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The Effect of the Alcoholic Essence of *Laurus nobilis L.* on Pro-inflammatoiry Cytokine Gene Expression in Synoviocytes and Macrophage/Monocyte

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Abstract: Osteoarthritis (OA) is a chronic degenerative joint disease with an inflammatory component. It is associated with progressive histological alterations and disabling symptoms. Today, drugs such as glucocorticoids (GCs) and nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly employed for the treatment of osteoarthritis but have serious and life-threatening side effects. The current study aims to evaluate the effects of alcoholic essences of *Laurus nobilis L.* (AELN) on pro-inflammatory cytokines such as cyclooxygenase-2 (COX-2, isoform), inducible nitric oxide synthase (iNOS), interleukin-1β (IL-1β), interleukin-18 (IL-18), tumor necrosis factor-alpha (TNF-α), and nitric oxide (NO), as well as prostaglandin E2 (PGE2) on inflammatory cells, similar osteoarthritis in synoviocytes, and monocytes/ macrophages, and to compare it with dexamethasone (DEX) and ibuprofen (IBP). After collecting the leaves of the *Laurus nobilis L.* (LN) and after drying, the essences were collected by the Center for Genetic and Biological Resources of Iran. Synovial cells were isolated from the synovial membrane of the radiocarpal joint cartilage of an 8-month-old Holstein cow. THP-1 cells were prepared from the Pasteur Institute of Iran. Cells were cultivated and exposed to lipopolysaccharide (LPS) stimulation without, or in the presence of, DEX, IBP, or alcoholic essences of *Laurus nobilis L.* (AELN) The gene expressions of IL-1β, TNF-α, IL-18, COX-2, and iNOS were evaluated by real-time PCR. Concentrations of NO and PGE2 were measured by ELISA methods. Treatment of the studied cell with alcoholic essences of Laurus nobilis L, before stimulation with lipopolysaccharide, reduces the expression of proinflammatory cytokine genes such as cyclooxygenase-2, nitric oxide synthase, interleukin-6, interleukin-1 beta, interleukin-18, tumor necrosis factor-alpha, and It also reduces the production of nitric oxide and prostaglandin E2 by almost 50%. This reduction is significant compared to the 90% reduction due to treatment with dexamethasone and ibuprofen. Significant reduction in the expression of pro-inflammatory cytokines by alcoholic essences of Laurus nobilis L can be considered as a new drug in the treatment of osteoarthritis and requires further studies in laboratory animals and clinical studies.

Keywords: Osteoarthritis, *Laurus nobilis L.*, Synoviocytes, Monocytes/Macrophages, Proinflammatory Cytokine

1. Introduction

Osteoarthritis, or degenerative arthritis, is primarily a chronic musculoskeletal disorder of the mobile joints, such as the knee and hip, as the prevalence of osteoarthritis increases with age and, as the world's population ages, osteoarthritis progresses and It has become an inescapable issue which should be considered as a public health issue in a society that is aging [1, 2]. It affects about 250 million people (much more common in the elderly) worldwide [3]. In the COPCORD study in Iran conducted by Dr. Davatchi et al., The prevalence of osteoarthritis between the ages of 15-82

years in urban communities in the knee, hand, and hip areas was reported to be 15.3%, 9.9%, and 32.32%, respectively [4] Osteoarthritis is one of the most common causes of disability and inability to work in men over the age of 50 in the United States, after osteoarthritis. More than rheumatoid arthritis, it causes people to be hospitalized [5]. There are many causes for osteoarthritis, the most important of which are metabolic causes: such as acromegaly, diabetes, and high cholesterol, anatomical causes: such as joint deformity and traumatic causes: such as fractures, dislocations, joint and meniscus lesions, and inflammatory causes: such as rheumatoid arthritis. Name purulent arthritis which is the most common type [6]. Changes in joint tissue following trauma, removal of part of the cartilage, and exposure to joint fluid are considered antigenic factors and lead to increased production of matrix cytokines and metalloproteinases (MMPs) in the joint space. In addition, reducing chondrocyte growth factors and responsiveness leads to less matrix synthesis and repair [7]. In addition to aging, environmental, biomechanical, and biochemical factors may also play a role in the onset of osteoarthritis. Osteoarthritis affects the entire structure of the joints, including articular cartilage, subcutaneous bone, meniscus, synovial membrane, and patellar fat layer, Infrapatellar Fat Pad (IFP). Common structural features of osteoarthritis include cartilage destruction, subcutaneous bone degeneration, osteophyte formation, and changes in the synovium and joint capsule [8]. Patients with osteoarthritis have common clinical symptoms such as severe joint pain, stiffness, and significant loss of mobility, which leads to decreased productivity and quality of life among patients, as well as an increased socioeconomic burden for patients and the community [9]. The involvement of immune cells in the development and progression of osteoarthritis has been highlighted in recent studies [10]. Inflammatory components such as cytokines and chemokines are produced by chondrocytes and synoviocytes in the joints of patients with osteoarthritis [11]. Synovial fibroblasts are also a source of proinflammatory cytokines and matrix-degrading enzymes in osteoarthritis. In addition, IFP has been shown to contain significant amounts of immune cells such as macrophages and T cells. As a result, IFP acts as a local inflammatory mediator in knees with osteoarthritis. These inflammatory mediators alter cellular signaling pathways, gene expression, and joint tissue behavior [12]. Changes in cellular signal transduction lead to increased activation of the inflammatory pathway. Thus, inflammatory compounds and enzymes are further released. As a result, the anatomical and physiological functions of the joint change [13]. Immune cells such as activated neutrophils and macrophages can secrete cytokines such as IL-6 and IL-1β, which potentiate the inflammatory process in osteoarthritis [14]. Increased infiltration of leukocytes (macrophages, T lymphocytes, B lymphocytes, and neutrophils) in the synovium, especially in the underlying layer, is characteristic of osteoarthritis [15]. Cytokines secreted by immune cells are major players in inflammatory disease, including osteoarthritis. Proinflammatory cytokines, such as IL-1β and TNF-α, are mediators secreted in primary osteoarthritis and direct the inflammatory cascade independently or in association with other cytokines. They are produced by active cartilage cells, synoviocytes, and mononuclear cells [16]. IL-1β is involved in several cellular activities such as cell proliferation, differentiation, and apoptosis. By affecting the activity of cartilage cells in the joint, it disrupts the production of essential structural proteins such as collagen type II and aggrecan. In addition, IL-1β affects the synthesis of MMPs by cartilage cells, including MMP-1 and MMP-13, which in turn destroys articular cartilage [17]. A study conducted among patients with osteoarthritis showed that IL-1β, IL-6, IL-18, IL-17, IL-22, and beta-1 converting growth factor (TGFβ1) were compared in inflamed synovial tissues. Increased with non-inflamed tissues [18]. They are secreted by helper T cells, mast cells, and myeloid cells, and release IL-6, IL-8, and TNF-α by synovial fibroblasts and chondrocytes, leading to inflammation and cartilage breakdown [19]. IL-17 is present in synovial fluid in several end-stage osteoarthritis patients. Elevated levels of IL-17 and IL-22 have also been reported in the synovial fluid of the temporomandibular joint in patients with osteoarthritis. The increase in these two cytokines is accompanied by an increase in nuclear kappa-β ligand-receptor activator (RANKL), which differentiates osteoclasts and degrades subcutaneous bone, a layer of bone beneath the cartilage of the joints [20]. IL-22 stimulates synovial cell proliferation and increases the expression of MMPs in fibroblast-like synoviocytes (FLS) [21]. Interleukin 6 (IL-6) is known as a proinflammatory cytokine in chronic inflammatory diseases. In osteoarthritis, IL-6 released by joint tissue binds to the soluble IL-6 (IL-6R) receptor, leading to signal transmission [22]. In addition to the above cytokines, several other cytokines are involved in the pathogenesis of osteoarthritis. Some cytokines that increase in osteoarthritis include TGFβ1 [18], IL-1 receptor (IL-1R) [23], and L-1 alpha (IL-1α) [24]. Shan and his colleagues showed serum levels of IL-21, IL-17A, and IFN-γ in patients with osteoarthritis compared with controls [25].

Laurus Nobilis L. is an industrial plant that is used in food, medicine, and cosmetics. Dried leaves and their oil are widely used in the food industry for seasoning meat soups and fish. Antimicrobial and insecticidal activities are used in the food industry as preservatives. Fruits, which are alcoholic essences that are mainly used in making soups, have been used experimentally in rheumatism, skin inflammations, and intestinal problems such as bloating, indigestion, belching, and bloating. Its aqueous extract has been mentioned in Turkish medical culture as anti-hemorrhoids, anti-rheumatism, diuretic, an antidote for snake bites and stomach pain, and more recently as a cure for diabetes and prevention of migraine. The results of studies have shown that the Laurel has been effective in neutralizing free radicals and lipid peroxidation, and according to the results, it can be concluded that the extract of this LAUREL has more supportive effects on the liver than blood parameters. The therapeutic combination of the extract with carbon tetrachloride has shown positive and negative

synergies [26]. In general, the plants of this genus have many medicinal and therapeutic properties due to their alcoholic essences. Some of them are camphor producers. The leaves of this plant have been used in folk medicine as mosaics and to relieve colds. Its leaves have a decongestant, emetic, diuretic, anticonvulsant and regularizing effect Its fruit is eaten dried and has a stronger therapeutic effect than the leaves and its consumption has been used in the past to relieve anorexia and stomach weakness. It is also recommended in chronic bronchitis. Its alcoholic essences are used in sprains of joints, hemorrhoids, and rheumatic pains, by rubbing on the organ [27]. Today, drugs such as dexamethasone and nonsteroidal anti-inflammatory drugs are commonly used to treat osteoarthritis, but they have serious and life-threatening side effects [28]. As a result, the change in approach in the treatment of osteoarthritis has attracted the attention of many researchers working in this field. Based on this and several findings of the LAURELy plant, this group investigated the anti-inflammatory effect of the LAURELy alcoholic essences in a model similar to osteoarthritis.

2. Materials and Methods

RPMI-1640, DMEM-F12, Cell culture media, L-glutamine, Bovine fetal serum, Sodium Bicarbonate, Glucose-4- (2-hydroxyl) piperazine-1-ethane sulfonic acid (HEPES), MTT Assay Kit, Grace Reagent, Lipopolysaccharide (Escherichia coli serotype O127: B8) sodium pyruvate, Trypan Blue, Beta mercaptoethanol (Sigma Aldrich, UK), Gentamysin, Penicillin and streptomycin from Idea Bio Recombinant Company (Iran), and RNA extraction kit, RNA to cDNA conversion kit, RT-PCR Master Mix and primers from Sinagen Company (Iran). Amphotericin-B was prepared from Ciplus (India).

2.1. Preparation of Fragrant Laurel Alcoholic Essences

LAURELy alcoholic essences were prepared and gas chromatography was performed by the National Center for Genetic and Biological Resources of Iran.

2.2. Culture of Fibroblast-like Fibroblast Cells

The metacarpal joint of healthy eight-month-old calves was used to isolate synoviocyte cells. After draining the joint fluid into a flask containing DMEM-F12 medium enriched with 10% fetal bovine serum, 50 µg/mL ascorbic acid, and a combination of antibiotics including 100 U/mL penicillin, streptomycin 100 µg/ml, gentamicin 50 mg/ml, amphotericin 25µg/mL was stored in 25 cm^2 flasks in an incubator with 5% CO_2, 37°C and 95% humidity. After reaching a cell density of more than 80%, celluloid was counted with a trypan blue solution of 0.4% and the cell viability percentage was determined and cells with 95% viability were selected for the next culture [29]. In previous studies in this group, synoviocytes were confirmed by the vitamin antibody [30].

2.3. Culture of THP-1 Cells

THP-1 cells were prepared from Pasteur Institute and cultured in RPMI-1640 cell culture medium enriched with 2 mM L glutamine, 10% FBS, 1.5 g/l sodium bicarbonate. 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 0.05 mM β-mercaptoethanol, penicillin 100 U/mL, streptomycin 100 µg/ml, gentamicin 50 µg/mL and amphotericin b 25 µg/mL And kept in an incubator with 5% CO_2, 37°C and 95% humidity. After reaching the cell density of 95%, the cells were counted with a trypan blue solution of 0.4% and the cell viability percentage was determined and cells with 95% viability were selected for the next steps [31].

2.4. Division of Study Groups and Cell Treatment

The study groups were divided into seven groups and Table 1 shows how they are grouped. Culture of 10x10 synoviocytes and THP-1 cells in 75 cm^2 flasks in eight groups and storage for 1-2 hours in an incubator and then injection of 16.22µg/mL alcoholic essences into all flasks for 5 minutes. They were placed on a shaker with a rotational motion of 50 rpm/m and kept in the incubator again for 72 hours. Culture of $10x10^6$ synoviocytes and THP-1 cells in 75 cm^2 flasks in eight groups and storage for 1-2 hours in the incubator and then injection of 16.22µg/mL alcoholic essences into all flasks for 5 minutes They were placed on a shaker with a rotational motion of 50 rpm/m and kept in the incubator again for 72 hours. LPS was used at 100 ng/ml to provide conditions similar to osteoarthritis and to increase the expression of proinflammatory cytokines. Sodium dexamethasone 4 mg/mL [31], ibuprofen (non-steroidal anti-inflammatory drug) (10 mg/mL) [32] as positive control and their effect with alcoholic essences, DMSO as negative control were used in the study groups [33].

Table 1. *Classification of study groups.*

	Cells $6*10^6$	LPS 100ng/ml	Alcoholic essences of Laurus nobilis L	Dexamethazone	Ibuprofen	DMSO		
1	Seeded	Untreated	Untreated	Untreated	Untreated	Untreated	Negative Control (Untreated Cells)	Group 1
2	Seeded	Treated	Treated	Untreated	Untreated	Untreated	The effectiveness AELN on untreated cells	Group 2
3	Seeded	Treated	Untreated	Treated	Untreated	Untreated	The effectiveness of Dexamethazone on LPS treated cells	Group 3
4	Seeded	Treated	Untreated	Untreated	Treated	Untreated	The effectiveness AELN on LPS treated cells	Group 4
5	Seeded	Untreated	Untreated	Untreated	Untreated	Treated	The effectiveness of DMSO on untreated cells	Group 5
6	Seeded	Untreated	Treated	Untreated	Untreated	Untreated	The effectiveness AELN on untreated cells	Group 6
7	Seeded	Treated	Untreated	Untreated	Untreated	Untreated	Positive Control (Treated cells)	Group 7

2.5. Cytotoxicity Study by MTT Assay Method

MTT and Trypan Blue methods were used to evaluate the cytotoxic effect of alcoholic essences of *Laurus nobilis L.* and its LC50. MTT, a yellow tetrazole, is reduced by succinate dehydrogenase (SDH) in the mitochondria of living cells to purple formats. Dimethyl sulfoxide (DMSO) was added to dissolve the insoluble form of purple formazan in the dye solution. The absorbance of this dye solution was measured by a spectrophotometer at a wavelength between 500 and 600 nm [34]. As the odor of alcoholic essence leaves decreases, the survival rate gradually decreases, as at concentrations of 50 µg/mL, 45 µg/mL, this percentage decreases by 50% in THP-1 cells and synoviocytes. Cell treatment averaged 14.16 µg/mL and 16.22 µg/mL for THP-1 and synoviocytes, respectively.

2.6. Measurement of Nitrit Content

NO_2 accumulation was used as an indicator of NO production in cell culture media [35]. 10^5 synoviocytes were transferred to 24 well plates and kept in an incubator for 1-2 hours. Injection of odorous Laurel alcoholic essences with increasing concentrations (indicated in the survival percentage) to each well and plate storage for In the incubator for 24 hours in the next stage of LPS injection at the rate of 100 ng/mL to each well and for 72 hours in the cell culture incubator. Add 100 µl of grease reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine hydrochloride in 5% phosphoric acid) to 100 µl of supernatant and mix gently at room temperature. Absorption was measured at 550 nm using a spectrophotometer (Beckman, CA, USA). Nitrate concentration was calculated by comparing standard A550 sodium nitrate solution prepared in the same culture medium.

2.7. Measurement of Prostaglandin E2 Levels

PGE2 immunoassay (Invitrogen, USA) was used to measure prostaglandin produced in a cell culture medium by ELISA according to the manufacturer's instructions [36]. In summary, 100 ml of the culture medium (three times) was transferred to 96 wells with polyclonal antibody microplates. Fifty microliters of high sensitivity PGE2 solution were added to each good sample and then 50 ml of PGE2 antibody solution was added. The microplate was stored for 18 to 24 hours at 2 to 8°C. The top solution of each well was then discarded, and the bottom layer of each well was washed three times with PGE2 wash buffer. Then, 200 ml of pNPP solution was added to the wells. After storage for 1 hour at 37°C, 50 ml of reaction-stopping solution was added to the wells. Optical density was measured immediately at 405 nm using the SCO DIANOGNOSTIC ELISA reader. Data were calculated using the formula.

$$\text{Logit (B/B0)=In (B/B0/1- (B/B0))}$$

2.8. Reverse Transcription-polymerase Chain Reaction (RT-PCR)

RNA isolation and conversion to cDNA were performed based on methods published in previous articles [37]. Lysis of cells from Triazole reagent and RNA extraction with chloroform was performed. RNA was converted to cDNA by 2-step RT-PCR. Semi-quantitative PCR was used for each of the studied cytokines using specific primers (Table 2) and the GAPDH gene as the housekeeping gene. The 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 the master mix. The results obtained from CTs (Threshold cycle) were evaluated by two standard curve methods and the Pfaff method. In principle, CTs are numerical numbers and their importance was first assessed using ANOVA and secondly by Student-Newman Keuls (SNK), REST-2000. Finally, the Pfaff method was used to determine the amount of gene expression.

Table 2. *Sequence of primers designed for proinflammatory cytokines for THP-1 and BFLS.*

European Nucleotide Archive (ENA)	Gene	Forward primer	Reverce primer	PCR product length
AAC05592.1	Bovine COX-2	AAAAGCTGGGAAGCCTTTTC	AAGCTGGTCCTCGTTCAAAA	250
AAC48479.1	Bovine iNOS	CCTCTACTGGGAGGAGATGCA	AGAGGTGACCTTGCTCCTCA	170
AAA30584.1	Bovine iL-1β	CAGCTGCAGATTTCTCACCA	CCAGGGATTTTTGCTCTCTG	250
AAI23578.1	Bovine iL-6	CACCCCAGGCAGACTACTTC	AGCAAATCGCCTGATTGAAC	215
AAB84087.1	Bovine TNF-α	GCCCTCTGGTTCAGACACTC	CCCTGAAGAGGACCTGTGAG	213
AAB47507.1	Bovine GAPDH	CCACCCAGAAGACTGTGGAT	CAACAGACACGTTGGGAGTG	180
CAA25650.1	Human TNF-α	CTGAAAGCATGATCCGGGAC	TTAGAGAGAGGTCCCTGGGG	201
CAA28268.1	Human iL-6	CAGCCACTCACCTCTTCAGA	ACCAGGCAAGTCTCCTCATT	206
AAC27787.1	Human iL-18	CAATTGCATCAACTTTGTGGC	TAAATATGGTCCGGGGTGCA	227
AAP88771.1	Human NF-kB	CTCTTGACCTCACTTGCAGC	GGTGAGGTTGTCTGTCGGTA	241
AAA52518.1	Human GAPDH	GAAGGTGAAGGTCGGAGTCA	TGACAAGCTTCCCGTTCTCA	200

2.9. Statistic Analysis

All data are expressed as mean ± SD. Statistical analysis was performed using ANOVA, Student – Newman – Keuls, REST-2000. Cts was used for analysis. $P < 0.05$ is considered statistically significant.

3. Research Findings

3.1. Gas Chromatography Results of Laurel Alcoholic Essences

In general, in the alcoholic essences of leaves harvested from the National Botanical Garden of Iran, 20 compounds were identified, of which 1,8 - cinnamon 47%, sabine 13.9%, and alpha-Terpineol acetate were major components. Table 3 shows the constituents of the alcoholic essences of the plant obtained by gas chromatography in this study. In the alcoholic essences of the mentioned plant, 25 compounds were identified, which constitute 97.4% of the alcoholic essences compounds. The main components are:

1,8 cineole 49.1%, 2-sabin 12.13%, alpha terpineol acetate 11%, beta-pinene 4.5%, alpha-pinene 4%, eugenol 2.1% and pi -Cement 2% compounds.

Table 3. *Ingredients of fragrant Bay laurel alcoholic essences.*

Row	chemical mixture	Level	Row	chemical mixture	Level
1	α-thujene	0.1%	14	α-terpineol	1.5%
2	α-painene	4%	15	y-terpineol	1.9%
3	Sabinene	12/13%	16	linalool acetate	0.1%
4	B-pinene	4.5%	17	bornyl acetate	0.2%
5	Myrecene	0.5%	18	carvacrol	0.3%
6	P-cymene	2%	19	α-terpinyl acetate	11%
7	1,8-cineole	49.1%	20	Eugenol	2.1%
8	y-terpinene	0.4%	21	α-ylangene	2.8%
9	Cis sabinene hydrate	0.3%	22	E-caryophyllene	0.3%
10	terpinolene	2.4%	23	germacrene D	0.6%
11	trans sabinene hydrate	0.2%	24	y-cadinene	0.2%
12	Endo-fenchol	0.1%	25	germacrene B	0.4%
13	Terpinene-4-ol	0.3%			

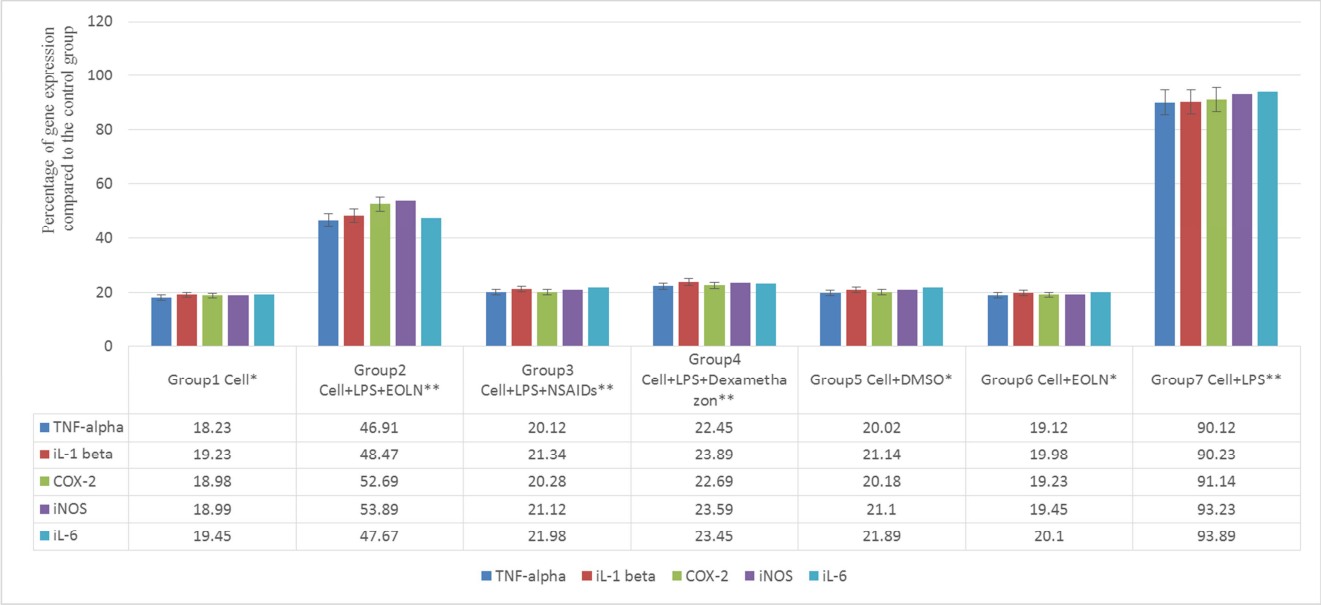

Figure 1. *Comparative effect of Laurel alcoholic essences with dexamethasone and ibuprofen on the expression of pro-inflammatory cytokines iL-6, iNOS, COX-2, iL-β, TNF-α in bovine Synoviocytes using RT-PCR. The statistically significant difference between the second group and other groups using Student-Newman-Keuls test (mean was analyzed (mean +/- 1 SD, n=3). P<0.001, P<0.05.*

3.2. Comparative Results of the Effect of Laurel Alcoholic Essences, Dexamethasone, Ibuprofen on the Expression of TNF-α, iNOS, iL-1β, iL-6 and COX-2

To compare the effect of Laurel alcoholic essences, dexamethasone, ibuprofen on the expression of pro-inflammatory cytokines (TNF-α, COX-2, iNOS, iL-1β, iL-6) in synoviocyte cells, in eight groups (Table 1) Comparison of quantitative analysis of RT-PCR results showed that the addition of LPS significantly increased the expression level of pro-inflammatory cytokines in synoviocytes (Figure 2, group 3). Treatment with dexamethasone (group 5) and ibuprofen (group 6) in cells stimulated by LPS significantly and significantly (P<0.001) significantly reduced the expression level of the studied proteins (90%). In the case of treatment with laurel alcoholic essences (group 4), this decrease in expression level (50%) was determined for all proteins studied (P<0.001). The expression of the studied proteins in the cells treated with alcoholic essences (group 2) was almost similar to the cells of the first group and DMSO did not change the expression of the studied proteins in the cells stimulated with LPS (group 7) and the cells without Did

not show stimulation (group 8). Culture of cells $10^6 \times 10^6$ in each flask for seven groups and storage in an incubator for 2-3 hours and then treatment of cells with dexamethasone, ibuprofen, alcoholic essences of leaves and DMSO, re-storage in the incubator for 72 hours and then injection LPS 100ng/ml for the desired groups, and re-incubate for 24 hours. The normal expression values of iL-6, iNOSs, COX-2, iL-β, TNF-α are shown in the first group (Figure 1).

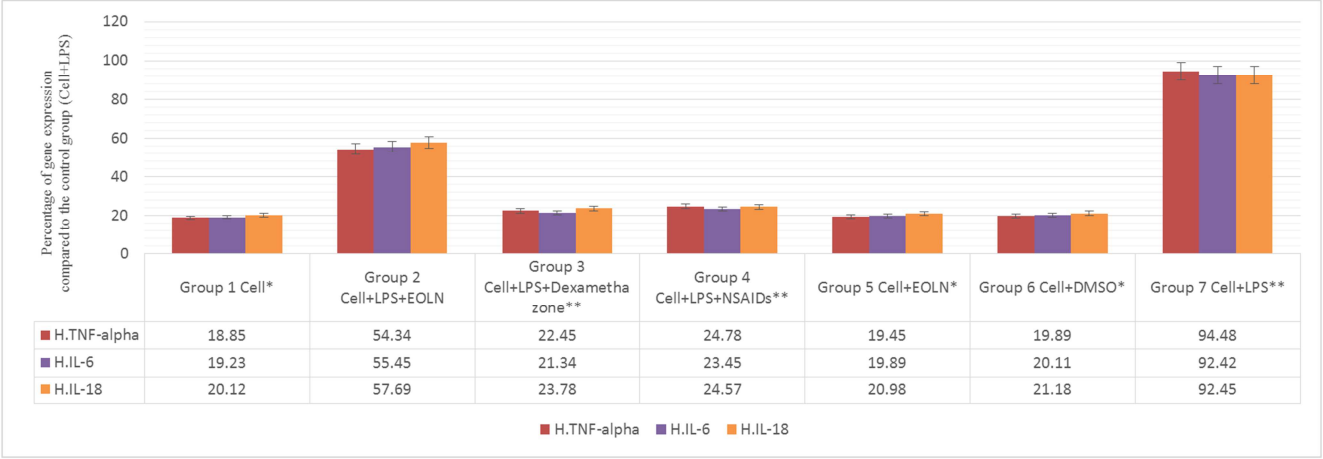

Figure 2. *Comparison of the effect of fragrant Laurel alcoholic essences dexamethasone and ibuprofen on LP-stimulated THP-1 cells on the expression of pro-inflammatory cytokines (IL-18, TNF-α) using Real-Time PCR. The statistically significant difference between the fourth group and other groups using the Student-Newman-Keuls test (mean was analyzed (mean +/- 1 SD, n=3). P<0.001, P<0.05.*

3.3. Comparative Results of the Effect of Laurel Alcoholic Essences, Dexamethasone, Ibuprofen on the Expression of TNF-α, iL-1β

To compare the effect of fragrant Laurel alcoholic essences, dexamethasone, ibuprofen on the expression of pro-inflammatory cytokines (TNF-α, iL-1β) in THP-1 cells, in eight groups (Table 1) and to compare the quantitative analysis of RT results PCR showed that the addition of LPS significantly increased the expression level of the studied pro-inflammatory cytokines in synoviocyte cells (Figure 3, group 3). Treatment with dexamethasone (group 5) and ibuprofen (group 6) in cells stimulated by LPS significantly and significantly (P<0.001) significantly reduced the expression level of the studied proteins (90%). In the case of treatment with Laurel alcoholic essences (group 4), this decrease in expression level (50%) was determined for all studied proteins (P<0.001). The expression of the studied proteins in the cells treated with alcoholic essences (group 2) was almost similar to the cells of the first group and DMSO had no change in the expression of the studied proteins in the cells stimulated with LPS (group 7) and the cells without Did not show stimulation (group 8). Culture cells at a rate of $10^6 \times 10^6$ per flask for seven groups and keep in an incubator for 2-3 hours, then treat the cells with dexamethasone, ibuprofen, Laurel alcoholic essences, and DMSO and keep in the incubator for 72 hours and then inject LPS. 100 ng/ml and keep in the incubator for 24 hours. The normal expression levels of iL-16, TNF-α are shown in the first group (Figure 2).

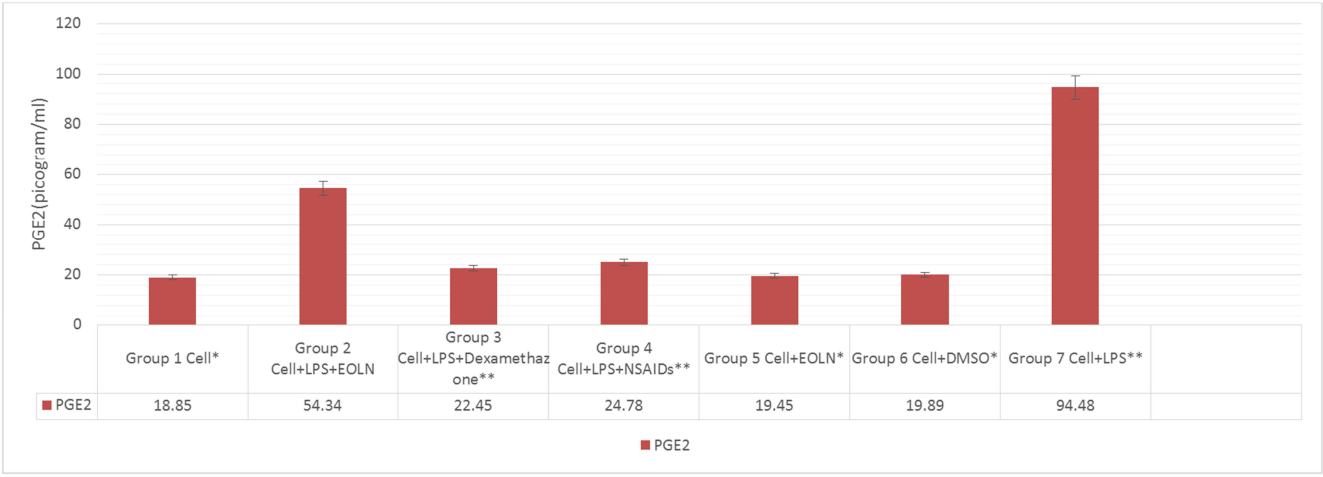

Figure 3. *The effect of Laurel alcoholic essences on the level of PGE2 production released in culture medium in THP-1 using ELISA. The statistically significant difference between the fourth group and other groups using the Student-Newman-Keuls test (mean was analyzed (mean +/- 1 SD, n=3). P<0.001, P<0.05.*

3.4. Comparative Results of the Effect of Laurel Alcoholic Essences, Dexamethasone, Ibuprofen on the Production of PGE2, NO

To determine whether fragrant LAUREL alcoholic essences such as dexamethasone and ibuprofen are effective inhibitors of the release of inflammatory factors, THP-1 cells were examined in eight study groups. Comparison of quantitative analysis of the results showed that the addition of LPS significantly increased the production of PGE2, NO in THP-1 cells. Culture cells at a rate of $10^6 \times 10^6$ per flask for eight groups and keep in the incubator for 2-3 hours, then treat the cells with dexamethasone, ibuprofen, Laurel essential oil, and DMSO and keep in the incubator for 72 hours and then inject. LPS 100 ng/ml for all groups except the first group and incubation for 24 h, mean PGE2 level released in cell supernatant is shown as active control percentage. (Figure 3, Group 3). Treatment with dexamethasone (group 5) and ibuprofen (group 6) significantly ($P<0.001$) reduced PGE2 production of NO (90%) in LPS-stimulated cells. In the case of treatment with Laurel alcoholic essences (group 4), this decrease in expression level (50%) was determined for all studied proteins ($P<0.001$). The production of PGE2, NO in alcoholic essences-treated cells (group 2) was almost similar to that of the first group, and DMSO had no effect on reducing the production of PGE2, NO in LPS-stimulated cells (group 7) and unstimulated cells (Did not show group 8). Culture of cells $10^6 \times 10^6$ per flask for eight groups and storage in an incubator for 2-3 hours and then treatment of cells with dexamethasone, ibuprofen, Laurel essential oil and DMSO and re-storage in the incubator for 72 hours and then LPS injection of 100 ng/ml for all groups except the first group and re-storage in the incubator for 24 hours, the mean level of NO emitted in cell supernatant is shown as the percentage of active control (Figures 4, 3).

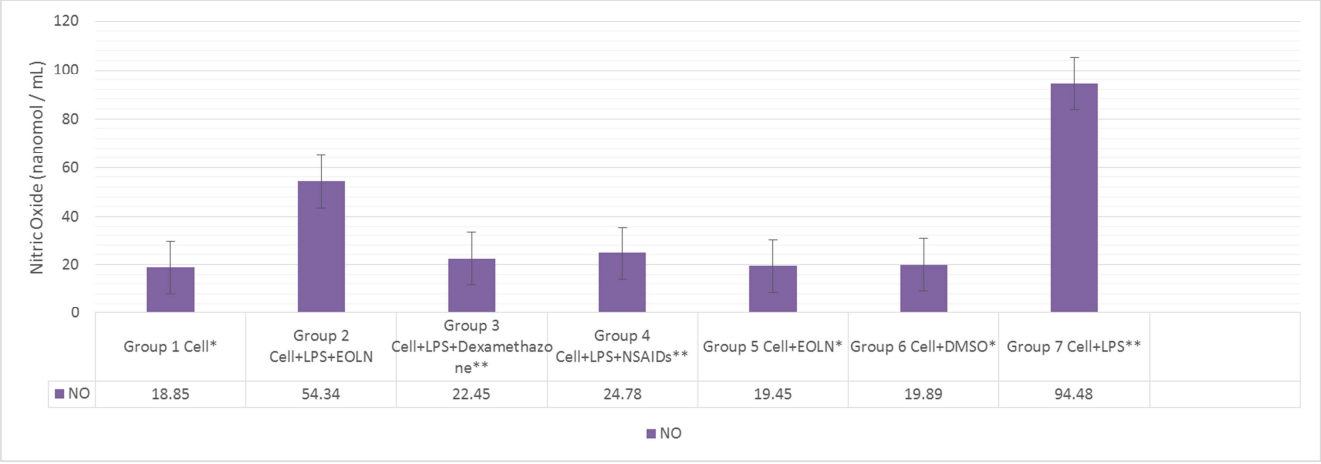

Figure 4. *The effect of Laurel alcoholic essences on no production level published in THP-1 medium using ELISA. There was a statistically significant difference between the fourth group and other groups using the student-Newman-Keuls test (mean was analyzed (mean +/- 1 SD, n=3). P<0.001, P<0.05.*

4. Discussion

There are several studies on the chemical composition of *Laurus nobilis L.* leaves [28, 38]. The study of Cherat et al. (2014) showed the main components of Laurus nobilis 8.1-cineole (31.81%) and 2-carene (13.08%). According to a recent report by Captuto et al. (2017), 1,8-cineole (31.9%), sabinene (12.2%), and linalool (10.2%) were the main components. According to these reports, Ozcan et al. (2005) suggested that 1,8-cineole (51.73-68.48%) is a major component of *Laurus nobilis L.* 1,8-Cineol has been shown to exert anti-inflammatory and antioxidant effects in acute pancreatitis and protect against influenza viral infection in mice [39-41]. In the present study, we show for the first time that the anti-inflammatory effects of Laurel alcoholic essences are not limited to synoviocytes and fibroblasts but also THP-1 cells. We proved that Bay laurel alcoholic essences expresses strong pro-inflammatory cytokine gene expression, TNF-a and IL-1b in an LPS-stimulated monocyte/macrophage cell model as well as suppresses TNF-a, IL-1b, COX-2 gene expression, Suppresses iL-6, and iNOS in cultured chondrocytes. Recently, several studies have been conducted to investigate the reduction of chemical consumption and change to herbal products and herbal medicines [42]. 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 [43]. Of course, the possible disadvantages and their interaction with other drugs should be considered. The present study investigated the anti-inflammatory effects of Bay laurel alcoholic essences and whether it could play a significant role in the treatment of patients with osteoarthritis. 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 [44]. In this study, an increase in NO production and an increase in iNOS

expression following cell stimulation by LPS, and a decrease in its expression by odorous LAUREL alcoholic essences were proved. Extensive studies have concluded that PGE2 is the main product of inflammatory cells and acts as a pro-inflammatory mediator [45, 46]. Independently, and is involved in the acute phase reaction in inflammation [47]. In several studies, osteoarthritis joints reported high levels of IL-1 in synovial fluid, synovial membrane, cartilage, and subcutaneous bone layer [48]. In another study, increased IL-1R1 receptor expression was reported in patients with OA at the level. Considering these two cases, it can be stated that the increase in interleukin-1β expression in LPS-activated synoviocytes was reduced by Laurel alcoholic essences. This is probably since the biological activation of cartilage cells and joint fluid cells by IL-1β through interaction with the membrane receptor IL-1R2, IL-1R1, which can bind to IL-1α and IL-1Rα, done [1, 49] formation of an inactive ligand-receptor complex, which leads to inability and activation of the intracellular signal. In the pathophysiological processes of OA, TNF-α together with IL-1β is considered a key inflammatory cytokine. It has also been reported that TNF-α can 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 TNF activation in OA molecular processes. TNF-R1 expression is also increased in FLS cells. In this study, an increase in TNF-α expression following cellular stimulation by LPS and a decrease in its expression by ASEL were demonstrated and iNOS increase NO and PGE2 levels, destroying cartilage, suppressing matrix production, and inhibiting cartilage cell synthesis. Inhibition of iNOS and COX-2 production can be a useful approach to reduce joint pain and inflammation in a patient with OA [50, 51]. Our study showed that the anti-inflammatory effect of odorous LAUREL alcoholic essences suppresses PGE2 and NO production in synoviocytes and macrophage/monocyte cells, and odorous alcoholic essences act as a potent suppressor of cytokines, COX-2, and iNOS gene expression. These mediators cause OA and reducing these mediators leads to improved cartilage destruction. Laurel alcoholic essences help reduce the expression and production of these inflammatory mediators in the cells under study. The effects of odorous LAUREL alcoholic essences evaluation on both BFLS and THP-1 cell types have shown the anti-inflammatory effects of odorous LAUREL alcoholic essences, which is not limited to cartilage cells but affects monocyte/macrophage cells associated with the synovial membrane Due to the anti-inflammatory effect of Laurel alcoholic essences in reducing cytokine gene expression and inflammation along with the side effects of chemical treatments such as dexamethasone and ibuprofen in the treatment of OA, the use of Laurel alcoholic essences in the treatment of OA can be a good alternative [52, 53].

5. Conclusion

The present study showed that the ability of Laurel alcoholic essences to reduce inflammation and pain supports its potential as an effective agent in the treatment of osteoarthritis. Laurel alcoholic essences 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 the alcoholic essences of the leaves can be somewhat similar to the medicinal effects of dexamethasone and ibuprofen. Therefore, considering the side effects of these synthetic drugs, it seems that odorous Laurel alcoholic essences can be considered as an effective factor in reducing inflammation and pain in patients with osteoarthritis, but more research is needed to elucidate the mechanism of action. This material is especially needed in animal models.

Contribution of Authors

All authors participated in the experiments, discussed the data, and commented on the writing of the article.

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