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*Original Research Article*

# Aqueous extract of *Nigella sativa* L suppress proinflamatory cytokine gene expression

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## ABSTRACT

Due to the side effects of current therapies for osteoarthritis one of the alternative medicine is using herbal medicine such as *Nigella sativa* L. We examined that alcoholic extract of Nigella sativa (AENS) has an anti-inflammatory activity. Cells were activated with 100 ng/ml lipopolysaccharide for 24 h and cell supernatants were analyzed for $PGE_2$ and nitrite content. One set of cells was activated for 1 h with LPS (100 ng/ml) for both reverse-transcriptase PCR and real-time PCR analysis of TNF-a, IL-1b , COX-2, and iNOS expression. AENS also reduced TNF-α and IL-1b expression in LPS-activated THP-1 cells. The present study demonstrates that the anti-inflammatory activity of AENS is not restricted to synoviocytes, but also affects monocyte macrophage-like cells that serve as a prototype for macrophages in the synovial membrane. These observations provide a scientific rationale for the pain-reducing and anti-inflammatory effects of AENS observed in osteoarthritis patients.

***Keywords:*** Nigella sativa .L, bovine fibroblast-like, osteoarthritis, proinflammatory cytokine

## INTRODUCTION

Osteoarthritis (OA), also known as degenerative joint disease, is characterized

by cartilage degeneration and osseous overgrowth. OA is commonly encountered in today clinical practice. The incidence of OA increases with age, and it is one of the most prevalent diseases in older people. In the USA alone, 10% of men and 13% of women aged 60 and older have been diagnosed with knee OA . The symptoms of OA include joint pain, swelling, tenderness, stiffness, and sometimes locking, which may lead to disability and severely affect the life quality of patients [1]. Progression of OA can be characterized by changes in ECM composition and structure. Natural, healthy cartilage matrix is mainly composed of collagen type II which provides tensile support for the tissue. Aggrecan, a negatively charged proteoglycan that attracts water molecules, provides the compressive resistant and shock absorbing capability of cartilage under loading [2]. It has been shown that during OA, there are sequential events that affect the integrity of homeostatic ECM; aggrecan content is decreased, while collagen content is increased [2]. This change in ECM composition predisposes the tissue for mechanical fault resulting in significantly altered mechanical environments of the cells within the cartilage matrix. Inflammatory mediators such as cytokines, lipid derivatives, reactive oxygen species or advanced-glycation end products can be produced and activate cells from joint tissues (mainly synovium, cartilage and subchondral bone), thus leading to the release of matrix metalloproteinases (MMPs) into the joint cavity and eventually cartilage degradation [3], which cause pain in daily activities. Therefore, our primary aim in this study is finding a way to decrease the inflammatory effect of OA in joint, consequently, it reduces swelling and pain in joints, and it prevent the demolition of articular cartilage. Series of biochemical events such as overproduction of pro-inflammatory cytokine IL1β , tumor necrosis alpha TNFα cause inflammation in the joint [3]. Many studies have been illustrating that some Interleukin play important role in OA, for example, Over-expression of IL-1β in cartilage cells of the proximal cause OA [4-7]. Overexpression of TNF-α, such as IL-1β, also have been found in OA [8]. In addition, TNF-α has been found as obstacle of the synthesis of proteoglycan [9]. IL-6 is an interleukin that acts as both a pro-inflammatory cytokine and an anti-inflammatory myokine. IL-6 activity also found in synovial of OA patients [10]. TNF-α is considered as the main inflammation mediators and it’s

because of involving in pro-inflammatory cytokines during the development of OA. TNF-α also stimulates the production of some of inflammatory mediators in the osteoarthritis disease. For instance, it causes increasing in genes expression of iNOS and COX-2, and consequently, increase of amount of NO and PGE2. Currently, Steroidal and nonsteroidal anti-inflammatory drugs (NSAIDs) suggested to patient for treatment, but these drugs have wide range of side effects. NSAIDs may cause a nonspecific colitis, liver damage [11], kidney damage [12], and Non-infectious meningitis [13], interfere with bone healing [14] and Gastric and intestinal disorders [15]. In this study for comparing the effect of Nigella Sativa with NSAIDs Ibuprofen has been used. Herbal medicine has no side effect, therefore medical communities tend to use herbal medicine instead of chemical which has many side effects. Nigella sativa, often called black cumin, is an annual flowering plant in the family Ranunculaceae, native to south and southwest Asia. Many active compounds have been isolated, identified and reported so far in different varieties of black seeds. The most important active compounds are thymoquinone (30%-48%), thymohydroquinone, dithymoquinone, p-cymene (7%-15%), carvacrol (6%-12%), 4-terpineol (2%-7%), t-anethol (1%-4%), sesquiterpene longifolene (1%-8%) α-pinene and thymol. Black seeds also contain some other compounds in trace amounts. Seeds contain two different types of alkaloids; isoquinoline alkaloids, nigellicimine and nigellicimine-N-oxide, and pyrazol alkaloids or indazole ring bearing alkaloids which include nigellidine and nigellicine. Moreover, sativa seeds also contain alpha-hederin, a water soluble pentacyclic triterpene and saponin, a potential anticancer agent [16]. The extensive researches using modern scientific techniques were carried out by various researchers on N. sativa. A number of pharmacological actions of N. sativa have been investigated in the past few decades, for instance, Antibacterial activity [7], Antifungal activity [8], Anti-schistosomiasis activity [9], Antioxidant activity[3], Antidiabetic activity [2], Anticancer activity[6], Immunomodulatory activity [4], Cardiovascular activity [5], Gastro-protective activity [6], Hepato-protective activity [8], Nephroprotective activity [7], Pulmonary-protective activity and anti-asthmatic effects [9], Testicular-protective activity [10], Neuro-pharmacolgical activities [10], Anticonvulsant activity [11], Contraceptive

and anti-fertility activity [12], Antioxytocic activity [13] and Anti-inflammatory and analgesic activity [14-17]. Besides, as it known inflammation paly important role in OA, therefore its good choice to use black seed for inflammation. The aim of this experiment is survey effect of ethanol extract of black seed in decreased expression Citrullus colocynthis inflammatory cytokine TNF-α and iNOS and IL1β and COX-2 and PGE2 , NO at the molecular level on chondrocytes and monocytes macrophages.

## MATERIALS AND METHODS

### *Nigella sativa* L extract preparation

*Nigella sativa* L was initially obtained from Iran center of genetic resources. The seeds were screened manually to remove bad ones. The seeds were washed to remove sand and other debris and air-dried. In the first step the seeds (100 g each time) were extracted in a soxhlet extractor with distilled water (for 4 h) and then the solvent was evaporated in vacuum with a rotatory evaporator, which yielded a black brown concentrate (28.1 g from 100 g of seeds). On average, this extract contained 11.5% water. It was kept at 4 °C before administration, for less than three days.

### BFL and THP-1 cell culture

Synovial fluid was punctured from the radiocarpal joint cartilage of an 8-month-old Holstein cow and washed out three times by 1 molar PBS buffer (PH = 7.2). Then, it was incubated in collagenase type II at 37 $^{o}$C for 16 h. After incubation, it was filtered through 1mm Wire Strainer Screen, which was sterilized , and the waste resulted from the effect of the collagenase type II were isolated from BFL cells. The tube was centrifuged for 3 min, the supernatant was discarded and the pellet cells were washed four times with HBSS. The supernatant was removed with a pipette and finally deposited cells were incubated in the medium containing DMEM-F12 supplemented with FBS, 50 μg/ml ascorbic acid, 100 u/ml penicillin and 0.25 μg/ml streptomycin, with a density of 5 × 105 cell in the 22.2 cm plates at a temperature of 37 $^{o}$C, the humidity of 90% and 5% $CO_2$ to reach cell density of 80-85%. [17]. THP-1 cells were obtained from the Pasteur Institute of Iran, and were amplified in a sterile medium to the extent necessary. The next steps were accomplished completely identical and separately in the two groups of BFL cells and THP-1 cells. Following pretreatment, cells were activated with LPS (20 ng/ml) for 1 h for gene expression analysis using reverse-transcriptase PCR (RT-PCR) and 24

h to measure secreted $PGE_2$ and nitrite levels using immunoassay [19].

**Determination the toxicity**

Toxicity of AENS excelsior assessed by MTT Assay, trypan blue assay and LC50 were determined.

**MTT assay**

MTT assay was conducted to determine the cytotoxicity of the test compounds. Briefly, BFLs and THP-1 cells ($3 \times 10^4$ cells/well) were seeded on a 96-well plate and pretreated with AENS and the component extracts (0.001–100 $\mu$g/mL) for 24 h; after that, 200 $\mu$L of MTT (200 $\mu$g/mL) was added to each well and incubated for 1 h. To dissolve formazan, 100 $\mu$L of DMSO solution was added to each well and measured using a spectrophotometer (SpectraMAX M5, Molecular Devices, CA) at 595 nm. [20].

**LC50 determination for AENS:**

LC50 is defined as amount of compound to induce death in 50% of cell population. $5x10^5$ cells incubated in 12 wells with 1 ml of DMEMF-12 media enriched 10% FBS, 50 µg/ml ascorbic acid, 100 unites 50 µg/ml, 100 µg streptomycin and 0.25 µg/ml amphotericin. Plates rotated 2 min then kept in incubator 37 $^oC$, 5% $CO_2$, 90% humidity about 20 min. AENS in 0.01, 0.09, 0.1, 0.9, 1 , 9, 18 , 27, 36, 45, 54, 63, 72, 81, 90 and 100 µg/ml added to 12 wells plates, followed by keeping in incubator 37 $^oC$, 5% $CO_2$, 90% humidity. After 24 h, plates check out for LC50 determination. Accumulation of cell mass is sign of cell lysis by AENS injection to media nearly 50% of cell population died. In order to avoid error, median LC50 estimated 5.62 mcg/ml.

**Cell stimulation and treatment:**

**Expression analysis**

RNA was isolated and RNA concentration was determined. In the following, isolated RNA was employed to produce cDNA using RT-PCR method, PCR was used to amplify cDNA and finally Real Time PCR was used to determine the expression levels of IL-1B, TNF-α, PGE2 and NO genes by specific primers.

**$PGE_2$ high sensitivity immunoassay**

A commercial $PGE_2$ immunoassay (R&D Systems, Minneapolis, MN, USA) was used to quantify secreted $PGE_2$ levels in the cellular supernatant, according to the manufacturer instructions (Invitrogene, Eliza

kit, Carlsbad, California, United States). A $PGE_2$ standard was run in parallel to the supernatant samples. Briefly, 100 µl of each supernatant sample was assayed in triplicates on a 96-well microplate coated with a goat anti-mouse polyclonal antibody. 50 µl of $PGE_2$ high sensitivity conjugate was added to each sample well. Next, 50 µl of $PGE_2$ antibody solution was added to each sample well. The microplate was incubated for 24 h at 8 °C. After the incubation period, the microplate wells were aspirated and washed with $PGE_2$ wash buffer for a total of three washes. After the last wash, 200 µl of pNPP substrate was added to the microplate wells. After incubation for 1 h at 37 °C, 50 µl of stop solution was added to the sample wells. Optical density was measured immediately using the SpectraMAX 340 microplate reader (Molecular Devices) at 405 nm with wavelength correction set between 570 nm and 590 nm [21].

**Nitrite determination assay**

Production of NO was assayed by measuring the levels of the stable NO metabolite, nitrite, using sodium nitrite resuspended in distilled $H_2O$ as the standard. 100 µl of the culture supernatant was allowed to react with an equal volume of Griess reagent (one part 0.1% naphthylethylenediamine and one part 1% sulfanilamide in 5% $H_3PO_4$) in a flat bottom 96-well microplate for 10 min at room temperature in the dark. Nitrite levels were determined at 540 nm using a spectrophotometer (SpectraMAX 340; Molecular Devices; Sunnyvale, CA, USA). Levels of nitrite were normalized to standard values [19].

**Statistical analysis**

Data were presented as mean ± standard deviation. All experiments were performed in triplicate, and their results were analyzed by one-way analysis of variance (ANOVA), software version 20 followed by Dunnett post hoc test using GraphPad Prism version 5 for Windows (GraphPad Software Inc., San Diego, CA, USA). The statistically significantly value was set at $p < 0.05$.

## RESULTS

**The effect of AENS on TNF-α and iL-18 gene expression**

Bovine sinoviocytes cultured for 72 h with control media alone and AENS alone expressed low levels of TNF-α and IL-1β relative to LPS-activated chondrocytes is shown in Table 1. Sinoviocytes activated for 1 h with 100 ng/ml LPS expressed increased levels of TNF-α and il-18. AENS suppressed

TNF-α and il-18expression by approximately 60% in activated chondrocytes when compared to the activated control. TNF- α and il-18 expression were reduced by approximately 40% when compared to activated control, iL-18 and TNF- α expression levels in LPS-stimulated cells and treated with dexamethasone and NSAID respectively were 35% and 45%, which reduced expression by about 80% and 75%, respectively. Due to the fact that the DMSO has an anti-inflammatory effect, in this study was used as a control. As a result, DMSO treatment has no effect on the reduction of expression in stimulated cells as well as in the increasing expression in unstimulated cells.

**Table 1.** The effect of aqueous extract of Nigella sativa (AENS) on proinflamtroy gene expression using semiquantitative RT-PCR . Bovine Fibroblast synoviocytes were incubated with AENS for 72 h then activated with LPS for one h. Normalized gen expression is shown as percent of activated control

| | Cell* | Cell+Vehicle** | Cell+LPS** | Cell+LPS+Dexamethazone** | Cell+LPS+NSAID** | CelLPS+Vehicle** | Cell+LPS+AENS** |
|---|---|---|---|---|---|---|---|
| Bovine TNF-alpha** | 20.25±2.9 | 20.25±2.9 | 100 | 26.34±2.9 | 30.12±2.3 | 100 | 57.16±1.9 |
| Bovimne iL-18** | 21.03±3.8 | 21.03±3.8 | 100 | 24.45±4.9 | 31.24±23 | 100 | 59.55±1.3 |
| Bovine iNOS** | 22.01±1.9 | 22.01±1.9 | 100 | 25.12±3.7 | 31.78±1.9 | 100 | 61.46±4.6 |
| Bovine COX-2** | 20.98±2.9 | 20.98±2.9 | 100 | 25.56±2.6 | 32.26±2.7 | 100 | 63.36±1.7 |

**The effect of AENS on cytokine gene expression in human THP-1 cells**

Human THP-1 cells incubated for 72 h with control media alone and AENS alone expressed low levels of TNF-α and IL-1β relative to LPS-activated cells is shown in Figure 1. Cells activated with 100 ng/ml LPS showed a significant upregulation of TNF-α and IL-1β expression. In activated THP-1 cells pretreated with AENS, TNF-α was reduced by 35% when compared to activated control cells. Pretreatment with ASU suppressed IL-1β expression by approximately 35%. AENS suppressed TNF-α and IL-1β expression to levels similar to nonactivated control levels.

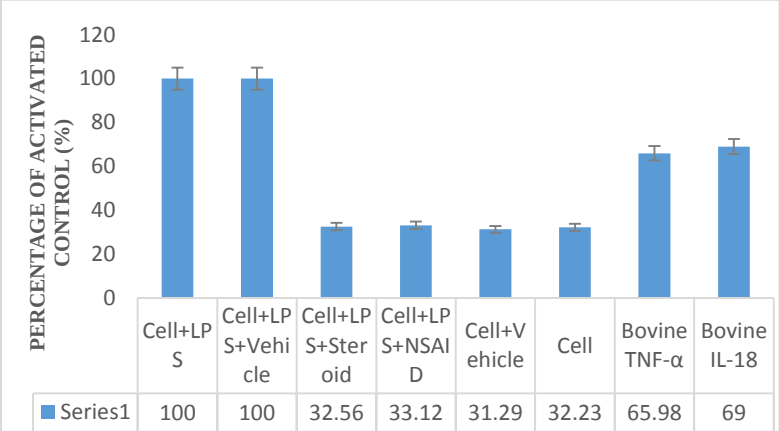


**Figure 1.** The effect of AENS on gene expression

## DISCUSSION

Osteoarthritis (OA) is a degenerative joint disease which mostly is common for old age people. Mechanical factor plays important role in this disease, it is changing both structure and function of joint. OA affected on all of the section in joints, for instance, bone, cartilage, muscle, rabat, capsule. In addition, it is intensified by age, genetics, trauma, obesity, biomechanical stress on the joints. During the growth of cartilage in the presence of osteoarthritis chondrocytes is very complex mechanism. However, researcher believe that the unbalancing between anabolic and metabolic mechanisms (which is hold Homeostasis extracellular matrix (ECM)) cause destruction of joint cartilage and its source of creation of osteoarthritis. For treatment, because of the analgesic and anti-inflammatory effects of nonsteroidal anti-inflammatory drug (NSAIDs), NSAIDs suggest to patient [22]. However, it has been approved that the treatment methods are often ineffective in some patients [23], and there is no cure for OA [14]. In addition, the mechanism of the disease and its progression is unknown [15], consequently the main goal of our study is to reduce symptoms of the disease.

OA treatment options are fall into 4 major categories: non-pharmacological, pharmacologic, complementary and alternative, and surgical. Generally,

treatment should start with non-invasive and safest one before proceeding to more invasive and expensive one [16]. Non-pharmacological therapy often starts with physical exercise. The exercise program consisted of muscle strengthening and range of motion exercises. Pharmacologic treatment start with is acetaminophen. It is inexpensive, safe, and effective [17]. Researchers suggested that acetaminophen is better than placebo for treating mild osteoarthritis, and equal to nonsteroidal anti-inflammatory drugs (NSAIDs), but with fewer gastrointestinal adverse effects. Acupuncture is a good example of complementary and alternative treatment [18], however the effect of it stable only for short time. Our treatment classified in this group of treatment. Surgery should be reserved for patients whose symptoms have not responded to other treatments. The well-accepted indication for surgery is continued pain and disability despite conservative treatment [19].

In the next, Nigella sativa feature will be considering and the reason for selecting this plant. Nigella sativa (N. sativa) is an herbaceous plant which is known as the black seed. It has been used as a natural food additive. Traditionally these seeds are also used for the prevention and cure of many ailments in the middle East and south East Asia [50]. Nigella sativa seeds contain 36%–38% fixed oils, proteins, alkaloids, saponin and 0.4%–2.5% essential oil. Many component has been found in the black seed, but major one is thymoquinone (27.8%–57.0%), ρ -cymene (7.1%–15.5%), carvacrol(5.8%–11.6%), t-anethole (0.25%–2.3%), 4-terpineol (2.0%–6.6%) and longifoline (1.0%–8.0%) . Many studies have been proved that black seed has anti-inflamatory effect [13]. In addition to all of benefit of black seeds, this plant is native of Iran and it's very in-expensive [54]. In addition, in previously studies we have been proved that alcholic extract of *Nigella sative* L. suppress proinflamatory cytokine gene expression in the model like osteoarthritis [15]. Therefore, it's an intelligent choice to test the effect of black seed on inflammation caused by OA.

## CONCLUSION

During the study was conducted on the pathophysiology of OA that increased expression of inflammatory cytokines in this disease, including IL-1β and TNF-α PGE2, NO, iNOS, COX-2, catabolic pathways that degradation joint cartilage and thus inducing apoptosis and activate the immune system. The best way to prevent symptoms is to reduce the synthesis of the cytokine.

Nowdays, there are synthetic drugs with adverse side effects for reducing inflammation and arthritis pain for arthritis patients. For reducing side effects of chemical drugs can be as effective drugs with little side effects from medicinal seeds for the treatment this disease. One of the alternative solution is black seed. Studies were conducted in the past black seeds was observed the effect anti-inflammation and reduction of apoptosis and necrosis. Our research examined on the effect of ethanol extract of black seed on inflammatory cytokine expression in inflamed cells and monocytes with LPS20 both cartilage cells macrophage. Our tests showed that ethanol extract of this seed reduce the amount of IL-1β and it can affect very high levels of expression TNF-α, PGE2, NO, iNOS , COX-2 and it can reduce the expression of cartilage cells and monocytes macrophage. We suggest that the future of this seed as a medicine for reducing the expression of inflammatory cytokines and reduce inflammation and joint pain and swelling caused by the expression of these cytokines used in people with osteoarthritis.

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