreased the oxidative stress and inflammatory markers in the CIA rat model. Thangam et al[15] showed that the aqueous and alcoholic extracts of *T. ammi* decreased the volume of edema in the toe of the rat in acute inflammation and granuloma weight under acute inflammation. These anti–inflammatory effects were comparable with those of aspirin and phenylbutazone.

Yu et al[13] reported that thymol (one of the most important compounds found in *T. ammi*) reduced the inflammatory factors including CRP, IL-1β, IL-6, TNF-α, TNF-β, and related indexes of atherosclerosis including vascular cell

adhesion molecule-1, monocyte chemotactic protein-1, and MMP9 in a hyperlipidemic rabbit model. This study demonstrated the antioxidant and anti–inflammatory roles of thymol.

The effect of ibuprofen on the mRNA expression level of the *COX2* and *iNOS* genes is investigated in several studies. Stratman et al[9] reported that ibuprofen reduced the activity of iNOS in glial cells treated with LPS and INF-γ, by reducing the expression level of the *iNOS* mRNA. In this study, ibuprofen did not have an effect on the level of *COX2* mRNA but reduced its activity. In the study conducted by Vandivier et al,[20] ibuprofen reduced the production of NO in normal individuals and volunteers who received endotoxin but did not indicate its mechanism of action. Alvarez Soria et -al[21] reported that the long-term use of NSAIDs, celecoxib and aceclofenac, inhibited the synthesis of COX2 and iNOS in the articular cartilage of patients with osteoarthritis.

The results of the study by Liu et al[22] showed that chronic treatment with ibuprofen prevented learning and memory loss and loss of hippocampal neurons in diabetic rats. Additionally, ibuprofen significantly reduced the level of COX2 and iNOS proteins in the temporal cortex and hippocampus and IL-1β in the serum of diabetic rats but increased the PPARγ protein synthesis and mRNA expression. Heneka et al[23] reported that NSAIDs bound to PPARγ nuclear receptors and activated them. PPARγ also inhibited the expression of pro-inflammatory genes. In humans, ibuprofen, and pioglitazone, a PPARγ agonist, reduce inflammation and the expression of *COX2* and *iNOS* genes in the hippocampus and cortex of mice with Alzheimer's disease. Crofford et al[24] showed that the expression of *COX2* gene in the synovial tissue of patients with RA increased by IL-1 and cytokines. These cytokines stimulate transcription of *COX2* by activating NFκB transcription factors. On the other hand, the study by D'Acquisto[25] showed that ibuprofen inhibited the activation of NFκB in T-cells and expression of *COX2* and *PGE2* in macrophages. NF-κB plays a key role in regulating the immune response and plays an important role in *COX2* gene expression. It should be noted that the promoter of the *COX2* gene has regions for binding to NFκB and PPARγ.[26] Probably, in the current study, ibuprofen also inhibited inflammatory genes by NFκB and PPARγ messengers.

Conclusion

The results of the current study showed that the aqueous extract of *T.* ammi seeds similar to ibuprofen can reduce paw thickness, arthritis score, and expression of the genes involved in the inflammation process and may possibly be considered as a suitable candidate to manufacture an anti–inflammatory drug as an alternative or in combination with ibuprofen. However, more studies should be conducted in this regard.

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Disclosure

The authors declared no conflicts of interest.

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