



## Evaluation of the Anti-Inflammatory Properties of D-limonene Compared with Dexamethasone and Ibuprofen in Bovine Synoviocyte

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### Article Info

#### Article type:

Research Article

#### Article history:

Received: 22 July 2021

Revised: 11 June 2022

Accepted: 3 September 2022

Published online: 15 June 2023

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### ABSTRACT

**Introduction:** Osteoarthritis is a public health concern that leads to disability due to chronic pain, stiffness, sleep disturbance, and depression. D-limonene is a racemic mixture of limonene, a natural cyclic monoterpene, and the major component of the oil extracted from the citrus rind with chemo-preventive and anti-inflammatory activities. The purpose of this study was to investigate the anti-inflammatory properties of D-limonene compared with dexamethasone and ibuprofen in bovine fibroblast-like synoviocyte cells (BFLSc) as well as Human THP-1 monocyte/macrophage-like cells.

**Materials and Methods:** BFLSc was isolated from the synovial membrane of the metacarpal joint cartilage of an 8-month-old Holstein. THP-1 cells were prepared by the Pasteur Institute of Iran. Cells were cultivated and exposed to 20 ng/ml lipopolysaccharide (LPS) stimulation without, or in the presence of, ibuprofen, dexamethasone, or D-limonene (7.5 µg/ml). The gene expressions of cyclooxygenase-2 (COX-2), Interleukin-1β (IL-1β), inducible nitric oxide synthase (iNOS), and Tumor necrosis factor alpha (TNF-α) were evaluated by real-time PCR. Concentrations of Nitric Oxide (NO), and Prostaglandin-E<sub>2</sub> (PGE<sub>2</sub>) were measured by ELISA methods.

**Results:** The inhibitory effects of D-limonene included dose-dependent decreases in the expression of iNOS and COX-2 proteins ( $P < 0.05$ ). The treated cells secreted increased amounts of COX-2, IL-1β, iNOS, TNF-α, NO, and PGE<sub>2</sub> in response to LPS stimulation in all conditions. D-limonene quenched the gene expression of COX-2, IL-1β, iNOS, and TNF-α, in BFLSc, and the production of PGE<sub>2</sub> and NO in monocyte/macrophage cells alike dexamethasone, and ibuprofen.

**Conclusion:** The results obtained in this study show that D-limonene probably exerts anti-inflammatory effects through the suppression of TNF-α, IL-1β, COX-2, and iNOS and NO, PGE<sub>2</sub> production.

**Keywords:** D-limonene, Osteoarthritis, Proinflammatory cytokine, THP-1, Bovine fibroblast-like synoviocytes cells

**How to cite this article:** Maghsoudi H, Yazadanpanah E. Evaluation of the Anti-Inflammatory Properties of D-limonene Compared with Dexamethasone and Ibuprofen in Bovine Synoviocyte. *J Bas Res Med Sci.* 2023; 10(1):1-14.



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Publisher: Ilam University of Medical Sciences

### Introduction

Osteoarthritis (OA) is one of the most common musculoskeletal disorders, and it is estimated to be the eleventh leading cause of disability worldwide. OA, which is directly related to aging, is less well-

known in Asia than in the Caucasus population (1). In Asia, OA has a particular prevalence. It usually occurs after mid-life, and various factors can affect the condition. However, according to scientific issues, OA is caused by aging, age, weight, or excessive use of joints (2). The most

common joints involved in the disease are joints in the hands, the spine, and the weight-bearing joints such as the pelvis and knees (3). If OA is not caused by physical harm or disease, it is called primary osteoarthritis, which is often caused by age. As age increases, the protein in the cartilage is lost, resulting in layering. They create fine cracks in the cartilage area, which ultimately lead to the destruction of the cartilage (4). The repeated use of particularly diluted articular cartilage will gradually lead to inflammation of the cartilage, joint pain, and swelling. With the loss of cartilage function, it develops friction between the bones that cause pain and limitation, and dryness in movement. To be cartilage inflammation stimulates bone formation resulting from the process of causing bone marrow or osteophytes (4). Secondary osteoarthritis is due to several factors such as obesity, recurrent bouts, frequent surgery, metabolic disorders, congenital malformations, gout, diabetes, hormonal disorders, and problems with special occupations (5). Earlier OA can also be seen in weightlifters, footballers, and army personnel due to repeated blows to the joint tissue (ligaments, bones, and cartilage) (6). The degenerative process of osteoarthritis cannot be reversed by current treatments. Corticosteroids, nonsteroidal anti-inflammatory drugs (NSAIDs), and hyaluronic are usually used in the treatment of osteoarthritis (7). Glucocorticoids a many inflammatory, immunologic, allergic, OA and malignant disorders, and the toxicity of glucocorticoids is one of the commonest causes of iatrogenic illness associated with chronic inflammatory (8). Corticosteroids have genomic and non-genomic side effects on cells and many of their genomic side effects are caused by transactivation and transrepression (9). Considering that NSAIDs are a very effective drug for pain and inflammation, low dosage of NSAIDs can lead to significant side effects, including gastrointestinal bleeding, cardiovascular complications, and kidney toxicity.

Therefore, according to the secondary side effects, it is very important to pay attention to herbal medicines and to prevent such secondary side effects. As a result, complementary approaches for pain control as well as improving performance and quality of life have been considered for the treatment of musculoskeletal disorders.

The use of D-limonene is an option for alternative therapeutic approaches (10). One of the best sources of natural antioxidants is the phenolic compounds found in plant samples, and in plants, citrus fruits also have activity as important flavonoid reserves (11). Citrus fruits are one of the most important commercial fruit crops that grow all over the world. The production of essential oil from citrus peel has a significant economic value. Orange peel essential oil is one of the most common and important essential oils produced all over the world, and its great fame is due to its very pleasant and pleasant fragrance, which makes it easy for people to accept and benefit from its therapeutic properties (12). The main components of orange peel essential oil can be mentioned as limonene, myrcene, linalool, octanal, and decanal (13). D-limonene (Limonene, (+/-) - (Pub Chem CID:22311) also known as 1-methyl-4-(1-methylethynyl) cyclohexane, is a naturally occurring cyclic monoterpene with a lemony odor that is considered the main compound in essential oils of orange and grapefruit, jasmine, and peppermint (14). D-limonene has a proven effect on many cancers, and also has antioxidant, anti-diabetic, anti-apoptotic, and lipid peroxidation effects, preventing mitochondrial dysfunction, ROS inhibition, and preventing post-translational modification of signal transduction proteins, thus stopping the G1 cell cycle arrest and also causing the differential expression of cell cycle-related genes (15, 16). Recent studies have demonstrated the reduction of the gene expression of TNF- $\alpha$ , I-6, and IL-1 $\beta$ , and reducing the production of PGE2, NO following the treatment of LPS-stimulated RAW 264.7 cells with D-

limonene (17). In another study, the antioxidant and anti-inflammatory properties of D-limonene was proven through the regulation of iNOS, COX-2, PGE2 and ERK mouse ulcerative colitis models (18). In vivo, and in-silicon studies have proven that D-limonene has an anxiolytic-like activity which can be attributed to the involvement of GABAA receptors (19). However, the anti-inflammatory effects of D-limonene have not yet been identified, therefore, we conducted a detailed study to investigate the anti-inflammatory properties of D-limonene compared with dexamethasone and ibuprofen in BFLSc and THP-1 cells. The anti-inflammatory effect of D-limonene has been proven by researchers by inhibiting pro-inflammatory mediators and also the production of cytokines (20). Luo et al (21), demonstrated that essential oils isolated from the Lamiaceae species had anti-inflammatory activities and remarkably gene expression of TNF- $\alpha$ , nuclear NF- $\kappa$ B, IL-6, and COX-2 were suppressed.

## Materials and Methods

### Reagents

D-limonene, DMEM-F12 medium, RPMI-1640 medium, L glutamine, FBS, sodium bicarbonate, 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  $\beta$ -mercaptoethanol provided by Sigma-Aldrich, UK. PGE2 immunoassay provided by Invitrogen USA, Gentamycin provided by Daropakhsh, Tehran, Iran. Penicillin and Streptomycin provided by BIO IDEA Tehran, Iran. RNA Extraction kite, M-MLV Reverse Transcriptase, and Taq polymerase, all of the primers were ordered to the CinnaGene Iran. Amphotericin B provided by Cipla, Mumbai, India. TPP tissue culture flask TPP Techno Plastic Product provided by AG Zollatrasse Switzerland. Penicillin and

streptomycin, Trypsin EDTA 0.5%, provided by IDEABIOIDEA (BIO IDEA, Tehran, Iran), Gentamycin from Daropakhsh (Iran), Amphotericin B (Cipla, Mumbai, INDIA), Dimethyl sulfoxide, RNA isolation kit, RT-PCR, (CinaClone, and Tehran, Iran)

### Preparation of D-limonene

D-limonene provided by Sigma (Sigma Aldrich, UK) company. D-limonene with a concentration of 20 mM was prepared by dimethyl sulfoxide (DMSO) and stored at -20oC until used.

### Cell Culture

The method used for the isolation of BFLSc from synovial fluid and cultivation of THP-1 cells was modified from a method previously described (22).

### Trypan Blue Assay

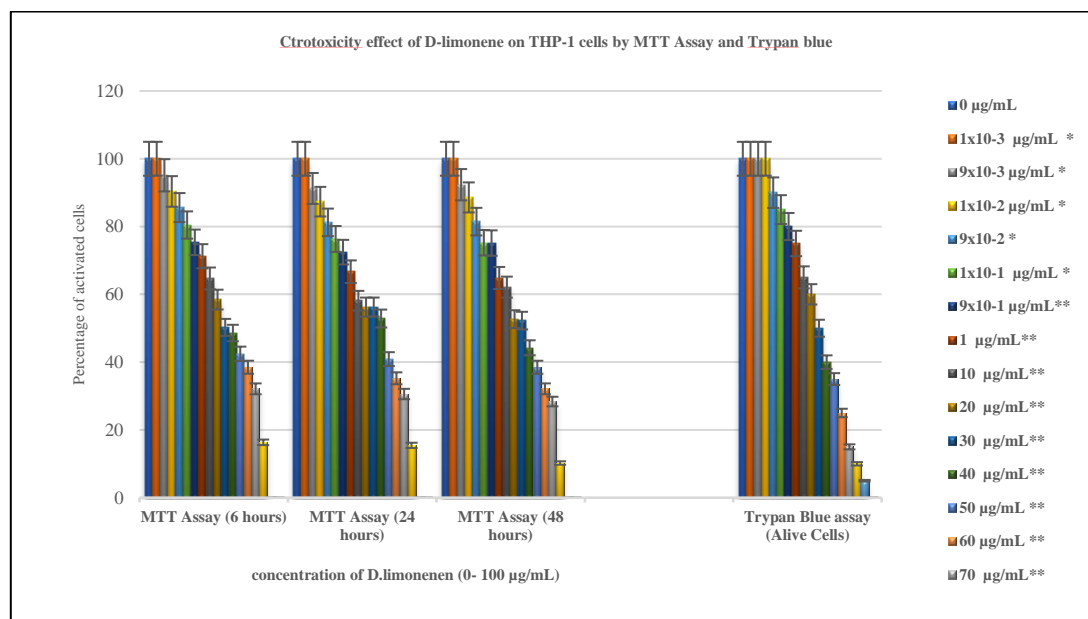
Trypan blue is an azo dye derived from toluidine. Trypan blue is used as a vital dye to distinguish living cells from non-living cells. This dye selectively turns dead tissues and cells blue (23). With increasing concentration of D-limonene, the viability of BFLSc, as well as THP-1 cells, gradually decreased, at a concentration of 20  $\mu$ g/mL and 30  $\mu$ g/mL of D-limonene in BFLSc and THP-1 cells, the viability was set at 50%, respectively (Figures 1).

### MTT Assay

The MTT assay is done at a wavelength of 570 nm to evaluate the cell viability of BFLSc and THP-1 cells. The amount of color produced is directly proportional to the number of living cells. Figure 2 shows the dose- and time-dependent cytotoxicity of the D-limonene on BFLSc and THP-1 cells over a 6- to 48-h period using the MTT assay. As shown in the figure, increasing the concentration of D-limonene gradually reduces absorption so that in the concentrations of 30 and 20  $\mu$ g/mL of D-limonene in BFLSc and THP-1 cells, the

viability was set at 50%, respectively (Figure 2), and the average of them is

respectively 6.21  $\mu\text{g/mL}$ , and 3.56  $\mu\text{g/mL}$  which are used in our study.



**Figure 1.** Cytotoxicity effect of D-limonene on THP-1 cells by Trypan blue and MTT Assay. Cells treated with increasing concentration of D-limonene over 6 to 48 hours. Cellular viability decreased in a dose-dependent manner in THP-1 cells treated with D-limonene. Data are presented as the mean  $\pm$  SE from four independent duplicate experiments. \* $P < 0.01$  and \*\* $P < 0.001$ .

### Treatment Procedure

D-limonene with a concentration of 10 mg/mL was prepared using DMSO, and the non-enriched culture medium was used to prepare the desired final concentrations. To determine the optimal concentration of D-limonene by incubation of LPS-stimulated BFLS, and THP-1 ( $5 \times 10^5$  cells/well) for 72 hours with the increasing concentration of D-limonene at (0,  $1 \times 10^{-3}$ ,  $5 \times 10^{-1}$ ,  $1 \times 10^{-2}$ ,  $5 \times 10^{-2}$ ,  $1 \times 10^{-1}$ ,  $5 \times 10^{-1}$ , 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 95 and 100  $\mu\text{g/mL}$ ), then treated with Dexamethasone sodium sulfate (4mg/mL) (24), NSAID 10 mg/mL (25), for 24 hours, and consumable culture medium in the respective flasks was analyzed for secreted PGE2 and NO.

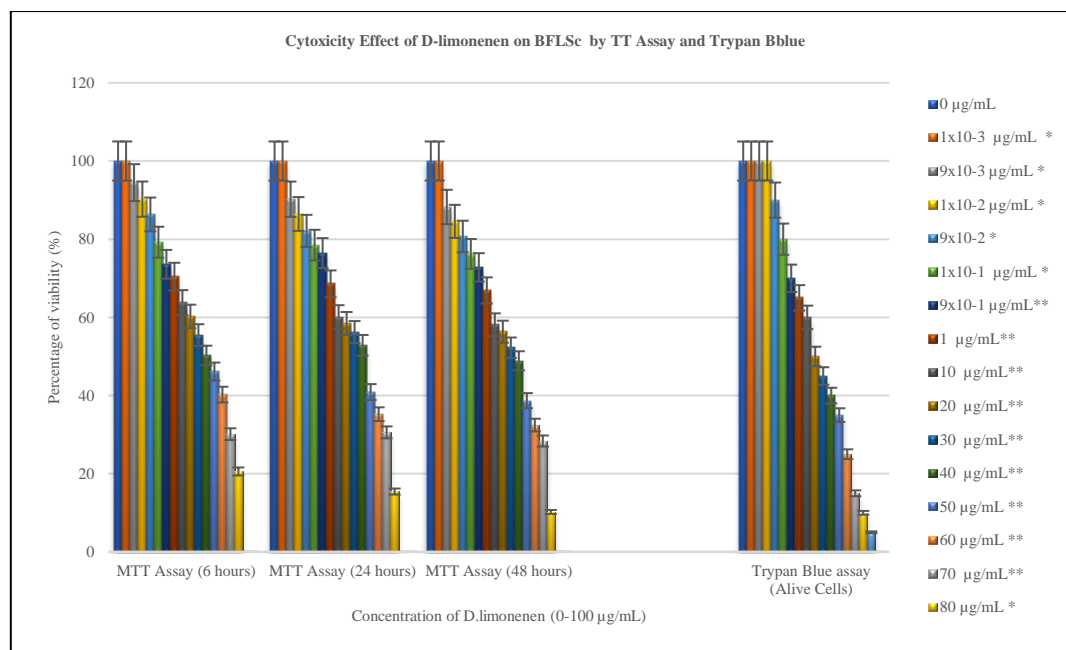
### Nitrite Determination Assay

THP-1 cells ( $1.5 \times 10^5$  cells/mL) were pre-incubated for 18 h then activated with LPS

(100 ng/mL), finally treated with various concentrations of D-limonene for a further 24 h, finally adding 100 $\mu\text{L}$  of Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthyl] ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 $\mu\text{L}$  samples of the medium. All measurements were performed in triplicate. Determination of concentration of NO<sub>2</sub><sup>-</sup> in each sample was determined by using a curve prepared from standard concentrations of NaNO<sub>2</sub> (26).

### PGE2 High-sensitivity Immunoassay

THP-1 cells ( $1.5 \times 10^5$  cells/mL) were pre-incubated for 18 h then activated with LPS (100 ng/mL), and finally treated with various concentrations of D-limonene for a further 24 h, the supernatant was centrifuged at 15322 (RCF) or g for 3 min at +4°C to pellet cells. Culture supernatants were harvested for PGE2 measurement using ELISA kit (27).



**Figure 2.** Cytotoxicity effect of D-limonene on BSLSc cells by Trypan blue, and MTT Assay. Cells treated with increasing concentration of D-limonene over 6 to 48 hours. Cellular viability decreased in a dose-dependent manner in THP-1 cells treated with D-limonene. Data are presented as the mean  $\pm$  SE from four independent duplicate experiments. \* $P < 0.01$  and \*\* $P < 0.001$ .

### Quantitative Real-Time PCR (RT-PCR)

RNA isolation and conversion to cDNA were performed based on methods published in previous articles (28). Lysis of cells with 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 iNOS, TNF- $\alpha$ , COX-2, IL-1 $\beta$ , and iIL-6 with specific primers, and the GAPDH gene as the housekeeping gene (Table 2).

### Division of Study Groups

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

**Table 1.** Classification of the studied groups.

		DMSO	Ibuprofen	Dexamethasone	D-limonene	LPS 100	
Group 1	Negative Control (Untreated cells)	U	U	U	U	U	Seeded
Group 2	The effectiveness of D-limonene on Un-treated cells	U	U	U	T	U	Seeded
Group 3	Positive Control (Treated Cells)	U	U	U	U	T	Seeded
Group 4	LPS-treated	U	U	U	T	T	Seeded
Group 5	Comparison of the effectiveness of Dexamethasone with D-limonene in LPS-treated cells	U	U	T	U	T	Seeded
Group 6	Comparison of the effectiveness of NSAIDs with D-limonene in LPS-treated cells	U	T	U	U	T	Seeded
Group 7	LPS-treated	T	U	U	U	T	Seeded
Group 8	Comparison of the effectiveness of DMSO in Un-treated cells	T	U	U	U	U	Seeded

U: untreated, T: treated, DMSO: dimethyl sulfoxide, LPS: lipopolysaccharide, NSAID: nonsteroidal anti-inflammatory drugs.

### Statistical Analysis

The Student's-test, ANOVA and REST were used to determine the statistical

significance of differences between values for a variety of experimental and control groups. Data are expressed as the mean  $\pm$  standard error of the mean (SEM) for at

least three independent experiments performed in triplicate. P values of 0.05 and 0.001 or less were considered statistically significant. The IC<sub>50</sub> values were calculated by scatter plot in an Excel graph.

## Results

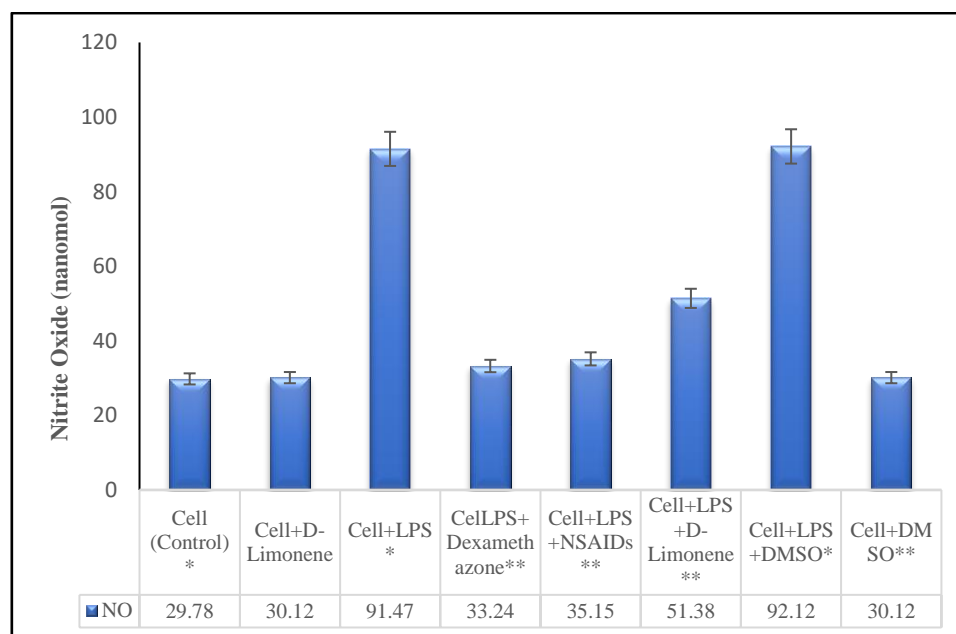
### Inhibition of NO Production by D-limonene

The production of NO in the case of treatment of cells with D-limonene (group 2) and treatment with DMSO did not affect oxide production. In the case of stimulation of the cells by LPS, a very significant increase of NO released into the culture medium was observed (group 3) and the treatment of LPS-stimulated cells by DMSO did not show any effect on reducing its production. On the other hand, the treatment of cells LPS-stimulated by dexamethasone (group 4), and ibuprofen (group 5) showed a decrease in nitrite oxide production by about 75%, and the treatment

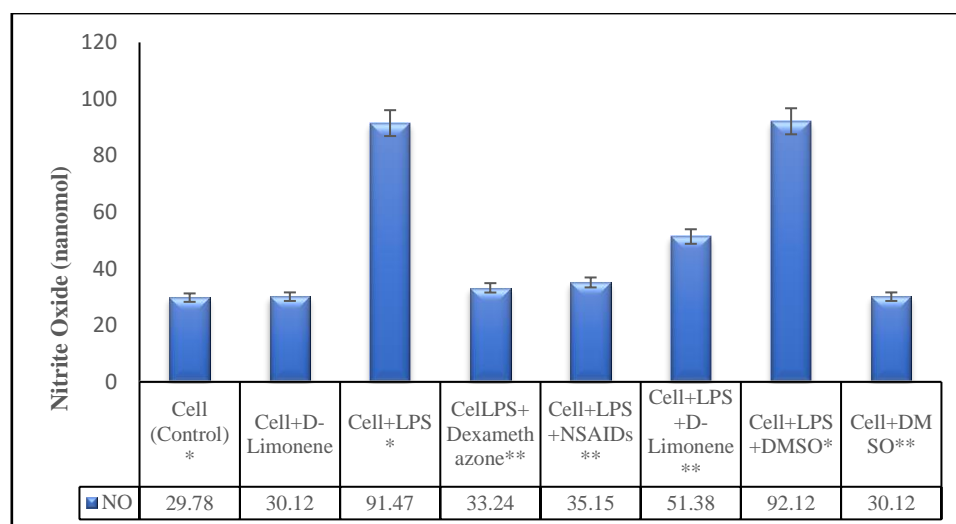
of cells LPS-stimulated by D-limonene decreased the production of nitrite oxide in about 50% specified (Figure 3).

### Decreasing PGE2 Production by D-limonene

PGE2 was measured in the supernatant of LPS-P-stimulated THP-1 for 16-18 hours in the presence of D-limonene. While PGE2 was secreted into media by unstimulated cells (treatment with D-limonene group 2, treatment with DMSO group 8 (was low, and the treatment of LPS-stimulated cells by DMSO did not show any effect on reducing its production. LPS stimulated (group 3) an increase in PGE2 production. On the other hand, the treatment of cells LPS-stimulated by dexamethasone (group 4), and ibuprofen (group 5) showed a decrease in nitrite oxide production by about 75%, and the treatment of cells LPS-stimulated by D-limonene decreased the production of PGE2 in about 50% specified (Figure 4).



**Figure 3.** The effect of D-limonene on NO levels of THP-1 cells. Cells were incubated with D-limonene for 72 hand activated with LPS for 24 h. Mean NO levels released into the cellular supernatant are shown as a percent of activated control. Statistical significances between activated control and other groups were analyze using the Student–Newman–Keuls test (mean  $\pm$  1 SD, n + 3).



**Figure 4.** The effect of D-limonene on PGE2 levels in THP-1 cells. Cells were incubated with D-limonene for 72h and activated with LPS for 24 h. Mean PGE2 levels released into the cellular supernatant are shown as a percent of activated control. Statistical significances between activated control and other groups were analyzed using the Student–Newman–Keuls test (mean  $\pm$  1 SD, n = 3).

#### Downregulation of iNOS gene expression by D-limonene

To determine the amount of D-limonene to inhibit iNOS expression, NO was examined in the supernatant of LPS-stimulated THP-1. Cells incubated with control media (group 1), D-limonene (group 2), and DMSO (group 8) displayed low levels of iNOS expression relatively to stimulated cells (Figure 3). A significant increase in the gene expression of the iNOS in case of stimulation of the cells by LPS is quite clear, and this amount is estimated to be three times that of untreated cells. On the other hand, the induction of iNOS expression in LPS-stimulated cells was significantly prevented by D-limonene treatment. (Table 2 and Figure 5). Therefore, it can be concluded that the inhibition of the production of NO (Figure 2) by D-limonene can be caused by the inhibition of iNOS expression in LPS-stimulated cells.

#### Downregulation of COX-2 Gene Expression by D-limonene

To determine whether the decrease in PGE2 production was the result of the effect of inhibiting the expression of COX-2 by D-limonene, LPS-activated BFLSc treated with D-limonene for 18 h were subjected to

RT-PCR analysis. The gene expression level of COX-2 in the first group (media alone) and the second group (D-limonene), and the eighth group (DMSO) compared to BFLSC activated were not significant (Table 2 and Figure 3). The remarkable upregulation of iNOS in LPS-stimulated cells is quite clear, and this amount is estimated to be four times that of untreated cells. Dexamethasone (group 5) and NSAIDs (group 6) suppress gene expression of COX-2, about 75% respective to cells treated with LPS. DMSO did not affect mRNA gene expression of LPS-treated (group 7). D-limonene (group 4) when compared to the activated cells (LPS) (group 3) and treated cells with Dexamethasone (group 5) and NSAIDs (group 6) suppressed mRNA gene expression of COX-2 in LPS-treated cells, by about 50%. (Table 2 and Figure 5). Therefore, it can be concluded that the inhibition of the production of PGE2 (Figure 4) by D-limonene can be caused by the inhibition of COX-2 expression in LPS-stimulated.

#### Suppression of TNF- $\alpha$ , and IL-1 $\beta$ Gene Expression by D-limonene

The gene expression level of TNF- $\alpha$  and IL-1 $\beta$  in the first group (media) and the second

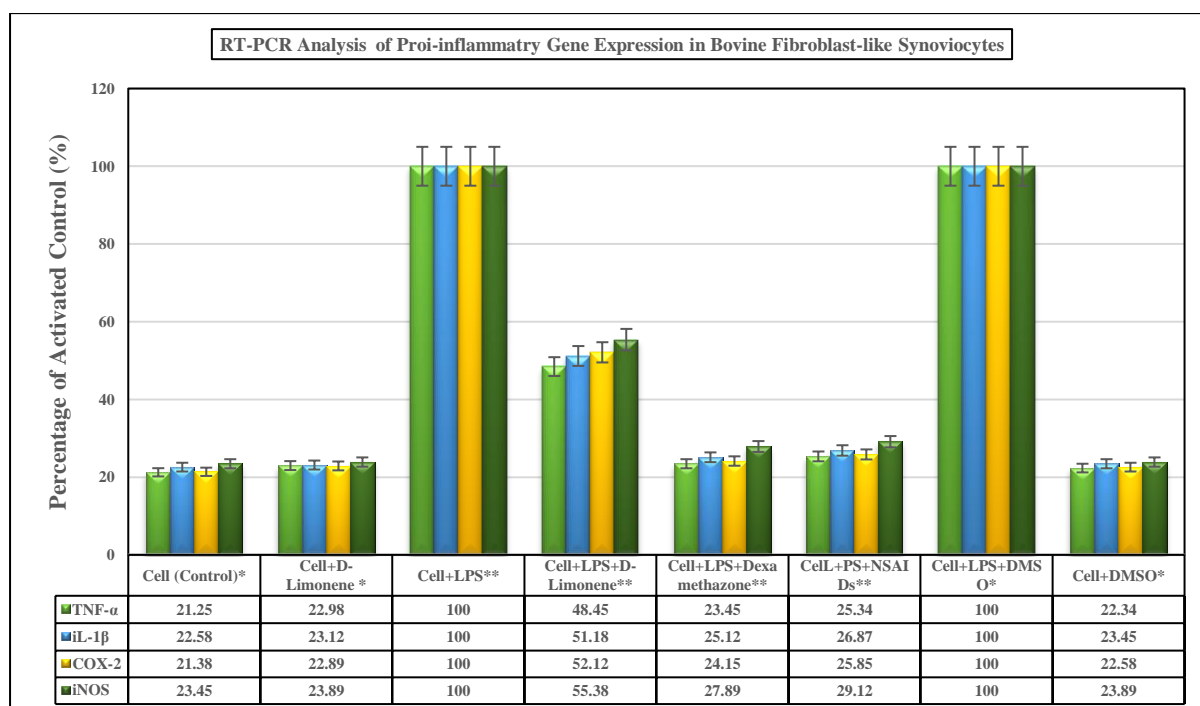
group (D-limonene), and the eighth group (DMSO) compare to those treated with LPS were not significant (Table 2 and Figure 5). The Remarkable upregulation of IL-1 $\beta$  and TNF- $\alpha$  in LPS-stimulated cells is quite, and this amount is estimated to be four times that of untreated cells. Dexamethasone (group 5) and NSAIDs (group 6) suppress gene expression of TNF- $\alpha$  and IL-1 $\beta$ , about 75% respective to cells treated with LPS.

DMSO did not affect mRNA gene expression of LPS-treated (group 7). D-limonene (group 4) when compared to the activated cells (LPS) (group 3) and treated cells with Dexamethasone (group 5) and NSAIDs (group 6) suppressed mRNA gene expression of TNF- $\alpha$  and IL-1 $\beta$  in LPS treated cell, by about 50% when compared to activated control cells (Table 2 and Figure 5).

**Table 2.** The effect of D-limonene on pro-inflammatory gene expression in BFLSc, using semi-quantitative RT-PCR. BFLSc were incubated with D-limonene for 72 hours and activated with LPS for 1 h. Normalized gene expression is shown as the percent of activated control (C + LPS).

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
TNF- $\alpha$	21.25 $\pm$ 2.81	22.98 $\pm$ 2.74	100	48.45 $\pm$ 3.42	23.45 $\pm$ 3.15	25.34 $\pm$ 3.84	100	22.34 $\pm$ 3.10
IL-1 $\beta$	22.58 $\pm$ 3.22	23.12 $\pm$ 2.31	100	51.18 $\pm$ 3.22	25.12 $\pm$ 3.46	26.87 $\pm$ 3.44	100	23.45 $\pm$ 3.59
COX-2	21.38 $\pm$ 2.92	21.38 $\pm$ 2.95	100	52.12 $\pm$ 3.55	24.15 $\pm$ 3.31	25.85 $\pm$ 3.57	100	22.58 $\pm$ 3.34
iNOS	23.45 $\pm$ 3.13	23.89 $\pm$ 3.32	100	55.38 $\pm$ 2.86	27.89 $\pm$ 3.57	29.12 $\pm$ 3.18	100	23.89 $\pm$ 3.26

Group 1: Cell (Control), Group 2: Cell + D-limonene, Group 3: Cell + LPS, Group 4: Cell + LPS + D-limonene, Group 5: Cell + LPS + Dexamethasone, Group 6: Cell + LPS+ NSAIDs, Group 7: Cell + LPS + DMSO, Group 8: Cell + DMSO.



**Figure 5.** The effect of D-limonene on pro-inflammatory gene expression in BFLSc using real-time PCR. BFLSc were incubated with ASU for 72 h and activated with LPS for 24 h. Quantification of normalized TNF- $\alpha$ , IL-1 $\beta$ , COX-2, and iNOS expressions are shown. Statistical significances between activated control and other groups were analyzed using the Student–Newman–Keuls test (mean  $\pm$ 1 SD, n = 3).

#### Suppression of TNF- $\alpha$ , and IL-6 Gene Expression by D-limonene

THP-1 cells after cultivation for 3 days in the following conditions: media (group 1), D-limonene (group 2), and DMSO (group 8) expressed low levels of TNF- $\alpha$

expression relative to LPS-treated cells (Table 3 and Figure 6). A significant upregulation of TNF- $\alpha$  and IL-6 expression in LPS-stimulated cells is quite clear, and this amount is estimated to be three times that of untreated cells (group 3). Dexamethasone (group 5) and NSAIDs



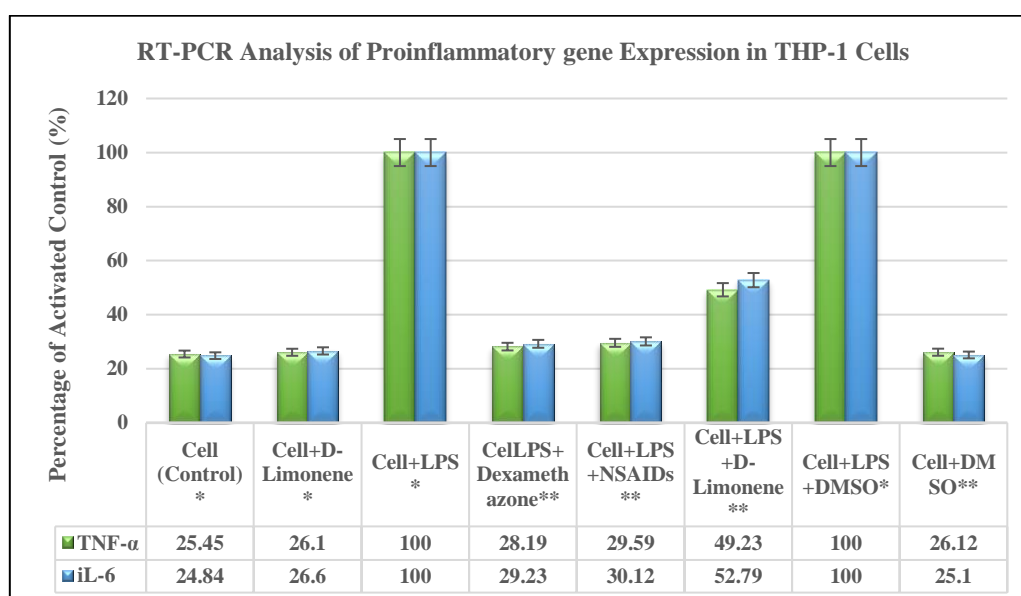
(group 6) suppress gene expression of TNF- $\alpha$  and IL-16, about 75% respective to cells treated with LPS. DMSO did not affect mRNA gene expression of LPS-treated (group 7). Treatment of stimulated cells

with D-limonene has led to a significant downregulation of TNF- $\alpha$ , and IL-6 expression by about 50% when compared to activated control cells (Table 3 and Figure 3).

**Table 3.** The effect of D--Limonene on pro-inflammatory gene expression of TNF- $\alpha$ , and IL-6 in THP-1 cells using semi-quantitative RT-PCR analysis. THP-1 cells were incubated with D-limonene for 72 h and activated with LPS for 1 h. Normalized gene expression is shown as a percent of activated control (C + LPS).

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
TNF- $\alpha$	25.45 $\pm$ 3.4	26.1 $\pm$ 2.9	100	28.19 $\pm$ 3.9	29.59 $\pm$ 3.1	49.23 $\pm$ 3.7	100	26.12 $\pm$ 3.4
IL-6	24.84 $\pm$ 3.9	26.6 $\pm$ 2.8	100	29.23 $\pm$ 3.5	30.12 $\pm$ 3.6	52.79 $\pm$ 3.1	100	25.1 $\pm$ 3.5

Group 1: Cell (Control), Group 2: Cell + D-limonene, Group 3: Cell + LPS, Group 4: Cell + LPS + D-limonene, Group 5: Cell + LPS + Dexamethasone, Group 6: Cell + LPS+ NSAIDs, Group 7: Cell + LPS + DMSO, Group 8: Cell + DMSO.



**Figure 6.** The effect of ASEL on cytokine gene expression in THP-1 cells using real-time PCR. THP-1 cells were incubated with ASEL for 72 h and activated with LPS for 24 h. Quantification of normalized TNF- $\alpha$  and IL-1b expression is shown. Statistical significances between activated control and other groups were analyzed using the Student–Newman–Keuls test (mean  $\pm$  1 SD, n = 3).

## Discussion

Due to the prominent role of pro-inflammatory mediators and cytokines in the process of osteoarthritis, which has been proven by other researchers (29), the present study aimed to investigate the effect of D-limonene on the expression of proinflammatory cytokines, and inflammatory mediators in BFLSc, and THP-1 cells stimulated by LPS were investigated. The inflammatory process is caused by a series of cellular activities, including cellular and vascular actions along with the secretion of specific

hormones (30). Primary osteoarthritis is a chronic degenerative process that leads to articular cartilage damage and the occurrence of secondary inflammation in the synovium causes rapid cartilage destruction and as a result increases the expression and production of pro-inflammatory cytokines and other inflammatory mediators, such as IL-1, IL-6, TNF- $\alpha$ , nitric oxide (NO) synthesized by inducible NO synthase, and prostaglandin E2 synthesized by cyclooxygenase-2 is enhanced (31). And the regulation of the expression of pro-inflammatory genes in the process of inflammation is regulated by

the Nuclear factor kappa B (NF- $\kappa$ B) (32). Numerous isolated bioactive terpenes compounds have shown the potential to reduce inflammation by broad mechanisms (33). Stimulation of pro-inflammatory cytokines such as IL-1 and TNF- $\alpha$ , in the process of inflammation, causes the activation of NF- $\kappa$ B and its translocate to the cell nucleus and leads to the stimulation of the production of pro-inflammatory genes (34). Researchers have proven the reduction of the expression of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1, and IL-6 by D-limonene in the Raw 264.7 macrophage cell line (35, 36, 37, 38). Similar results were obtained in experiments using in vivo models such as Swiss mice, Wistar rats, and albino mice (BALB/C) (39,40). These studies have led to the inhibitory effects of D-limonene, which includes the inhibition of the NF- $\kappa$ B signaling pathway, but the mechanism of the effect of reducing the effects of the expression of pro-inflammatory cytokines is not fully known. The results obtained in this study showed that the anti-inflammatory effect of D-limonene may be obtained by suppressing the expression of TNF- $\alpha$ , IL-1 $\beta$ , COX-2, and iNOS gene expression, and NO, PGE2 production. In the process of inflammation, inflammatory mediators such, as cytokines have a very skillful and prominent role. Inducible nitric oxide synthase, nitric oxide is derived from arginine. Inducible nitric oxide synthase plays a very prominent and important role in regulating the body's immunity and defense against pathogens. PGE2 production in LPS-treated macrophages is primarily due to the activation of COX-2 gene transcription. The production of prostaglandins is catalyzed by cyclooxygenase-2, which represents their prominent role in the inflammation process. Therefore, one of the potential anti-inflammatory agents is the inhibitor of NO, and COX-2(41). After exposure to LPS, THP-1, and BFLSc, leading to increasing and expression of pro-inflammatory cytokines and inflammatory mediators. D-

limonene, at concentrations of 3.5  $\mu$ g/mL, and 6.2  $\mu$ g/mL did not produce a cytotoxicity effect, significantly prevented the production of PGE2, NO, as well as gene expression of iNOS, COX-2, IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , in LPS-treated THP-1, and BFLSc, suggesting that it down-regulated inflammatory mediators and proinflammatory cytokines. Considering that the anti-inflammatory effects of D-limonene have been identified, further studies should be evaluated to determine this underlying mechanism. Given this fact, many intracellular signaling pathways are involved in LPS-induced macrophage activation and production of proinflammatory cytokines, and inflammatory mediators. Of course, we must pay special attention to the pivotal role of nuclear factor- $\kappa$ B, and mitogen-activated protein kinase (MAPK) pathways (42). NF- $\kappa$ B is One of the most effective transcription factors, which plays a very important role in the expression of pro-inflammatory mediators and cytokines, and as a result, it has become a very suitable target for the development of strong inhibitors as new anti-inflammatory drugs (43). Given the role of NF- $\kappa$ B in modulating the expression of inflammatory mediators, it is the inhibitory effects of D-limonene may involve in the inhibition of the NF- $\kappa$ B signaling pathway. However, we cannot exclude the involvement of other transcription factors. Considering the prominent role of pro-inflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$  in OA, increasing the production of these cytokines by macrophages and synoviocytes play a significant role in the development of OA. On the other hand, upregulation of iNOS, and COX-2 leads to increasing the production of NO, and PGE2, and as a result, these changes speed up the process of OA (44).

## Conclusion

The results obtained in this study show that D-limonene probably exerts anti-inflammatory effects through the

suppression of TNF- $\alpha$ , IL-1 $\beta$ , COX-2, and iNOS and NO, PGE2 production.

### Author Contribution

All stages of this project from design to analyzing data were done by H.M U P and E.Y and H.M. wrote the paper and many

thanks to Professor Petty for her valuable comments

### Conflict of Interest

The authors had no conflict of interest in this study.

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