Suppression of Proinflammatory cytokine gene expression with Aqueous Extract of Berberine

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Abstract:
Based on knowledge from traditional Iranian herbal medicine, this ex vitro study aims to examine the anti-inflammatory mechanism of Berberis vulgaris L by measuring the expression and release of pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α), cyclooxygenase-1 (COX-1), and interleukine-18 (IL-6), IL-1β and inducible nitric oxide synthase (iNOS) in THP-1. The effects were assessed by measuring the levels of secretory proteins and mRNA of TNF-α and IL-6, the levels of nitric oxide (NO) secretion and the expression of iNOS in THP-1 cells. Cells were treated with 20 ng lipopolysaccharide/ml (LPS) in the presence and absence of concentrations of extracts from the Berberis vulgaris L. During the entire experimental period, we used extract concentrations (0.3 μg/mL) that had no cytotoxic effects, as measured with MTT, MTT and LDH assays. Berberis vulgaris extracts remarkably suppressed the LPS-induced NO release, significantly attenuated the LPS-induced transcription of iNOS and inhibited in a dose-dependent manner the expression and release of TNF-α. Significant effects were observed on the release of IL-6. Taken together, these results suggest that Berberis vulgaris probably exerts anti-inflammatory effects through the suppression of TNF-α and iNOS expressions.

Key words: Berberis vulgaris L, Osteoarthritis, Bovine fibroblast – like Synoviocyte, iNOS, TNF-α, IL-18, IL-1β

1. Introduction:
Osteoarthritis (OA) is a degenerative joint disease characterized by progressive loss of articular cartilage, subchondral bone sclerosis, osteophyte formation, and synovial inflammation. In the USA, an estimated 27 million adults have clinical OA [1]. Data from 2007 to 2009 show that one in five, or 50 million, U.S. adults reported doctor-diagnosed arthritis in that period; one in nine, or 21 million, had arthritis-attributable activity limitations [2]. In 2003 the costs attributable to arthritis and other rheumatic conditions were $128 billion ($81 billion in medical expenditures and $47 billion in earnings losses), which represented nearly 1% of that...
year’s U.S. gross domestic product [3]. The number of U.S. adults with arthritis is projected to rise to 67 million by 2030[4]. The large urban COPCORD study in Iran showed a high prevalence of rheumatic complaints in the population over the age of 15 years, 41.9%. Knee OA and low back pain were the most frequent complaints [5]. Osteoarthritis refers to a clinical syndrome of joint pain accompanied by varying degrees of functional limitation and reduced quality of life [6]. It is the most common form of arthritis. OA is the leading musculoskeletal cause of disability in most Western countries [8] and one of the leading causes of pain and disability worldwide [9]. The most commonly affected peripheral joints are the knees, hips and small hand joints. Although pain, reduced function and effects on a person’s ability to carry out their daily-to-day activities can be important consequences of osteoarthritis, pain in itself is of course a complex biopsychosocial issue, related in part to person expectations and self-efficacy, and associated with changes in mood, sleep and coping abilities [10]. The inflammatory component of OA which is marked by symptoms such as joint pain, swelling and stiffness, is now well recognized with a variety of pro-inflammatory mediators being identified in the OA joint, including the interleukins (ILs) and tumor necrosis factor alpha (TNF-alpha). Infiltration of B cells in the synovium and activation of T cells have also been reported [9,10]. Increased levels of inflammatory cytokines have been noted in both early and late OA, and are produced by the synovium, from activated chondrocytes and osteoblasts [11,12]. Inflammation within the OA joint is at least in part the result of a cyclical interaction between damaged cartilage and inflamed synovium. Products of cartilage breakdown are phagocytosed by synovial cells, resulting in an inflamed synovium, which then produces pro-inflammatory mediators. This results in further release of proteolytic enzymes that result in breakdown of cartilage [12].

IL-1 and TNF-alpha appear to drive the increased levels of catabolic enzymes, prostaglandins and nitric oxide in the OA joint [13] with recent work suggesting this is due to the activation of synovial macrophages [14]. Current treatment options used to manage OA are not curative and fail to reverse the degenerative process of OA. Among the commonly used pharmacologic agents are nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and hyaluronan preparations [15-18]. NSAIDs, in particular, are widely used but their prolonged consumption is associated with serious adverse side effects such as gastrointestinal ulcerations [17]. The need for effective treatment modalities with fewer side effects has prompted OA patients to consider complementary approaches to control pain as well as to improve function and quality of life. The understanding of osteoarthritis and its manifestations has expanded in recent years; so have the therapeutic and treatment options to manage the disease [18]. The major goals of treatment are pain control with minimal adverse effects, maintenance or improvement of joint mobility and function, and improved health related quality of life [19]. Treatment should be tailored to each individual. Because no single therapy is adequate, the major clinical guidelines for disease management generally agree that therapy should involve a combination of non-pharmacologic and pharmacologic therapies [20]. Plant extract have been used as a source of medicines for a wide variety of human disorders. Herbal and natural products have recently received increased attention because of their biological and pharmacological activities [21].

Berberis vulgaris grows in the wild in much of Europe and West Asia. It produces large crops of edible berries, rich in vitamin C, but with a sharp acid flavor. In Europe for many centuries the berries were used for culinary purposes in ways comparable to how citrus peel might be used. Today in Europe they are very infrequently used. The country in which they are used the most frequently today is Iran where they are referred to as “Zereshk in Persian. The berries are common in Persian cuisine such as in rice pilafs (known as “Zereshk Polo”) and as a flavoring for poultry meat. Due to their inherent sour flavor, they are sometimes cooked with sugar before being added to Persian rice [22]. Berberine is an alkaloid plant with a long history of medicinal use in both Ayurvedic and Chinese medicine. It is present in Hydrastis Canadensis (goldenseal), Coptis chinensis (Coptis or goldenthread), Berberis aquifolium (Oregon grape), Berberis vulgaris (barberry), and Berberis aristata (tree turmeric). The berberine alkaloid can be found in the roots, rhizomes, and stem bark of the plants. Berberine extracts and decoctions have demonstrated significant antimicrobial activity against a variety microorganisms including bacteria, viruses, fungi, protozoans, helminths, and chlamydia. Currently, the predominant clinical uses of berberine include bacterial diarrhea, intestinal parasite infections, and ocular trachoma infections [23,24].
The specific use of Rasaut is for curing eye diseases and indolent ulcers earned a great fame. In the British Pharmacopoeia, the alkaloid berberine- the active principle in several species of Berberis and Mahonia, has been incorporated for its successful use in the treatment of oriental sore. The roots of Berberis species are employed as an anti-periodic, diaphoretic and antipyretic, and its action was believed to be as powerful as quinine [24]. Berberine (BBR) is a well-known drug used in traditional medicine and has been shown to possess anti-inflammatory properties. Whether it can affect the production of inflammatory cytokines by RPE cells is not yet clear and was therefore the subject of our study. Berberine (BBR), an alkaloid derived from Chinese goldthread (bark of cork tree), has long been used in the treatment of diarrhea and other gastrointestinal disorders [25-29]. Recent studies have shown that it has antitumor, antimicrobial, and even antibiotic effects [35]. A recent study has suggested that BBR may be effective in the treatment of experimental type I diabetes [36], and experimental autoimmune encephalomyelitis (EAE), raising the possibility of its use in the treatment of autoimmune or autoinflammatory diseases. In view of the inhibitory effect of BBR on inflammation, we examined whether it could also affect cytokine production by Bovine Fibroblast-Like Dynoivioocyte as well as THP-1 cells. The purpose of this study was to determine whether berberine acts upon multiple cell types involved in joint inflammation, other than the cell types previously studied. In particular, we investigated whether the mode of action of roots of berberin involves monocyte/macrophages, in addition to chondrocytes and fibroblast-like synoviocytes found in the joint. Macrophages are bone marrow-derived cells of the monocyte system found in abundance in the synovial lining during inflammation [30]. Previous studies have reported that synovial membrane cells, such as macrophages, are important regulators of cytokine synthesis, inflammation, and cartilage degradation in osteoarthritic joints [40,41]. In the present study, we used a monocyte/macrophage-like cell model to evaluate the effects of berberin on biomarkers that are important in the inflammatory process. We evaluated the effect of berberin on IL-1b, TNF-α, COX-2, and iNOS gene expression as well as PGE2 and NO production in cultured chondrocytes and monocytes. By elucidating the cellular targets of berberin, we can further clarify how this extract provides symptomatic relief in patients suffering from inflammatory disorders such as OA. Berberine was able to inhibit cell proliferation at G0/G1 phase (thought to be from reducing mitochondrial membrane potential) [42]. In chondrocytes, Berberine was able to attenuate MMP concentrations (seen as being involved in osteoarthritic) [43] and increase TIMP-1 at 25-100uM, which worked against IL-1b actions and exerted an anti-osteoarthritic effect; 50uM showed almost normalization of these levels to control values [44]. An acute injection of 50-100uM Berberine into a rat knee three hours prior to inflammatory insult was able to abolish the effects of IL-1b at 100uM Berberine [44]. In a rat model of adjuvant-induced Arthritis, 10mg/kg Berberine (injected) daily 9 days was able to attenuate the paw edema (marker of disease progression) in mice while a shorter supplement time frame of 3 days exerted nonsignificant benefit and irregular injections actually exacerbated paw edema [45].

Oral ingestion of Berberine for 4 weeks can dose-dependently reduce the serum rise of 8-isoprostane in response to the pro-inflammatory LPS, with oral ingestion of 30mg/kg effectively abolishing the LPS-induced rise in 8-isoprostane. The LPS-induced increase of TNF-α (53-86%), IFN-γ (74-88%), and IL-1α (68-93%) was also attenuated with 10-30mg/kg ingestion of Berberine. An attenuation of these proinflammatory biomarkers has also been noted in response to dextran sulfate sodium (research chemical to induce colitis) [46], and in response to TNBS-induced colitis, where the degree of cytokine reductions following 20mg/kg reached 100% (TNF-α), 78% (IL-1β), and 98% (IL-6) while the levels of IL-10 (reduced to 11% in colitis control) had the reductions attenuated to 53% [47].

In vitro studies utilizing human cell lines demonstrated that berberine inhibited activator protein 1 (AP-1), a key transcription factor in inflammation and carcinogenesis [48]. Another study, utilizing human peripheral lymphocytes, showed berberine to exert a significant inhibitory effect on lymphocyte transformation, concluding that its anti-inflammatory action may be due to inhibition of DNA synthesis in activated lymphocytes [49]. A third study concluded that during platelet activation in response to tissue injury, berberine had a direct effect on several aspects of the inflammatory process. It exhibited dose-dependent inhibition of arachidonic acid release from cell membrane phospholipids, inhibition of thromboxane A2 from platelets, and inhibition of thrombus formation [50].
2. Methods:

2.1. Preparation of Plant Extracts. Wild samples of barberry roots were collected from the Birjand (32°53′N and 59°13′E) which is the east Iranian provincial capital of South Khorasan and the centre of the county Birjand resp. Quhestan, known for its saffron, barberry. A specimen of plant was submitted at herbarium of faculty of science of urmia university (Iran), and was identified by the botany department. Roots after washing with cold water were dried in the shade then were powdered by using mechanical grinding. The aqueous extract was prepared by cold maceration of 150 g of powdered root barks in 500 mL of distilled water for 72 h. Then the extract was filtered through a whatman No.1 filter paper to obtain a clear extract. The filtrate was concentrated by water bath (65 °C) for 48 h, dried in vacuum (yield 10 g) and the residue was stored in a refrigerator at 2-8 °C. Powder (1mg) was dissolved in dimethyl sulfoxide (DMSO) and stored as aliquots (20 mM) at -20°C until used. Vehicle (distilled water containing 10% DMSO) was used as a negative, dexamethazone and ibuprofen control were used as a positive control.

2.2. CELL CULTURE. Isolation and Cultivation of Synovial Cell: Synovial cells were isolated from either biopsy of synovial membrane or samples of radiocarpal joints fluid from 8 months old Holstein Friesians cattle from slaughter house. Tissue biopsies were minced into pieces with a length of 3mm along their longest axis. The amount of joint fluid that had been taken, ranging from 0.5 to 50 mL, was centrifuged at RCF 500×g at 20° C, and the sediment was transferred to a T-25 tissue culture flask control media containing: Dulbecco’s Modified Eagle Medium: Nutrient Mix F-12 (GIBCO®, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (GIBCO®, Grand Island, NY, USA), 50 mg/ml ascorbic acid (Sigma-Aldrich,UK), 50 mg/ml Gentamycin (Daropakhsh, Tehran, IRAN), Penicillin 100 U/ml, Streptomycin 100 µ/ml ( B.IO IDEA, Tehran, IRAN), Amphotericin B 0.25 µg/ml (Cipla, Mumbai, INDIA). The flasks were placed in a humidified atmosphere with 5% carbon dioxide at 37°C, and the medium was entirely replaced with fresh medium with intervals of seven days. The adherent cells were harvested after detachment with Trypsin EDTA 0.5% when the culture had reached 75% confluence, frozen in FBS with 10% dimethyl sulfoxide at a cooling rate of 1°C/min, and stored in liquid nitrogen. For the experiments, cryopreserved cell isolates which had been passed 1 or 2 times before they were frozen were selected. These cryo-preserved cell isolates were rapidly thawed and gently mixed with the previously mentioned medium, grown to 75% confluence, detached, split, replated, and again grown until a minimum of 150 cm2 flask bottom area was covered with a 90% confluent layer of cells with good fibroblast morphology, corresponding to minimum 1.5×107 cells. This propagation required four to six passages[52]. Human THP-1 monocyte/macrophage-like cells (Pasteur Institute of Iran) defrost in laboratory (were propagated in control media containing: RPMI-1640 medium (GIBCO®, Grand Island, NY, USA) supplemented with 2 mM L-glutamine (Sigma-Aldrich,St. UK), 10% FBS (Sigma-Aldrich,UK), 1.5 g/l sodium bicarbonate(Sigma-Aldrich, UK), 4.5 g/l glucose (Sigma-Aldrich, UK), 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, Sigma-Aldrich,UK), 1.0mM sodium pyruvate (Sigma-Aldrich,UK) and 0.05 mM β-mercaptoethanol (Sigma-Aldrich,UK), and 50 mg/ml Gentamycin (Daropakhsh, Tehran, IRAN), Penicillin 100 U/ml, Streptomycin 100 µ/ml ( B.IO IDEA, Tehran, IRAN), Amphotericin B 0.25 µg/ml (Cipla, Mumbai, INDIA) Cells were pelleted via centrifugation and assessed for viability using the Trypan-blue exclusion method[53].

2.3. LDH assay Lactate dehydrogenase (LDH) is an oxidoreductase (EC 1.1.1.27) present in a wide variety of organisms. It catalyses the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD+. In the lactate dehydrogenase (LDH) assay, the leakage of the cytoplasm-located enzyme LDH into the extracellular medium is measured. The presence of the exclusively cytosolic enzyme, LDH, in the cell culture medium was indicative of cell membrane damage[54]. For the LDH assay, 5 × 105 THP-1 and bovine fibroblast-like synoviocyte were seeded per well in 96-well microtiter plates. Twenty-four hours after cell seeding, cells were exposed to varying concentrations Berberis vulgaris extract (BVE) (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg/ml). After 24 hours of treatment, the supernatants were collected from each well. Cell monolayers were then treated with a cell lysis solution for 30 minutes at room temperature. The cells and the lysate were collected. LDH activity was measured in both the supernatants and the cell lysate.

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fractions using LDH activity assay kit (Catalog Number MAK066), a non-radioactive cytotoxicity assay kit (Sigma, Aldrich, UK.), in accordance with the manufacturer’s instruction. The absorbance was determined at 490 nm using 96-well plate ELISA reader (elaizareader,Sco DIAGNOSTIC).

When disease or injury or toxic material damages tissues, cells release LDH into the bloodstream. Since LDH is a fairly stable enzyme, LDH has been widely used to evaluate the presence of damage and toxicity of tissue and cells. Quantification of LDH has broad range of applications. In this colorimetric LDH quantification assay, LDH reduces NAD to NADH, which then interacts with a specific probe to produce a color ($\lambda_{max} = 450$ nm). In the lactate dehydrogenase (LDH) assay, the leakage of the cytoplasm-located enzyme LDH into the extracellular medium is measured. The presence of the exclusively cytosolic enzyme, LDH, in the cell culture medium is indicative of cell membrane damage [55, 56], with some modifications. Briefly, $5 \times 10^3$ THP-1 was seeded per well in 96-well microtiter plates. Twenty-four hours after cell seeding, cells were exposed to varying concentrations of alcoholic extract BVE, after 24 hours of treatment; the supernatants were collected from each well. Cell monolayers were then treated with a cell lysis solution for 30 minutes at room temperature. The cells and the lysate were collected. LDH activity was measured in both the supernatants and the cell lysate fractions using Lactate Dehydrogenase Activity Assay Kit Sigma-Aldrich,UK), in accordance with the manufacturer’s instruction. The absorbance was determined at 490 nm using 96-well plate ELISA reader. The percent of LDH released from the cells was determined using the formula: LDH release $= \frac{\text{absorbance of the supernatant)}(\text{absorbance of the supernatant and cell lysate)} \times 100$

2.4. SDH assay. SDH activity assay Succinate dehydrogenase (SDH; EC 1.3.5.1) is a mitochondrial enzyme that catalyzes the oxidation of succinate to fumarate and carries electrons from FAD to Co Q in eukaryotes and bacteria. It has a central function in the maintenance of cellular energy metabolism via the tricarboxylic acid cycle and the electron transport chain [57, 58, 59]. SDH activity was measured in both the supernatants and the cell lysate fractions using Lactate Dehydrogenase Activity Assay Kit (Catalog Number MAK197), a non-radioactive cytotoxicity assay kit (Sigma, Aldrich, UK), in accordance with the manufacturer’s instruction. The absorbance was determined at 490 nm using 96-well plate ELISA reader (elaizareader,Sco DIAGNOSTIC).

For the SDH assay, $5 \times 10^3$ THP-1 and bovine fibroblast-like synoviocyte were seeded per well in 96-well microtiter plates. Twenty-four hours after cell seeding, cells were exposed to varying concentrations of BVE (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 $\mu$g/ml). After 24 hours of treatment, the supernatants were collected from each well. Cell monolayer was with homogenized in 100 mL of ice cold SDH assay buffer, Keep on ice for 10 minutes. Centrifuge the samples at 10,000 $\times$ g for 5 minutes to remove insoluble material. Transfer supernatant to a fresh tube. Add 5–50 mL of the samples into duplicate wells. Bring samples to a final volume of 50 mL using SDH Assay Buffer. Add 50 mL of the appropriate Reaction Mix to each of the wells. Incubate the plate at 25 °C. Take the initial measurement. Measure the absorbance at 600 nm ([A600 initial] at the initial time (T initial). Continue to incubate the plate at 25 °C taking measurements (A600) every 5 minutes for 10–30 minutes.

2.5. MTT cell proliferation assay. The metabolic activity can be evaluated by measuring the activity of the mitochondrial enzyme succinate dehydrogenase using the MTT test. This test is widely used in the in vitro evaluation of the toxicity of plant extract. We applied the MTT test to evaluate the safety of extract from D. samaneh Haj-allahyari et al. Suppression of Proinflamatroy cytokine gene expression with Aqueous Extract of Berberin. One mg of BVE was dissolved in 10 ml of DMSO and diluted with cell media to achieve the required final concentration. The optimal experimental concentration of BVE was first determined by incubation bovine fibroblast – like Synoviocyte and THP-1 (5x10^3 cells/well) for 72 hours with control media supplemented with 10% FBS or BVE at concentration of 10, 15, 20, 25, 30,35, 40, 50, 60
nd 70 µg/ml. Cells were activated with lipopolysaccharide (LPS, 20 ng/ml: Sigma-Aldrich), Dexamethasone sodium sulphate (4mg/ml), NSAID (10mg/ml)[21, 26], for 24 hours and cellular supernatant was analyzed for secreted PGE2 and nitrite concentrations. There was no significance effect on PGE2 and nitrite levels at 10 µg/ml. There was a slight suppression of PGE2 and nitrite levels at 15 µg/ml. The optimal level of suppression was found at 7.5 µg/ml.

2.7. Nitrite Determination Assay. THP-1 cells were plated at a density of 2x10^5 per well in a 24-well plate. The cells were pretreated with the indicating concentration of TPAE for 1 hour and then incubated with 20 ng/ml LPS for an additional 1h. The concentration of NO was determined with a Nitrite/Nitrate Assay Kit (Sigma UK, Catalog Number 23479) as a sensitive assay. Centrifuge cell culture media at 1000 x g for 15 minutes to remove insoluble material because Cell culture medium that contains NO3- as a component (such as RPMI-1640) is not a suitable medium to detect [NO3- + NO2-]. Collect the supernatant. Add 10 μL of the Nitrate Reductase solution and 10 μL of the Enzyme Co-factors solution to the sample and standard wells for [NO3- + NO2-] Detection. Mix well using a horizontal shaker or by pipetting and incubate the plate at 25 °C for two hours. Then add 50 μL of Griess Reagent A to all wells. Mix well using a horizontal shaker or by pipetting and incubate the plate at 25 °C for five minutes. Add 50 μL of Griess Reagent B to all wells. Mix well using a horizontal shaker or by pipetting and incubate the plate at 25 °C for ten minutes. Measure the absorbance at 540 nm (A540) in a microplate reader (elaizareader,Sco DIAGNOSTIC).

2.8. PGE2 High Sensitivity Immunoassay. THP-1 cells were plated at a density of 2x10^5 per well in a 24-well plate. The cells were pretreated with the indicating concentration of TPAE for 1 hour and then incubated with 20 ng/ml LPS for an additional 1h. The concentration of PGE2 was determined using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacture’s instruction (invitrogen, human prostaglandin E2, ELISA kit Catalog # KHL1701). Briefly, 100 ml of each supernatant sample was assayed in triplicates on a 96-well microplate coated with a goat anti-mouse polyclonal antibody. Fifty microliters of PGE2 high sensitivity conjugate was added to each sample well. Next, 50 μl of PGE2 antibody solution was added to each sample well. Cover plate with a plate cover and incubate for two hours at room temperature on an orbital shaker. Add 200 μL pNPP solution to each wells. Cover the plate and allow to develop in the dark on an orbital shaker. This assay typically develops in approximately 60 to 90 minutes. Remove the plate cover, and read the plate at a wavelength of between 405 and 420 nm (elaizareader,Sco DIAGNOSTIC).

2.8. Total RNA Isolation. Bovine fibroblast-like synoviocyte cells were plated at a density of 6x10^6 per flask. The cells were pretreated with the indicating concentration of BVE for 1 hour and then incubated with 20 ng/ml LPS for 24 hours, (for RNA isolation we used Cinna Pure RNA Kit Cat. No.: PR891620, IRAN Tehran) then harvested the cells with Trypsin-EDTA 0.5%. Discard supernatant completely by pipetting to remove residual growth medium. Rinse the cell pellet by PBS and repeat the centrifugation step. Remove supernatant completely by pipetting. Dislodged cell pellet by soft hitting then add 400 μl Lysis solution. Disrupt and homogenize cells by vortexing in one minute. For sufficient homogenization, pass lysate through 20- Gauge needle at least for ten times. Incomplete homogenization leads to significantly reduced RNA yields. Add 300 μl Precipitation solution, and invert it for ten times. Transfer the solution to a spin column with collection tube (included) by pipetting. Centrifuge the tube at (12000 x g, 13000RPM) for 1 min. Discard collection tube. Place spin column in new collection tube (included) add 400 μl Wash buffer I to spin column. Centrifuge at 12000g(13000RPM) for 1 min. Discard flow-through. Wash the spin column with 400 μl of Wash buffer II centrifugation at 12000g (13000RPM) for 1 min. Discard flowthrough. Wash the spin column with 400 μl of Wash buffer II by centrifugation at 12000g (13000RPM) for 1 min. Discard flow-through. Place spin column in collection tube. Centrifuge at 12000g (13000RPM) for 2 min, carefully transfer the column to a new 1.5 or 2ml ml tube (not included). Place 100 μl 55°C pre heated RNase free water in the center of the column, close lid and incubate for 3-5 min at 55°C. Thereafter, centrifuge at

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12000g (13000RPM) for 1 min to elute the RNA. Total RNA was quantified with UV spectrophotometry (Spectrophotometer UV-2100 series, Unico, Spain) and evaluated for RNA concentration and integrity.

2.9. Complementary DNA Synthesis. For each sample, 1 µg of total RNA was converted to complementary DNA (cDNA) using The 2-step RT-PCR (Vivantis Selangor Darul Ehsan, Malaysia). RT was carried out at 42°C for 60 min followed by heating at 94°C for 5 min to stop the cDNA synthesis reaction and to destroy any DNase activity.

2.10. Semiquantitative PCR. Semi quantitative RT-PCR was performed using primers specific to bovine COX-2 (forward, GATGACTGCCAACACCCCAT; Reverse: reverse, CAAATGATTCTAGGGCTTCAGAG, BNF-α forward, GGTTGGAAGCTCGTATGCACAT; reverse ATAGTCCAGGTAGTCCGCA Bίνος forward, CGGAGCCACCCAGGGATCTATT; reverse, GGAGAGCT TTCACGCACACAT and bovine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward, ATT CCA CCC ACG GCA AGT T; reverse CGC TCC TGG AAG ATG GTG AT) as the housekeeping gene. Primers specific for human TNF-α (forward, AGATATGGCCACACACTGGG; reverse, AAAACGGGGTTGGGAGGAAA), and human GAPDH (forward, TCCCTGAGGGTTTTGTGC; reverse, GGTTGACTGCAAGAGCCGTAT) as the housekeeping gene were also used. Thermal cycling was performed on the MJ Research Tetrad Thermal Cycler (Bio-Rad, Hercules, CA, USA) using 2 ml cDNA template and reagents from the SuperTaq Plus Kit (Ambion, Austin, TX, USA). Each sample was analyzed in duplicate. Amplified PCR products were visualized under UV illumination on a 1.5% agarose gel containing ethidium bromide (5 mg/ml, Sigma-Aldrich). The band intensity of the PCR products was quantified using Adobe Photoshop C2 software and normalized to the GAPDH housekeeping gene. Three independent experiments (n=3) were performed and the mean ±1 SD are shown in the figures. Reverse transcription-polymerase chain reaction (RT-PCR) was instructions.

2.11. Statistical Analysis. Error limits cited and error bars plotted represent simple standard deviation of the mean. When comparing different samples, results were considered to be statistically different when P<0.05, P<0.001 (student’s t-test, ANOVA test and REST test) for unpaired samples.

3. Results:

![Relative gene expression analysis of proinflammatory cytokine](image-url)
3.1 The effect of EOFE on TNF-α and IL-18 gene expression

Bovine sinoviocytes cultured for 72 h with control media alone and EOFE alone expressed low levels of TNF-α and IL-1β relative to LPS-activated chondrocytes (Table 1, Fig.1). Sinoviocytes activated for 1 h with 100 ng/ml LPS expressed increased levels of TNF-α and IL-18. EOFE suppressed TNF-α and IL-18 expression by approximately 35% in activated chondrocytes when compared to the activated control. TNF-α and IL-18 expression were reduced by approximately 65% when compared to activated control. IL-18 and TNF-α expression levels in LPS-stimulated cells and treated with dexamethasone and NSAID respectively were 21.54% and 21.789.89%, which reduced expression by about 80% and 80% 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.

![Graph showing PGE2 production in THP1 cells](image)

**Fig. 2. The effect of AEB on PGE2 levels in Chondrocytes. Bovine Chondrocytes were incubated with BVE for 72 h. Mean PGE2 levels released into the cellular supernatant are shown as percent of activated control. Statistical significances between activated control and other groups were analyzed using the Student-Newman-Keuls test (mean ± 1 SD, n = 3, *P<0.05, ** P<0.001).**
3.2 The effect of EOFE on COX-2 gene expression and PGE2 production in bovine Sinoviocytes

Bovine sinoviocytes incubated with control media alone and E.O.F.E alone expressed low levels of COX-2 relative to activated sinoviocytes (Table 1, Fig.1). Sinoviocytes also secreted low levels of PGE2 in the cellular supernatant (Fig.2). Sinoviocytes activated with 100 ng/ml LPS expressed high levels of COX-2 and secreted a significant increase in PGE2 in the cellular supernatant. AENS downregulated COX-2 expression by greater than 35% when compared to activated control levels (Table 1, Fig.1). Pretreatment with EOFE in activated sinoviocytes reduced PGE2 levels by 35% when compared to activated control (Fig.2). COX-2 expression and PGE2 production in EOFE-treated activated sinoviocytes was reduced to levels similar to non-activated control levels (Table 1, Fig.1).

![Graph showing the effect of COX-2 on PGE2 production in bovine Sinoviocytes](image)

3.3 The effect of EOFE on iNOS gene expression and nitrite production in bovine Sinoviocytes

Bovine chondrocytes incubated with control media alone and E.O.F.E alone displayed low levels of iNOS expression (Table 1, Fig.1), and nitrite production (Fig.3), compared to activated chondrocytes. Activated chondrocytes expressed high levels of iNOS expression. Nitrite levels increased three-fold in activated chondrocytes compared to nonactivated cells. Activated chondrocytes pretreated with EOFE showed significant downregulation of iNOS expression by greater than 40% (Table 1, Fig.1). Pretreatment with EOFE suppressed nitrite secretion by 30% in activated chondrocytes relative to activated control levels (Fig.3).

![Table showing the effect of EOFE on iNOS expression and nitrite production](image)
3.4 Effect of EOE on cytokine gene expression in human THP-1 cells:

Human THP-1 cells incubated for 72 h with control media alone and E.O.F.E alone expressed low levels of TNF-α and IL-1β relative to LPS-activated cells (TABLE 2, Fig 4). 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 EOE, TNF-α was reduced by 35% when compared to activated control cells. Pretreatment with EOE suppressed IL-1β expression by approximately 35%. EOE suppressed TNF-α and IL-1β expression to levels similar to nonactivated control levels (Table 2, Fig 4).

4. Discussion

B. vulgaris (barberry), a well-known medicinal plant in Iran and also a food, belongs to Berberidaceae family. As a shrub with 1 to 3 meters in height, B. vulgaris grows in many regions of the world, including Iran (especially Khorasan) [62]. Fruit, leaves, and stem have medical usages including hepatoprotection. B. vulgaris fruit extract contains various flavonoids that act as antioxidant [63]. BBR, a well-known drug with potential anti-inflammatory and immunoregulatory properties used in traditional medicine, [64, 65]. It has been shown to markedly down-regulate the expression of IL-6, IL-8, and MCP-1 by ARPE-19 cells [66]. Our study suggests that BBR exerts suppresses the gene expression of potent proinflammatory cytokines, TNF-α and IL-1β, in an LPS-stimulated monocyte/macrophage-like cell model in addition to suppressing gene expression of TNF-α, IL-1β, COX-2 and iNOS in cultured synoviocytes. Berberin has been used to treat trachoma [67, 68, 69], but whether it can also be used for the treatment of osteoarthritis not yet clear and was therefore the rationale for starting this ex-vivo experimental study. Osteoarthritis (OA) is associated with cartilage destruction, subchondral bone remodeling and inflammation of the synovial membrane, although the etiology and pathogenesis underlying this debilitating disease are poorly understood [70]. The joint degradation associated with OA involves inflammation of the surrounding cartilage, synovial membrane, and bone tissues. Various studies have documented the deleterious effects of synovial macrophages in inflamed joints [71]. Secreted inflammatory molecules, such as proinflammatory cytokines, are among the critical mediators of the disturbed processes implicated in OA pathophysiology. Interleukin (IL)-1β and tumor necrosis factor (TNF), in particular, control the degeneration of articular cartilage matrix, which makes them prime targets for therapeutic strategies [70]. Various studies have documented the deleterious effects of synovial macrophages in inflamed joints [71], relationship between macrophage density, duration of disease, and severity of disease in dogs afflicted with osteoarthritic joints [72], and macrophages has a primary source of proinflammatory cytokines that are found at elevated levels in the synovial tissue of
OA patients. These results collectively suggest that macrophages are crucial to the initiation and maintenance of synovial inflammation seen in many OA joints. Catabolic and proinflammatory mediators such as cytokines, nitric oxide, prostaglandin E₂ and neuropeptides are produced by the inflamed synovium and alter the balance of cartilage matrix degradation and repair, leading to excess production of the proteolytic enzymes responsible for cartilage breakdown. Cartilage alteration in turn amplifies synovial inflammation, creating a vicious circle. As synovitis is associated with clinical symptoms and also reflects joint degradation in OA, synovium-targeted therapy could help alleviate the symptoms of the disease and perhaps also prevent structural progression.

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