Alcoholic Extract *S. lavandulifolia Vahl* Suppress *TNF-α, IL-1β, COX-2, iNOS* Gene Expression, and *PGE2* and *NO* Production in Bovine Fibroblast-Like Synoviocyte and THP-1

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Hossein Maghsoudi[a*]
[a] Department of Biotechnology, Payame Noor UnDepartmentehran, 1395-4697, Iran.
[a*]Correspondence author: Dr. Hossein Maghsoudi Department of Biotechnology, Payame Noor University, Tehran, 1395-4697, Iran. Email: drhmaghsoudi@pnu.ac.ir hossein_m2002@yahoo.com. ORCID: 0000-0002-1524-6420

Postal Address: Payamenour University Tehran IRAN1865865399 Building Dr. Chamran, St Mafakhe, Taghiabad, Highwa Varamin, Shahrerey, Payamenour University, Tehran, Iran Zip Code: 1865865399.Tel: +9833416811-20 Extension:150

ABSTRACT

Background: Osteoarthritis (OA)[1], known as an inflammatory disease, affects the quality of life of countless people all around the world. In this ex vivo study, the anti-inflammatory effects of alcoholic extract *S. lavandulifolia Vahl* were investigated.
Purpose: Anti-inflammatory effects S. lavandulifolia *Vahl.* was evaluated based on measuring the levels of mRNA expression of pro-inflammatory genes including *TNF-α*[2], *iL-1β*[3], *COX-2*,[4] and *iNOS*[5] in bovine fibroblast-like synoviocytes[6] and the levels of *NO*[7] and PGE_2[8] in Human THP-1 cells.
Experimental Approach: Cells were treated with 100 ng/ml LPS in both the absence and presence of Alcoholic Extract *S. lavandulifolia Vahl* (AESL)[9] (7.5µg/ml) that had no cytotoxic effects, as measured with MTT and trypan blue assays.
Results: Results showed that *S. lavandulifolia Vahl.* downregulated *TNF-α* (41.24%), *iL-1β* (47.12%), *COX-2* (44.92%), and *iNOS* (45.34%) expression in BFLS, downregulated *TNF-α* (56.8%), *iL-6* (58.04%) and *iL-18* (52.59%) in THP-1 activated cells and this suppression was paralleled by a significant reduction in *PGE_2* (52.12%) and *NO* (51.06%) in cellular supernatant of THP-1.

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۱ . Osteoarthritis (OA)
۲ . tumor necrosis factor-alpha (TNFα)
۳. interleukin 1 beta (iL-1β)
۴. cyclooxygenase-2 (COX-2)
۵. inducible nitric oxide synthase (iNOS)
۶ . bovine fibroblast-like synoviocytes (BFLS)
۷. nitric oxide (NO)
۸ . prostaglandin E2 (PGE2)
۹ . Alcoholic Extract *S. lavandulifolia* (ASEL)
10. Matrix Metalloproteinase (MMPs)
11. Dimethyl sulfoxide (DMSO)

Conclusion: For the first time, our results suggested that *S. lavandulifolia Vahl* exerts has anti-inflammatory effects through the suppression of *TNF-α, IL-1β, COX-2*, and *iNOS* in bovine fibroblast synoviocytes along with the reduction in *NO* and PGE_2 production in THP-1 cells.

Keywords: *IL-1β, TNF-α, iNOS, Stachys lavandulifolia*, THP-1

1. Introduction

Osteoarthritis is a very common musculoskeletal rheumatic disorder and its prevalence in 2017 affected 303 million (James et al., 2017) and at least 50% of the elderly with osteoarthritis of the knee and pelvis (Sharma et al., 2006). Osteoarthritis can affect any joint, but preferably affects the joints of the knee, arms, pelvis, and spine, causing pain and disability. Its economic costs to patients and the community are also significant, and in 2016 it was described as a serious disease by the Osteoarthritis Research Society International (OARSI) (March et al., 2016). The main symptom of the disease is pain, which is more common than joint stiffness and disability (Trouvin et al., 2018). Pain can be caused by an inflammatory condition caused by pro-inflammatory cytokines, which leads to a neuropathic pain condition with the gradual onset of nerve damage, in turn (Orita et al., 2011). Inflammation of the synovial membrane, followed by the production of several cytokines, such as alpha tumor necrosis factor, interleukins, and nerve growth factor, play a major role in its pathogenesis. TNF-α activates sensory neurons directly through its receptors and initiates a series of inflammatory reactions through the production of interleukins (Ohtori et al., 2004: Pollock et al., 2002). The degenerative changes in osteoarthritic joints are triggered by a series of biochemical events including excess production of the proinflammatory cytokines interleukin-1- beta (IL-1b) and tumor necrosis factor-alpha (TNF-a). Overproduction of these cytokines stimulates cartilage matrix degradation by inhibiting the production of proteoglycans and type II collagen while upregulating the production of matrix-degrading enzymes such as matrix metalloproteinase (MMPs). These cytokines also upregulate the expression of COX-2 and iNOS, two potent proinflammatory enzymes that lead to increased synthesis of PGE2, and NO. Because cartilage degeneration is directly linked to elevated levels of iL-1b, TNF-a, COX-2, and iNOS expression, the use of compounds that inhibit the expression of these proinflammatory mediators is a promising treatment strategy for managing the catabolic symptoms of OA (Au et al., 2007). The morphological changes observed in OA include cartilage erosion as well as varying degrees of joint inflammation. These changes are related to a complex network of biochemical factors, including proteolytic enzymes, which lead to the breakdown of cartilage macromolecules. The exact mechanisms involved in the pathogenesis of OA are not yet well understood. OA pathogenicity appears to be the result of complex interactions between mechanical, cellular, and inflammatory factors (Chen et al., 2020). in response to environmental stimuli, macrophages (resident and inflammatory macrophages) can be classified based on activation: classical active macrophages (proinflammatoryM1) and alternative activated macrophages (anti-inflammatory M2). M1 macrophages are pro-inflammatory and have a central role in host defense against infection, while M2 macrophages (alternatively activated macrophages) are associated with responses to anti-inflammatory reactions and tissue remodeling, and they represent two terminals of the full spectrum of macrophage activation. Transformation of different phenotypes of macrophages regulates the initiation, development, and cessation of inflammatory diseases (Liu et al., 2020). M1 macrophages are stimulated by interferon-γ, lipopolysaccharide, granulocyte colony-stimulating factor, or TLR, to produce high levels of pro-inflammatory cytokines such as iL-1β. (Tan et al., 2016). M2 macrophages have anti-inflammatory action and help to repair tissue and eliminate inflammation. M2 and M1 macrophages can also regulate wound healing. M2 macrophages by iL-4 and iL-13 to produce inflammatory anti-inflammatory cytokines such as iL-

10, iL-1α, TGF-β and Rrg-1, activate the Th2 immune response and anti-inflammatory functions (Biswas and, Mantovani, 2010: Gu et al., 2017). Under normal conditions, most macrophages show the M2 phenotype to maintain tissue homeostasis (Dey et al., 2015). In inflammation, macrophages are activated and polarized to an M1 phenotype. These M1 macrophages have three main functions, delivery of antigens, phagocytosis and modulation of the immune system through the production of pro-inflammatory nitric oxide and cytokines, which can lead to tissue damage (Ma et al., 2019: Fujiwara and Kobayashi, 2005). During inflammation, macrophages become predominantly a polar M1 phenotype and can repair damaged tissues and restore tissue homeostasis by producing anti-inflammatory cytokines and cytokine antagonists (Nakkala et al., 2020). In the synovium of patients with OA, abundant pro-inflammatory cytokines have been found that are responsible for inflammation and cartilage destruction, and the accumulation of macrophages in the synovial mucosa can be identified as characteristic of synovitis (Shapouri-Moghaddam et al., 2018). Both inflammatory and destructive responses are to be highly dependent on macrophages, which play an important role in the pathogenesis of OA by inducing inflammatory mediators, growth factors, and proteinases (Takano et al., 2016: Bondenson et al., 2010). The balance between macrophages M1 and M2 may be disturbed in OA, and the degree of imbalance will be associated with severe OA levels. Decreased activity of synovial macrophages from subtypes M1 to M2 may contribute to the onset and progression of OA (Xue et al., 2019: Barboza et al., 2017). In the synovium of OA patients, M1 cytokines, including iL-12, iL-1β, and TNF-α, increase, while cytokine M2 decreases iL-1α. In OA, macrophage activation can be caused by cartilage damage through the secretion of Matrix Methaloproteinase-1, Methaloproteinase-3 and Methaloproteinase-9, cytokines, and growth factors. Possible mediators, including damage-associated molecular patterns (DAMPs), leak into damaged joint cartilage and activate synovial macrophages. Activation of synovial macrophages leads to the release of proinflammatory cytokines as well as catabolic and anabolic agents that can cause osteophyte formation (Liu-Bryan, 2013: Bloom et al., 2007).

Until now, therapies for OA commonly focused on the palliation of pain and discomfort, improvement of functional movement, and prevention of further degeneration. Thus, the primary approach to the clinical treatment of OA involves the extensive use of NSAIDs, analgesics, and hyaluronan which allows the brief symptomatic relief but provides no apparent disease-modifying effect (Abramoff and Caldera, 2020). Concerning pharmacologic treatment, NSAIDs are the most common prescription for pain relief in OA (Cho et al., 2015). Unfortunately, some patients cannot use NSAIDs due to their adverse effects on the gastrointestinal system, including dyspepsia, ulceration, upper Gl bleeding, and perforation of the stomach or duodenum (Turajane et al., 2009). Since respects plants have very few side effects, the use of medicinal plants is rising to overwhelm the world (Salmerón-Manzano et al., 2020). At current treatments, medicinal plants especially *S. lavandulifolia* in Iran are abundantly grown and traditionally used as a seasoning and spices for food. *S. lavandulifolia* L. is an aromatic plant that belongs to the Labiatae family (Modarresi et al., 2020). In Iran, *S. lavandulifolia* is widely found in Azerbaijan, Golestan, Khorasan, Mazandaran, and Tehran provinces (Morteza-Semnani et al., 2006). *S. lavandulifolia Vahl*. is a native plant that is widely distributed in different regions of Iran and known as "Chaye-e-Kohi" or "Lolopashmak" and is known as "Betony" in English (Mahzooni-kachapi et al., 2012). It is used as an herbal tea in gastrointestinal disorders, inflammatory diseases, anxiolytic, cough, sedative, antispasmodic, diuretic, ulcers, fevers, and diarrhea (Tomou et al., 2020). Its aerial parts are used as antipyretic, anti-inflammatory, spasmolytic, and sedative medicament (Bahrami Babaheydari et al., 2015). Pharmacological properties of this plant such as anti-inflammatory, anti-anxiety, antibacterial, antinephritic, anticancer, anti-Helicobacter pylori, antimicrobial, and antioxidant activities have also been reported (Noroozloo et al., 2015). Many studies have shown that *S. lavandulifolia* aerial

parts are a source of compounds such as tyrosinase, Butyrylcholinesterase inhibitors (HGNC symbol BCHE; EC 3.1.1.8), also known as BChE, BuChE, Acetylcholinesterase inhibitors (HGNC symbol ACHE; EC 3.1.1.7) , also known as AChE or acetyl hydrolase, which are used for the treatment of neurodegenerative disorders (Tundis et al., 2015). Researchers showed that *S. lavandulifolia* extracts protected gastric mucosa from the alcohol-induced gastric ulcer (Nabavizadeh et al., 2011). The role of aerial parts of wood betony (*S. lavandulifolia*) in the phytotherapeutic management of polycystic ovary syndrome is also investigated (Jalilian et al., 2013). The strong effect of hydroalcoholic extracts of *S. lavandulifolia* Vahl leaves on Leishmania major is proven (Asadi et al., 2015). Phytochemical studies demonstrated the presence of monoterpenes, sesquiterpenes, phenylethanoid glycosides, and iridoids as main constituents in *S. lavandulifolia* (Pirbalouti et al., 2013). Considering the growing interest in to use of herbal and phytochemical products in the treatment of OA, this study aimed to evaluate the anti-inflammatory effect of *S. lavandulifolia* on Osteoarthritis diseases.

2. Material and method

2.1. Reagents

DMEM-F12 medium, RPMI-1640 medium, L glutamine, FBS, sodium bicarbonate, vimentin antibody, glucose, 4-(2-hydroxyethyl) piperazine-1- ethane sulfonic acid (HEPES), sodium pyruvate, Ascorbic Acid, Trypan Blue, MTT assay kite, Griess reagent, LPS (Escherichia coli serotype O127:B8) and β-mercaptoethanol were purchased from Sigma-Aldrich, UK. PGE2 immunoassay was purchased from Invitrogen USA, Gentamycin was purchased from Daropakhsh, Tehran, Iran. Penicillin and Streptomycin were purchased from BIO IDEA Tehran, Iran. RNA Extraction kite, M-MLV Reverse Transcriptase, and Taq polymerase, all of the primers and PCR reagent were purchased from CinnaGene Iran. Amphotericin B was purchased from Cipla, Mumbai, India. TPP tissue culture flask TPP Techno Plastic Product AG Zollatrasse, was purchased from Switzerland. Penicillin and streptomycin, Trypsin EDTA 0.5%, were purchased from BIOIDEA (BIO IDEA, Tehran, IRAN), Gentamycin from Daropskhsh (Iran), Amphotericin B (Cipla, Mumbai, INDIA), Dimethyl sulfoxide, RNA isolation kit, RT-PCR, (CinaClone, and Tehran, Iran)

2.2. Ultrasonic extraction

In this method, ultrasonic waves with a frequency of 20 to 2000 kHz were used (Bajer et al., 2007). These waves increase the permeability of the cell wall and cause a gap. To extract this method, 10 g of powdered dried plant leaves, 100 ml of methanol, and a few drops of acetic acid were added to the plant sample and then mixed to obtain a nearly homogeneous solution. The container with the glass lid and parafilm tape was completely closed and the solution was placed in the ultrasonic device for 30 minutes. After 30 minutes when the mixture was irradiated with ultrasound, the mixture was smooth and after decontamination with a rotary evaporator and complete drying, a dark extract was obtained which was used to measure the biological properties. The weight of the obtained extract was 2.1 g. Powder (1mg) was dissolved in DMSO and stored as aliquots (20 mM) at -20°C until used. The chemical composition of the plant extract was determined using Gas Chromatograph (GC).

3. Cell culture

3.1. Isolation and cultivation of synovial cells

Articular fluid culture was obtained by an innovative method (Dr. Maghsoudi). Synovial cells were isolated from biopsy of synovial membrane of metacarpal joints fluid from 8 months old local cattle from slaughter house. After aspiration, the joint fluid was spread dropwise to a plate containing the culture medium. Dulbecco's Modified Eagle Medium supplemented with 15% fetal

bovine serum, 50 mg/ml ascorbic acid, and 50 mg/ml, Penicillin 100 U/ml, Streptomycin 100 µ/ml, Amphotericin B 0.25 µg/ml .The plates 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 21 days. Cell passage was performed for up to 3 generations so that the type A cells (macrophage / monocyte) were gradually removed from the culture medium and the type B cells (synoviocytes) were uniformly placed on the plate.

3.2. Characterization of bovine fibroblast like synoviocyte

Bovine fibroblast like synoviocytes were characterized by immune staining with vimentin, a specific cellular marker for mesenchymal cells (Eriksson et al., 2009). Whole cell lysates were prepared from approximately 2×10^5 cells by homogenization in the lysis buffer and subsequent centrifugation at 30678 g for 15 min. The protein concentration in the supernatant was determined using the Bradford method (Bio-Rad, Hercules, CA, USA). Protein samples were separated on 12% SDS-PAGE and then transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). The membranes were incubated with primary antibody 1× SDS, the loading buffer was added to the lysis sample and boiled for 10 min, and the samples were then loaded on 12% SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane and the surface of the membrane was blocked overnight in the blocking buffer (PBS, pH 7.3, 0.5% BSA, and 0.5% T-20). The filter was washed three times for 20 min each in the washing buffer (PBS, pH 7.3, 0.05% BSA, and 0.05% Tween-20). After washing, the filter was incubated with primary mouse antibody specific for the vimentin at 1:200 dilution for 1 h at 37 °C. After incubation, the filter was washed three times for 10 min each in the washing buffer. This was followed by incubation in secondary antibody (goat anti-mouse IgG, alka line phosphates conjugate) (Sigma, USA), and 1:5000 dilution was achieved in the washing buffer for 2 h at room temperature. After incubation, the filter was rinsed twice with alkaline phosphate buffer (Tris-HCl, pH = 9.5 100 mM, NaCl 100 mM, and MgCl2 50 mM) and then incubated in the substrate solution (66 µl of 50 mg/ml of nitro blue tetrazolium [NBT] and 33 µl of 50 mg/ml of bromochloroindiyl phosphate [BCIP] were added to 10 ml of alkaline phosphate buffer), at room temperature with gentle agitation out of the light until a band appeared. To confirm that fibroblast like synoviocyte cultures were not contaminated CD14 expression (Ceciliani et al., 2021) was evaluated by reverse transcription polymerase chain reaction (RT-PCR). Total RNA extraction was performed using a commercial kit (SinaClon kit IRAN). RNA conversion to cDNA was performed using the commercial kit Superscript FirstStrand Synthesis System for RT-PCR (two-step RT-PCR; Vivantis) and real-time PCR were performed with SinaClon. Two microliters of cDNA was amplified by specific oligonucleotide primers for CD 14 (forward: 3′-CTG GAA GCC GGC G-5′; reverse: 5′-AGC TGA GCA GGA ACC TGT GC-3′). Primer sequences were selected to amplify both human and bovine genomes and were from separate exons to exclude a possible genomic DNA contamination of the RNA samples. PCR reactions were performed in 25 ml containing 1 U Taq DNA polymerase (SinaClon, Iran), 25 pmol of each primer, and 200 Mm deoxynucleotide triphosphates (dNTPs) in 1× PCR buffer (10 mM Tris, pH 8.3, 50 mM KCl, and 1.5 mM MgCl2). Cycling parameters were as follows: 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C for CD14. The size of the amplified sequence was 403 bp. mRNA from human macrophages was used as a positive control for CD14 expression. PCR products were analyzed on 1.5% agarose gel and stained with ethidium bromide.

3.3. Human THP-1

Human THP-1 monocyte/macrophage-like cells (Pasteur Institute of Iran) defrost in the laboratory were propagated in control media containing: RPMI-1640 medium , supplemented with 2mM L-glutamine , 10% FBS, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10mM 4-(2-hydroxyethyl) piperazine-1-ethane sulfonic acid (HEPES), 1.0mM sodium pyruvate, 0.05mM β-

mercaptoethanol and 50 mg/ml Gentamycin, Penicillin 100 U/ml, Streptomycin 100 µ/, Amphotericin B 0.25 µg/ml . Cells were pelleted via centrifugation at 2236 g for 5 minutes, supernatant was throughout, pellet was homogenized in RPMI-1640 medium and assessed for viability using the Trypan blue exclusion method. Viable cells were plated in six-well plates at a density of 5 x 105 cells/well and treated the same day (Schildberger et al., 2010).

3.4. Cell viability

3.4.1. MTT Assay

MTT assay and trypan blue were used to investigate the cytotoxic effect of the aqueous extract of AESL. MTT, a yellow tetrazole, is reduced to purple formazan by succinate dehydrogenase (SDH) in the mitochondria of living cells. To dissolve the insoluble purple formazan product into a colored solution, DMSO was added. The absorbance of this colored solution was measured by a spectrophotometer at a wavelength between 500 and 600 nm (Garbison et al., 2004).

3.4.2. Trypan Blue Assay

One of the primary and common methods for measuring cell viability is the trypan block staining method. Trypan blue is an azo dye that is impermeable to the cell membrane and therefore only enters the cell with the damaged membrane. Upon entering the cell, trypan blue binds to intracellular proteins, leaving the cells blue. Trypan blue staining method allows direct identification and counting of living (unstained) and dead (blue) cells in a specific population [33]. In this method, a cell suspension is simply mixed with trypan blue and then visually examined. Is placed to determine if the cells are dying or not. Transfer 100µL (1×10^4 cells/ well) of THP-1 and BFLSc cells were seeded into, two 12- wells plates and treated with the following: vehicles control (DMa SO), AESL at the concentration (0, $1x10^{-3,}$ $5x10^{-1}$, $1x10^{-2}$, $5x10^{-2}$, $1x10^{-1}$, $5x10^{-1}$, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 95 and 100 ng/mL). The cells were treated for 24, 48, and 72 hours' exclusion assay has performed the procedure to assess cell viability. After trypsinization, counting the unstained (viable) and stained (nonviable) cells separately under the microscope in four 1 x 1 mm squares of one chamber of hemcytometer and determine the average number of cells per square. The viability assay was repeated three times (Strober, 2001).

3.5. Treatment procedure

Making 1000 µg solution of AESL was dissolved in 10 mL of DMSO and diluted with DMEM (without supplementation). The optimal experimental concentration of AESL was first determined by incubation of BFLS and THP-1 ($5x10^5$ cells/well) for 72 hours with supplemented DMEM media. AESL was added at increasing concentrations $1x10^{-3,}$ $5x10^{-3}$, $1x10^{-2}$, $5x10^{-2}$, $1x10^{-1}$, $5x10^{-1}$, 5, 10, 15, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 1nd 100 µg/mL. Cells were activated with lipopolysaccharide 100 ng/mL, Dexamethasone sodium sulfate (4mg/mL) (Jeong et al., 2016), NSAID 10 mg/mL (Padumadasa et al., 2016), for 24 hours, and cellular supernatant was analyzed for secreted *PGE2* and nitrite concentrations.

4. Division of study groups

The studied groups were divided into seven groups and Table 1 shows how they are grouped.

5. Reverse transcription-polymerase chain reaction (RT-PCR)

RNA isolation and converted to cDNA was based on methods published in previous articles (Maghsosudi et al., 2018). For lysis of cells from the Trizol reagent and extraction of RNA with chloroform was performed. RNA was converted to cDNA by 2-step RT-PCR. Semi quantitative

PCR was performed for each of the studied cytokines using specific primers (Table 2) and GAPDH gene was used as the housekeeping gene. And PCR product was controlled in 1.5% agarose gel. Real-time PCR was performed using the same primers used for qualitative PCR, and evergreen was used as mastermix. The obtained results of CTs (Threshold Cycle) were evaluated by two standard curve methods and Pfaffi Method. In principle, CTs are numerical numbers and their significance was first determined by ANOVA and in the second stage by Student-Newman Keuls (SNK), REST-2000 were evaluated. Finally, to determine the amount of gene expression, Pfaffi Method was used

6. Measurement of Nitrite (NO_2^-) by in Human THP-1 cell

After 24-hour incubation with ASEL, the synoviocytes culture media were harvested and analyzed with a nitrate/nitrite colorimetric method. Briefly, nitrate was converted to nitrite by nitrate reductase, and Griess reagent (Sigma) was added to convert nitrite to a deep-purple azo compound. The absorbance of the latter was measured at 540 nm using a plate reader (Gurunathan et al., 2019).

7. Measurement of PGE_2 production by ELISA in THP-1 cell

The quantify secreted PGE2 levels in the cellular supernatant by synoviocyte cells is measured by the ELISA assay and commercial PGE2 immunoassay (Invitrogen, USA) according to the manufacturer's instructions (Latorre et al., 2018).

8. Statistical Analysis

Data are presented as means. ANOVA analysis was done by REST software version.20 and Student-Newman-Keuls test. Differences with $P < 0.05$ were considered statistically significant. The null hypothesis states that pro-inflammatory gene expression or production will be different between control and AESL-treated samples.

9. RESULT

9.1. Chemical composition of the S. lavandulifolia Vahl

Totally, about 40 different components have been detected in the extract. The main chemicals were phenol (8.17%), sabinene (71.19), β-pinene (5.526%), myrcene (5.68%), 2- Furancarboxaldehyde (5.28%), 1-Methyl-pyrrolidine-2-carboxylic (5.44), pyran (4.42%) and myristicine (4.11%). Some basophilic chemicals were also observed in the extract such as enoic acid (1.73%), Benzoic acid (1.45%) and acetic acid (1.34%) (Table 3). There are some differences in the kind and percent of chemical composition of S. lavandulifolia Vahl in comparison to other reports (Javindnia et al., reported the main component of the S. lavandulifolia Vahl from Tehran province as germacrene-D (13.2%), β-phellandrene (12.7%), βpinene (10.2%), myrcene (9.4%), α-pinene (8.4%) and Z-β-ocimene (5.8%). Other researcher (Cataldo et al., 2020) stated that α-pinene, Sabinene, Bicyclogermacrene, β-pinene, Myrcene, Myristicine and Thymol are the major chemicals. The difference observed among different scientific reports may be because of the methodology in plant extraction, time of plant collection during the year as well as the differences in the ecological or even genetic properties of the plant in different area. So, it is highly recommended to test the exact chemical composition of the plants before using them

9.2. Trypan Blue Assay

Trypan blue is a ~960 Daltons molecule that is cell membrane-impermeable and therefore only enters cells with compromised membranes. Upon entry into the cell, trypan blue binds to intracellular proteins thereby rendering the cells a bluish color. Due to increasing concentration of

AESL, the viability of BFLSc, as well as THP-1 cells, gradually decreased, at a concentration of 30 µg/mL and 35 µg/mL of AESL in BFLSc and THP-1 cells, the viability was set at 50%, respectively (Fig. 1A, and 1B).

9.3. MTT ASSAY

The MTT assay is done at a wavelength of 570 nm to evaluate the cell viability of BFLSc and The amount of color produced is directly proportional to the number of viable cells. Figure 2 shows the dose- and time-dependent cytotoxicity of the AESL on BFLSc and THP-1 cells over a 6- to 48-h period using the MTT assay. Due to increasing the concentration of AESL gradually reduces absorption so that in the concentration 30 and 350 µg/mL of AESL in BFLSc and THP-1 cells, the viability was set at 50%, respectively (Fig. 2A, and 2B).

9.4. Effect of ASEL on TNF-α, IL-1β, iNOS, and COX-2 cytokine gene expression

Gene expression levels of TNF-α, IL-1β, iNOS, and COX-2 in the first group (supplemented medium), the second group (ASEL), and the third group (DMSO) after 72 hours, did not change significantly and were reported almost similar (Table3 and Figure 1). Therefore, additives to the culture medium did not alter gene expression. Increased levels of gene expression of TNF-α, IL-1β, iNOS and COX-2 in the third group due to the presence of LPS (100 ng/mL) were very significant and increased, and the reason is only the presence of LPS. Treatment of stimulated cells by dexamethasone (fifth group) and ibuprofen (sixth group) was significantly reduced gene expression of TNF-α, IL-1β, iNOS and COX-2 by approximately 80%. On the other hand, DMSO (seventh Group) does not affect reducing the gene expression of stimulated cells. The main issue is the effect of the ASEL (fourth group) on reducing the gene expression of TNF-α, IL-1β, iNOS, COX-2 in cells stimulated by LPS was approximately 50%, and this effect was significant compared to treatment with dexamethasone and ibuprofen (Table4, and Figure 3).

9.5. Effect of ASEL on PGE2 production

The amount of prostaglandin production and its transfer into the culture medium, in the first group (supplemented medium), the second group (ASEL), and the third group (DMSO) after 72 hours, did not change significantly and were reported almost similar (Figure 4). Therefore, additives to the culture medium did not alter gene expression. Increased levels of PGE2 production and secreted in the culture medium (figure 4) due to the presence of LPS (100 ng/mL) were very significant and increased and the reason is only the presence of LPS. Treatment of stimulated cells by dexamethasone (fifth group) and ibuprofen (sixth group) was significantly reduced. DMSO did not change the rate of PGE2 production and transfer to the culture medium (group 7). The important point is the effect of the ASEL (as a treatment) on the stimulated cells (fourth group), which reduces the production and transfer of PGE2 (fourth group) by about 52.12.pg/mL (Figure 4).

9.6. Effect of ASEL on NO production

The amount of NO production and its transfer into the culture medium, in the first group (supplemented medium), the second group (ASEL), and the third group (DMSO) after 72 hours, did not change significantly and were reported almost similar (Figure 4). Therefore, additives to the culture medium did not alter gene expression. Increased levels of NO production and secreted in the culture medium (figure 4) due to the presence of LPS (100 ng/mL) were very significant and increased and the reason is only the presence of LPS. Treatment of stimulated cells by dexamethasone (fifth group) and ibuprofen (sixth group) was significantly reduced. DMSO did not change the rate of NO production and transfer to the culture medium (group 7). The important

point is the effect of the ASEL (as a treatment) on the stimulated cells (fourth group), which reduces the NO production (fourth group) which reduces the production and transfer of NO (fourth group) by about 51.06 nanomol/mL (Figure 5).

9.7. Effect of ASEL on TNF-α, iL-6 and iL-18 gene expression

Gene expression levels of TNF-α, IL-6 and iL-18 in the first group (supplemented medium), the second group (ASEL), and the third group (DMSO) after 72 hours, did not change significantly and were reported almost similar (Table3 and Figure 6). Therefore, additives to the culture medium did not alter gene expression. Increased levels of gene expression of TNF-α, IL-6, and iL-18 in the third group due to the presence of LPS (100 ng/mL) were very significant and increased, and the reason is only the presence of LPS. Treatment of stimulated cells by dexamethasone (fifth group) and ibuprofen (sixth group) significantly reduced gene expression of TNF-α, iL-,6 and iL-18 by approximately 80%. On the other hand, DMSO (seventh Group) does not affect reducing the gene expression of stimulated cells. The main issue is the effect of the ASEL (fourth group) on reducing the gene expression of TNF-α, iL-6 and iL-18 in THP-1 cells stimulated by LPS was approximately 50%, and this effect was significant compared to treatment with dexamethasone and ibuprofen (Table 5, and Figure 6).

10. DISCUSSION

In the present study, we show for the first time that the anti-inflammatory effects of ASEL are not limited to synoviocytes but also THP-1 cells. ASEL suppresses gene expression of TNF-α and IL-1β in an LPS-stimulated monocyte / macrophage cell, as well as TNF-α, IL-1β, COX-2, and iNOS gene expression in cultured bovine synoviocyte. Recently, several studies have been conducted to investigate the reduction of chemical consumption and change to herbal products and herbal medicines (Zheng and Wang, 2001). It is generally believed that they can be more effective in cases where synthetic chemical drugs have not been successful enough. In addition, natural products may have fewer unwanted side effects (Craig, 1999), of course, the possible disadvantages and their interaction with other drugs should be considered. Macrophage activation is an important event in the onset and spread of inflammation. NO, PGE2, and other anti-inflammatory cytokines are released when macrophages are stimulated by microorganisms or damage (Raetz et al, 2002) LPS is widely used to study the mechanism of the anti-inflammatory effect. NO synthesis is greatly increased by LPS, and studies show that inflammation is associated with NO levels (Kuo and Schroeder, 1995). In this study, the increase in NO production and the increase in iNOS expression following cell stimulation by LPS, and the decrease in its expression by ASEL were proved. Extensive studies have concluded that PGE2 is the main product of inflammatory cells and acts as a pro-inflammatory mediator (Dubois et al., 1995: Ahmadi, 2017).IL-1β is another potent inflammatory cytokine that mediates a wide range of reactions, it induces inflammatory reactions and catabolic effect independently, and involved in the acute phase reaction in inflammation (Ferrero-Miliani et al., 2007). In several studies, osteoarthritic joints, reported high levels of IL-1β in synovial fluid, synovial membrane, cartilage, and subcutaneous bone layer (Chow and Chin, 2020: Raymuev, 2018). In another study, increased IL-1R1 receptor expression was reported in patients with OA at the level of fibroblast-like cartilage cells and synoviocytes (Kulkarni et al., 2021). Considering these two cases, it can be stated; the increased expression of interleukin-1-β in LPS-activated synoviocytes was reduced by the ASEL. This is probably due to the fact that, the biological activation of cartilage cells and fluid cells. Patients with OA have an elevated level of IL-1β in both the synovial fluid (Melchiorri et al., 1998), synovial membrane (Massicotte et al. 2002), cartilage, and the subchondral bone layer (Sohn et al., 2012). The biological activation of cells by IL-1β is mediated by interaction with the membrane receptor,

namely, the IL-1R1 (IL-1RI), which can also bind IL-1α and IL-1Ra (Symons et al., 1995). Another receptor capable of binding IL-1β is IL-1R2 (IL-1RII), which after binding a ligand, such as the IL-1α, IL-1β, or IL-1Ra, forms an inactive ligand-receptor complex, showing no ability to communicate and activate the intracellular signal (Boraschi and Tagliabue, 2015). In the pathophysiological processes of OA, TNFα- along with IL-1β is considered a key inflammatory cytokine (Wojdasiewicz et al., 2018). TNF-α is secreted by the same joint cells that synthesize IL-1 and its concentration increases. TNF-α is also reported to be able to bind to two membrane receptor isotypes located on the surface of almost every TNF-R1 and TNF-R2 nucleated cell, both receptors are involved in signal transduction after TNFα activation in OA molecular processes. The receptor TNF-R1 can be stimulated by the soluble as well as membrane forms ligand whereas receptor TNF-R2 only recognizes the membrane form of the ligand (Grell et al., 1995). So far, the participation of TNF-R1 seems to have a greater impact on the local loss of articular cartilage than TNF-R2; this, however, does not change the fact that both receptors are involved in signal transduction after being activated by TNFα in the processes occurring in OA (Westacott et al., 2000). Elevated expression of TNF-R1 has also been observed in Fibroblast-like synoviocyte. (Steenvoorden et al., 2007). In this study, the increase of TNF-α expression following cell stimulation by LPS and its decreased expression by ASEL was proved. COX-2, and iNOS raise NO and PGE_2 levels and cause cartilage destruction, suppression of matrix production, and prevention of cartilage cell synthesis. Inhibition of iNOS and COX-2 production could be a useful approach to alleviate joint pain and inflammation in a patient with OA (Ferrer et al., 2019). Our study revealed that the anti-inflammatory effect of AESL suppresses PGE_2 and nitric production in synoviocytes cells and macrophage/monocyte cells and AESL acts as a strong suppressive of cytokines, COX-2, and iNOS gene expression. These mediators cause OA disease and a decrease in these mediators result in the betterment of cartilage destruction. AESL assists to decrease the expression and production of these inflammatory mediators in the studied cells. The evaluating effects of AESL in both BFLS and THP-1 cell types have shown anti-inflammatory effects of AESL that were not confined to cartilage cells, but rather in the monocyte/macrophage cells that are associated with the synovial membrane. Because of the anti-inflammatory effect of AESL in the reduction of cytokines gene expression and inflammation along with side effects of chemical treatment such as dexamethasone and ibuprofen in OA therapy, the use of *S. lavandulifolia* in OA therapy can be a suitable alternative.

Conclusion:

ASEL can reduce the expression of IL-6, IL-18, and TNF-α, and the production of NO and PGE2 in THP-1 cells. It seems that ASEL can act somewhat similar to the medicinal effects of dexamethasone and ibuprofen. Therefore, considering the side effects of these synthetic drugs, it seems that ASEL can be considered an effective factor in reducing inflammation and pain in patients with osteoarthritis, but more research is needed to clarify the mechanism of action.

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All authors contributed to performing the experiments and discussed the data and also commented on the manuscript.

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CONFLICT OF INTEREST:

The author deny any conflict of interest in any terms or by any means during the study. All the fees are provided by the research center fund and disbursed accordingly.

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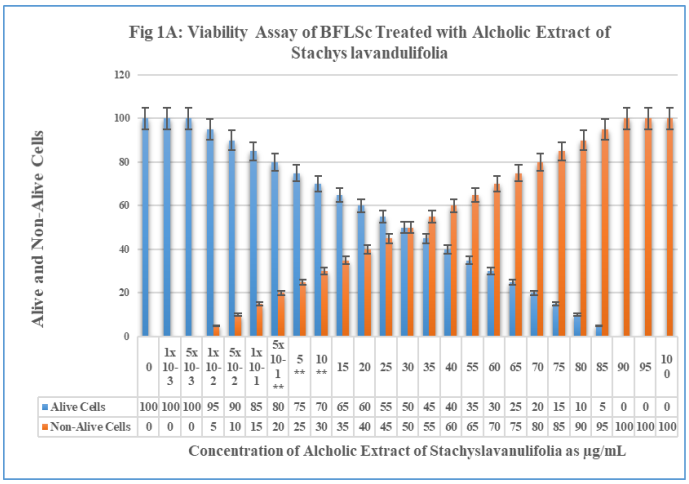
Figures:



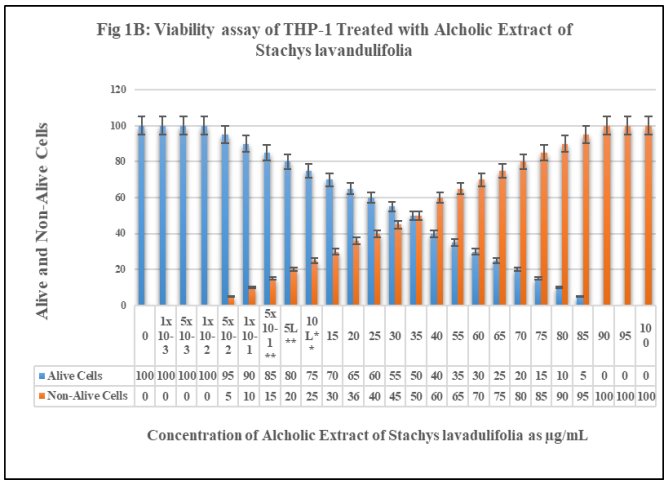
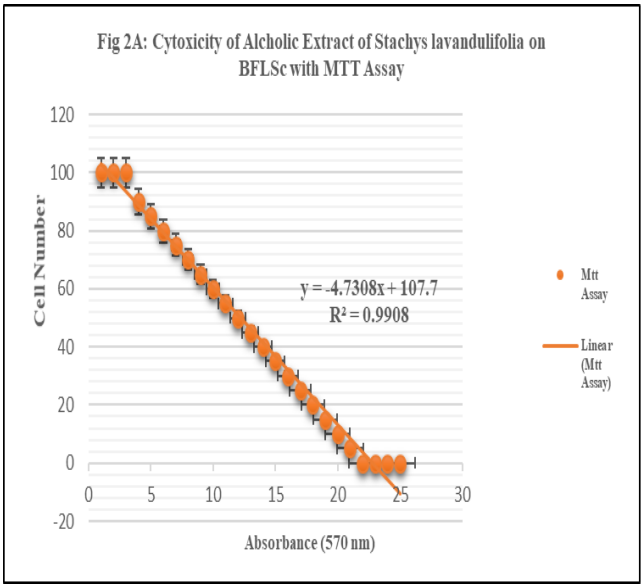


Figure 1: Trypan blue cellular viability assay for BFLSc (1A) and THP-1 (1B). Response of THP-1 and BFLS c cell treatment with increasing concentration of ASEL after 24 h. Cellular viability decreased in a dose-dependent manner in both cells treated with ASEL. Data are presented as the mean ± SE from four independent duplicate experiments. *p < 0.01 and **p < 0.001.



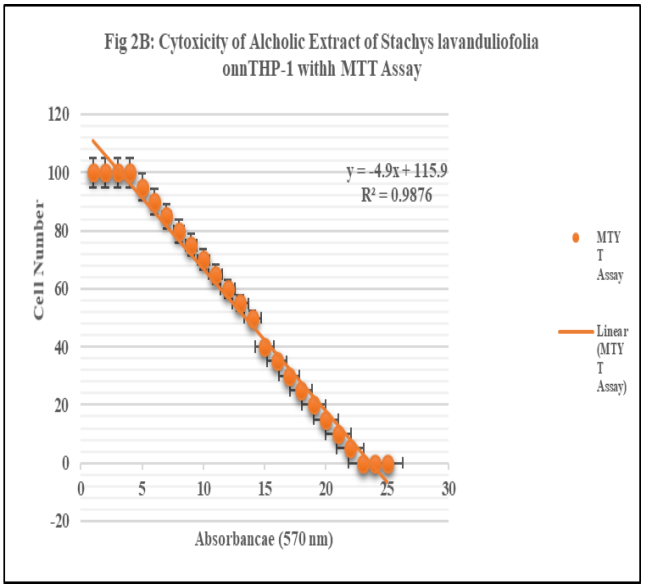


Figure 2: MTT Cell ProliferationAssay: BFLS cells(2A) ans THP-1 cell (2B) were grown in DMEM media supplemented with 10% FBS, Cells were exposed to increasing concentration of ASEL extract in the presence of LPS (1 µg/ml) or incubated with LPS (1 µg/ml) alone for 24 h. The cells were incubated with 20 µl MTT and placed in a 37 °C incubator for 4 h.After incubation, cells were treated with MTT Solvent for15min. at room temperature. Absorbance was measured at 570 nm. Inset graph is an expanded segment of the assay data at lower cell number per well

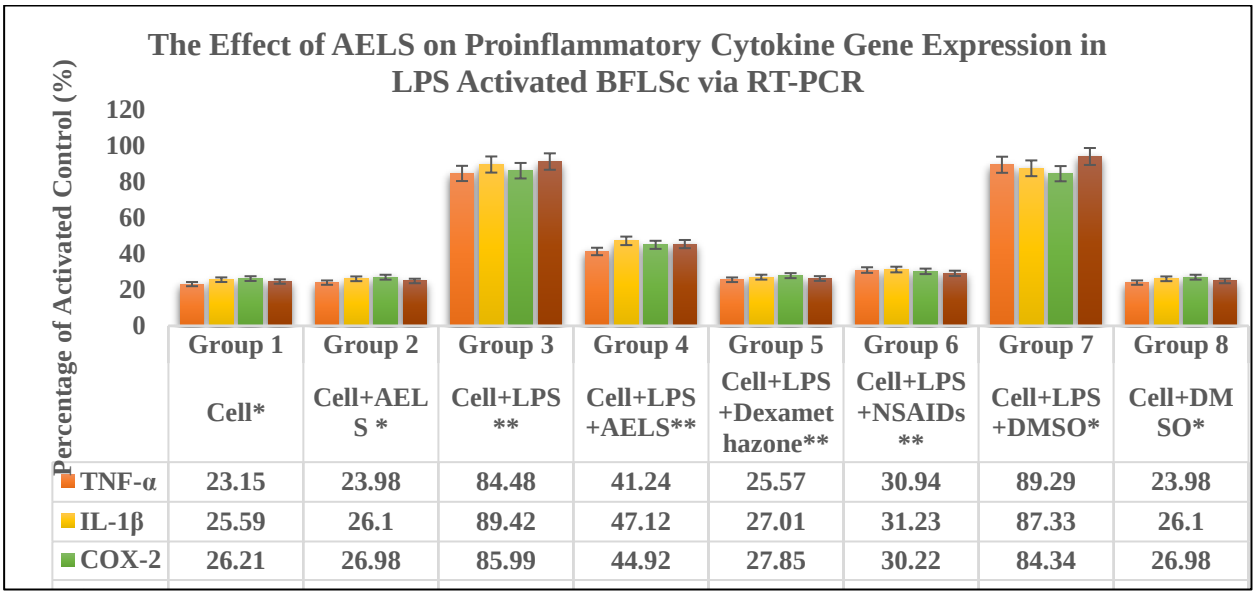


Fig. 3. The effect of AELS on proinflammatory gene expression in BFLSc using real-time PCR. BFLSc were incubated with AELS for 72 h and activated with LPS for 24 h. Quantification of normalized TNF-a, IL-1b, COX-2 and iNOS expression are shown. Statistical significances between activated control and other groups were analyzed using the Studente NewmaneKeuls test (mean±1 SD, n = 3).

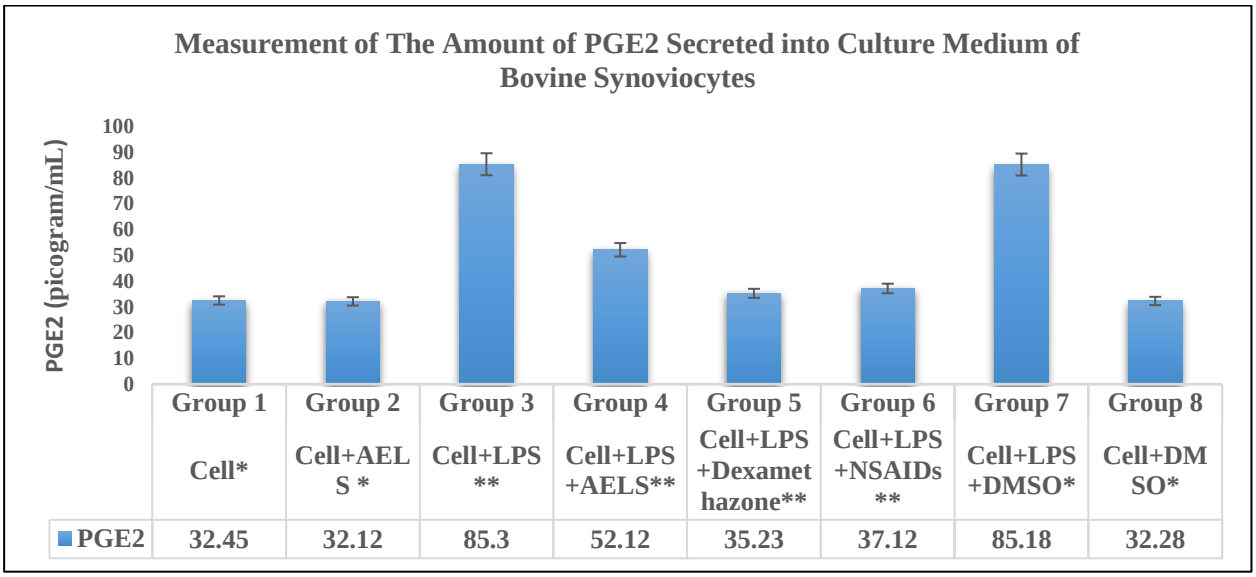


Fig. 4. The effect of ASEL on PGE2 levels in BFLSc. BFLSc were incubated with ASEL for 72 h and activated with LPS for 24 h. Mean PGE2 levels released into the cellular supernatant are shown as percent of activated control. Statistical significances between activated control and other groups were analyzed using the StudenteNewmaneKeuls test (mean ±±1 SD, n = 3)

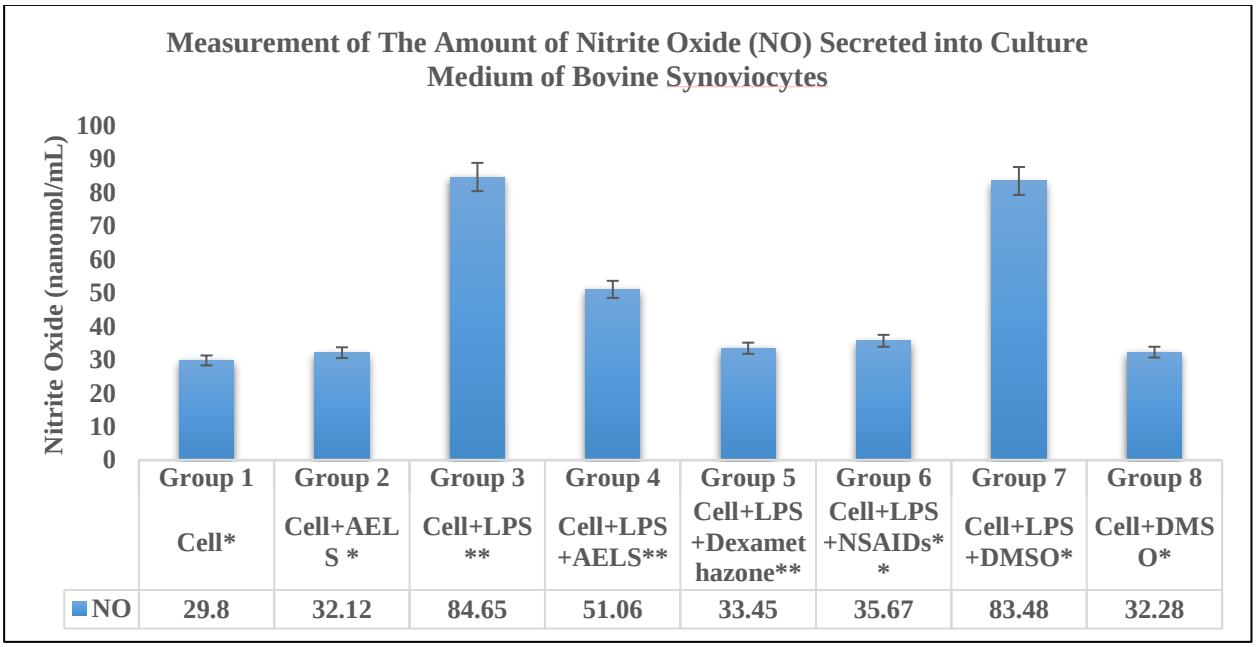


Fig. 5. The effect of ASEL on nitrite levels BFLS. BFLSc were incubated with ASEL for 72 h and activated with LPS for 24 h. Mean nitrite levels released into the cellular supernatant are shown as percent of activated control. Statistical significances between activated control and other groups were analyzed using the StudenteNewmaneKeuls test (mean ± 1 SD, n + 3).

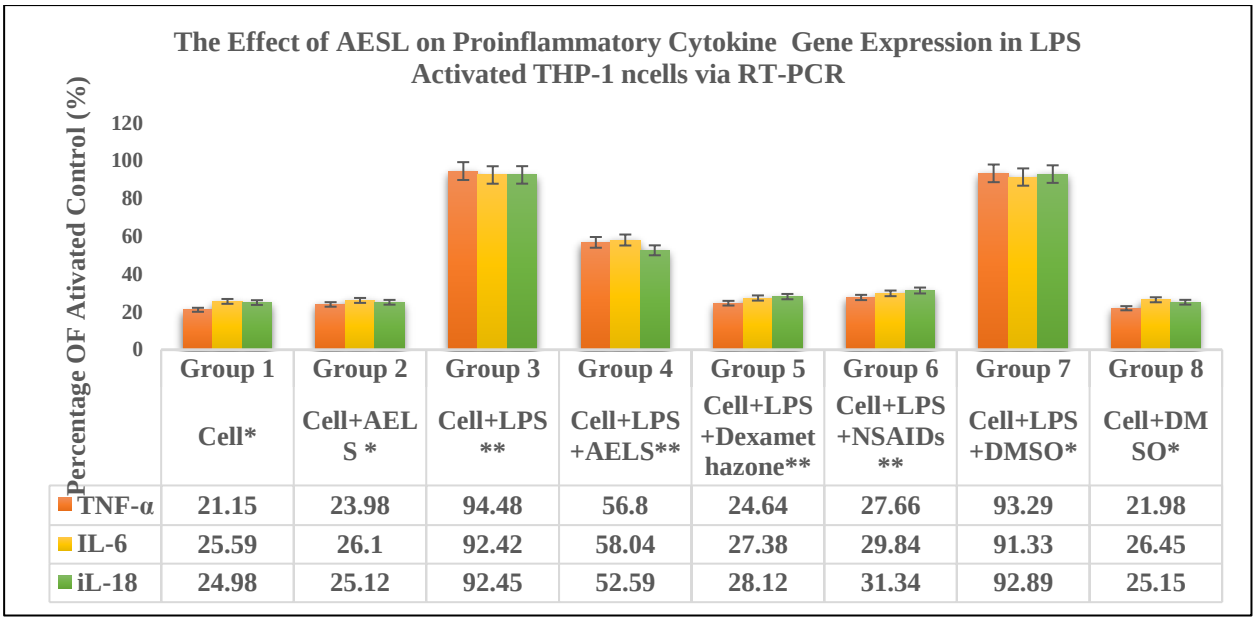


Fig. 6. The effect of ASEL on nitrite levels BFLSc . BFLSc were incubated with ASEL for 72 h and activated with LPS for 24 h. Mean nitrite levels released into the cellular supernatant are shown as percent of activated control. Statistical significances between activated control and other groups were analyzed using the StudenteNewmaneKeuls test (mean ± 1 SD, n + 3).

Tables:

Group number	Cells	LPS 100 ng/mL	Alcoholic extract of Stachys lavandulifolia	Dexamethasone	Ibuprofen	Dimethyl sulfoxide
1	$10x10^6$	Negative	Negative	Negative	Negative	Negative
2	$10x10^6$	Negative	positive	Negative	Negative	Negative
3	$10x10^6$	positive	Negative	Negative	Negative	Negative
4	$10x10^6$	positive	positive	Negative	Negative	Negative
5	$10x10^6$	positive	Negative	positive	Negative	Negative
6	$10x10^6$	positive	Negative	Negative	positive	Negative
7	$10x10^6$	positive	Negative	Negative	Negative	positive
8	$10x10^6$	Negative	Negative	Negative	Negative	positive

Table 1: Classification of study groups

Gene Bank id	Gene	Forward Primer	Reverse Primer	PCR Product Size
NM_174445.2	Bovine COX-2	GGTGCCTGGTCTGATGATGT	AGCCACTCAAGTGCTGTACG	175
DQ676956.1	Bovine iNOS	TCCCAAAAGGTGGACTTGGC	GTGACGTTTGGGGTCATCCT	105
NM_174093.1	Bovine iL-1β	TCTTCGAAACGTCCTCCGAC	AGCCAGCACCAGGGATTTTT	98
NM_173923.2	Bovine iL-6	GCGCATGGTCGACAAAATCT	CCAGTGTCTCCTTGCTGCTT	83
Z14137.1	Bivine TNF-α	ACTCCTTGAACTTCTGGGGC	TCCCATGTCCTAAGGGAGAGG	114
NM_001034034.2	Bovine GAPDH	AAGGTCGGAGTGAACGGATTC	ATTGATGGCGACGATGTCCA	90
X04430.1	Human TNF-α	GGACTGGAGATGTCTGAGGC	GCTCCTGGAGGGGAGATAGA	83
X12830.1	Human iL-6	CGTAACCGCACCCTGGGAC	CGGCTCTCTACACACACTGC	192
XM_011542805.2	Human iL-18	TGCAGTCTACACAGCTTCGG	AAGCAAAGAGCCATCTGCGA	97
X01677.1	Human GAPDH	CTGACTTCAACAGCGACACC	GTGGTCCAGGGGTCTTACTC	172

Table 2: The sequence of primer nucleotides for Bovine proinflammatiry cytokine COX-2, iNOS, iL-1 beta, iL-6, TNF-alpa and GAPDH and Human TF-α, iL-18, iL-6 and GAPDH

NO	Chemical Name	Percentage	NO	Chemical Name	Percentage
1	Phenol	8.17	20	Benzofuran	1.82
2	sabbinene	7.19	21	Enoic acid	1.73
3	1-Methyl-pyrrolidine-2-carboxylic	5.44	22	2-Pentylpyrazine	1.72
4	β-pinene	5.26	23	2-Propen-1-ol	1.63
5	Myrcene	5.68	24	Benzoic acid	1.45
6	2-Furancarboxaldehyde	5.42	25	4-Pyridinamine	1.41
7	Pyran	4.42	26	Phenic acid	1.39
8	Myristicine	4.11	27	1,2-Cyclopentanedione	1.34
9	Phenol,4-ethenyl-2- methoxy	3.39	28	Benzeneacetic acid	1.31
10	Vinyl phenol	3.16	29	Pyridinium	1.22
11	Myrcenen	3.11	30	Camphene	1.24
12	4,7-Methano	2.74	31	Benzenol	1.18
13	-Furanmethanol	2.51	32	1H-Pyrrole-2- carboxylic acid	1.18
14	2(3H)-Furanone	2.48	33	Acetic acid	1.34
15	α –pinene	2.35	34	Urea	1.09
16	Pamolyn	2.21	35	Phenylethanoid	0.87
17	Thymol	2.12	36	2-Hexadecen-1-ol	0.51
18	4H-Pyran-4-one	2.84	37	9-Octadecenoic acid	0.37
19	Hydrazine	1.92	38	Difluorobenzene,1-methoxy	0.12

Table3. Chemical composition of the hydroalchololic extract of S. lavandulifolia *Vahl* from Tehran province

Column1	Column2	Column	Column	Column	Column
		TNF-α	IL-1β	COX-2	iNOS
Cell*	Group 1	23.15±2.9	25.59±2.8	26.21±2.9	24.57±2.7
Cell+AELS *	Group 2	23.98±2.6	26.1±2.9	26.98±2.8	24.89±2.5
Cell+LPS**	Group 3	84.48±3.1	89.42±3.4	85.99±3.3	91.06±3.2
Cell+LPS+AELS**	Group 4	41.24±3.4	47.12±3.1	44.92±3.2	45.34±3.3
Cell+LPS+Dexamethazone**	Group 5	25.57±4.1	27.01±4.4	27.85±4.6	29.1±4.1
Cell+LPS+NSAIDs**	Group 6	30.94±4.3	40.9±4.2	30.22±4.6	34.54±4.3
Cell+LPS+DMSO*	Group 7	89.29±2.8	87.33±2.9	84.34±2.6	93.87±2.5
Cell+DMSO*	Group 8	23.98±2.7	26.1±2.8	26.98±2.9	24.89±2.6

Table 4: The effect of AELS on proinflammatory gene expression in BFLS, cusing semiquantitative RT-PCR. BFLSc were incubated with AELS for 72 h and activated with LPS for 1 h. Normalized gene expression is shown as percent of activated control (C + LPS). *P < 0.05; **P < 0.001.

Column1	Column2	Column	Column	Column
		TNF-α	IL-16	iL-18
Cell*	Group 1	21.15±4.4	25.59±4.3	24.98±3.8
Cell+AELS *	Group 2	23.98±3.9	26.1±3.8	25.12±3.5
Cell+LPS**	Group 3	94.48±3.5	92.42±3.6	92.45±3.9
Cell+LPS+AELS**	Group 4	56.8±4.1	58.04±4.2	52.59±4.8
l+LPS+Dexamethazon	Group 5	24.64±4.5	27.38±4.1	28.12±4.5
Cell+LPS+NSAIDs**	Group 6	27.66±3.6	29.84±3.5	31.34±3.9
Cell+LPS+DMSO*	Group 7	93.29±3.8	91.33±3.2	92.89±3.7
Cell+DMSO*	Group 8	21.98±3.1	26.45±3.4	25.15±3.6

Table 5: The effect of ASEL on proinflammatory gene expression in THP-1 cells using semiquantitative RT-PCR analysis. THP-1 cells were incubated with ASU for 72 h and activated with LPS for 1 h. Normalized gene expression is shown as percent of activated control (C + LPS). *P < 0.05; **P < 0.001.