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The Effect of *Malva neglecta* on the Reduction of Inflammatory Agents in Patients with Osteoarthritis

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Abstract

Osteoarthritis is a common joint disease in old ages. The current therapies are not often effective, since those are associated with some side effects such as peptic ulcers and gastrointestinal bleeding. Because of the degree of inflammation associated with pain in the patients with osteoarthritis, research on the plants with anti-inflammatory properties, although low, in addition to the analgesic effects, can make a substantial contribution to the patients with osteoarthritis, because the side effects caused by steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) are not induced by them.

In this study, we investigated the medicinal plants of Iran and found the role of Malva (*Malva neglecta*) in reducing bone-joint symptoms, therefore, the plant was investigated to determine the most effective dose and to study whether the clinical effects is associated with anti-inflammatory effect. Due to the role of chondrocytes and monocyte/ macrophage cells on the process of inflammation in the osteoarthritis, our study was performed on these two cell lines, as a model similar to what occurs in the human osteoarthritis.

Keywords: Osteoarthritis; Malva; Monocytes /macrophages; Chondrocytes

Introduction

Osteoarthritis is the most common joint disease in the world, which is common in the older adults. Nowadays, the osteoarthritis is not considered only as a degenerative disease but it is found to be induced by biomechanical, biochemical and cellular phenomenon [1,2]. The osteoarthritis treatment goals include: reducing swelling and pain in suffering joint and preventing the progression in the destruction of articular cartilage. The current therapies are not often effective since those are associated with some side effects such as peptic ulcers and gastrointestinal bleeding. Because of the degree of inflammation associated with pain in the patients with osteoarthritis, research on the plants with anti-inflammatory properties, although low, in addition to the analgesic effect, can make a substantial contribution to the patients with osteoarthritis, because the side effects caused by steroidal and nonsteroidal anti-inflammatory drugs (*NSAIDs*) are not induced with them.

Based on the researches, IL-1B and TNF-α have abnormal increase in the synovial joint fluid and the cartilage of osteoarthritis patients [2]. In vitro studies have shown that both IL-1B and TNF-α stimulate prostaglandin secretion and increase the matrix-degrading proteinases such as collagenase, gelatinase, proteoglycanase, and stromelysin and plasminogen activator [3]. Osteoarthritis is more a process than a disease, however, it occurs in all situations leading to the incompatibility between the mechanical stress against the joint cartilage and the joint's ability to withstand it. The high mechanical pressure, age, and inflammation increase the levels of inflammatory cytokines, which finally cause inflammation, pain, chondrocytes and cartilage destruction [4].

Several studies have stated the harmful effects of synovial macrophages in the inflammation of joints. Also, the studies have shown that the macrophages are a primary source of pro-inflammatory cytokines, which are found at high levels in the synovial tissue of osteoarthritis patients. Over all results showed that the macrophages are involved certainly in this disease, and are seen in the joint of osteoarthritis patients [4].

In this study, we investigated the effects of Malva (Malva neglecta) on chondrocytes and monocytes like cells/ human macrophages. Human THP-1 cells are a proper model for acting as a *replacement* model for monocytes/ macrophages which have been commonly employed in the experimental researches on the pro-inflammatory mediators present in the immune response. Due to the proliferation rate of THP-1 cells, an adequate population of them was applied for the examination to produce an adequate number of habitable cells for analysis. One of the most debilitating symptoms is pain, which is very difficult to control in advanced cases, and causes the long-term and the continuous consumption of analgesic. Eventually, Malva extract is an effective drug to reduce the pain, but the effects need to be studied more closely in order to standardize the usage.

Due to the role of chondrocytes and monocyte/ macrophage cells on the process of inflammation in the osteoarthritis, our study was performed on these two cell lines, as a model similar to what occurs in the human osteoarthritis.

The Study was based on stimulating the cells with lipopolysaccharide, and inducing them to produce inflammatory cytokines IL-1B, TNF-α, PGE2 and induced No.

Then, the effect of Malva on reducing the production of inflammatory mediators was evaluated. Since limb movement disorder is more observed in the anterior limb of cattle, *due to the large weight*

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bearing, as a result, the cartilage in this area was chosen as a model to study the anti-inflammatory effects. On the other hand, the use of large animals in such research due to the more mechanical similarity to human is more considered [5]. In this study, the preparation and the scientific extraction methods of plant and the culture and subculture of prokaryotic cells were studied. The effects of the aqueous extract of Malva were also evaluated in patients with osteoarthritis, as the most common joint disease in the world. In a model similar to osteoarthritis, the changes observed in two cartilage cell lines isolated from the metacarpal joint of Holstein cow and monocytes/macrophages stimulated with lipopolysaccharide were studied. The structure of cartilage joint is attacked in this disease. In the inflammatory diseases, which cause the destruction of cartilage structure, inflammatory cytokines including TNF-α, PGE2, IL-1B and induced NO are increased that trigger the exacerbation of inflammation in the joints.

Among the pro-inflammatory cytokines involved in the development of osteoarthritis, TNF-α is considered as the main inflammation agent. TNF-α also stimulates the production of a number of inflammatory mediators in the osteoarthritis disease. For example, the expression of iNOS and COX-2 genes increases with the addition of TNF-α to chondrocytes, and consequently, the amount of NO and PGE2 increases. Previous molecular studies performed on medicinal herbs with the anti-inflammatory effects, such as avocado, have indicated that the consumption of these plants reduces the expression of inflammatory cytokine IL-1B, TNF-α, PGE2 and induced NO [6].

Material and Methods

All steps of research was proceed in Rey's Payam Noor University biotechnology laboratory in Tehran. Malva neglecta was initially obtained from Iran's center of genetic resources, and then its aqueous extraction was prepared. On the other hand, chondrocyte cells were obtained from the healthy *radiocarpal joint cartilage* of *an 8-month-old Holstein* cow from Asia Meat Industrial Complex and monocytes/macrophages (THP-1) cells were obtained from the Pasteur Institute of Iran, and were amplified in a sterile medium to the extent necessary. The next steps were accomplished completely identical and separately in the two groups of chondrocyte cells and monocytes/macrophages cells. Following the investigation of Malva extract in different concentrations on the cell samples, the amount of LC50 was determined. Then the cells were treated with LPS to cause inflammatory conditions. Then, RNA was isolated and RNA concentration was determined. In the following, isolated RNA was employed to produce cDNA using RT-PCR method, PCR was used to amplify cDNA and finally Real Time PCR was used to determine the expression levels of IL-1B, TNF-α, PGE2 and NO genes by specific primers. ELISA system was used to determine the amount of prostaglandins in the required cells. The production of nitric oxide was determined using biochemical methods.

Chemicals needed

Absolute Ethanol, collagenaseII (SIGMA) , ascorbic acid , HCl , RPM I-1640 medium , DMEM-F12 medium , HBSS solution , citric acid , dextrose, sodium bicarbonate , sodium pyruvate , B mercaptoethanol, HEPES solution , Trypan blue, Tris Base , agarose , EDTA , PBS ,

Ethidium bromide , NaoH , NaCl , KCl , potassium dihydrogen phosphate (KH2PO4), monosodium dihydrogen phosphate , sodium dodecylsulfate , SDS , Xylene Syanvland bromophenolblue , lipopolysaccharide LPS , regulatory buffers (PH = 4,7,9), antibiotics including penicillin , streptomycin , amphotericin B ,and gentamicin , Dnase I , Taq DNA Polymerase 1M , HCl , methanol , hexane , ethyl acetate , RNA extraction Kit (Takapouzist).

List of primers

Nucleotide sequences, the melting Temperature of primers and the size of PCR products are as follows:

1-Specific primers for bovine – COX2

Forward : 3′-CTC TTC CTC CTG TGC CTG AT-5′

Reverse : 5′- TG AGT ATC TTT GAC TGT GGG A-3′

Tm forward: 52/9 °C, Tm reverse: 52°C, PCR product size : 100 bp

2- Specific primers for bovine TNF – α

Forward : 3′ -TAA CAA GCC GGT AGC CCA CG-5′

Reverse : 5′ -GCA AGG GCT CTT GAT GGC AGA-3′

Tm forward : 61°C, Tm reverse : 59/4 °C, PCR product size : 100 bp

3- Specific primers for bovine IL-1β

Forward : 3′-TTC TCT CCA GCC AAC CTT CTA T -5′

Reverse : 5′-ATC TGC AGC TGG ATG TTT CCA T -3′

Tm forward: 56/5°C, Tm reverse : 57/2 °C, PCR product size : 100 bp

4- Specific primers for bovine iNOS

Forward :3′-CGG TGC TGT ATT TCC TTA CGA GGC GAA

GAA GG-5′

Reverse : 5′-GGT GCT GCT TGT TAG CAG GTC AAG TAAAGG GC -3′

Tm forward: 71.5°C, Tm reverse : 70/4°C, PCR product size : 100 bp

5- Specific primers for bovine Glyceraldehyde 3- phosphate (GAPDH)

Forward : 3′-ATT CCA CCC ACG GCA AGT T -5′

Reverse: 5′-CGC TCC TGG AAG ATG GTG AT - 3′

Tm forward: 56/3°C, Tm reverse : 56/, PCR product size : 100 bp

6- Specific primers for Human TNF-α

Forward: 3′- GAG TGA CAA GCC TGT AGC CCA TGT TGTAGC -5′

Reverse : 5′ - GCA ATG ATC CCA AAG TAG ACC TGC CCAGAC T - 3′

Tm forward : 67/1°C, Tm reverse : 69/5°C , PCR product size : 100 bp

7- Specific primers for Human IL-1β

Forward: 3′- GAA GTA CCT GAG CTC GCC ATG GAA -5′

Reverse : 5′ - CGT GCA GTT CAG TGA TCG TAC AGG -3′

Tm forward : 65°C, Tm reverse : 60/4°C, PCR product size : 100 bp

8- Specific primers for Human Glyceraldehyde 3- phosphate (GAPDH)

Forward: 3′- TGA AGG TCG GAG TCA ACG GAT TTG GT -5′

Reverse : 5′ - CAT GTG GGC CAT GAG GTC CAC CAC -3′

Tm forward : 66/1°C, Tm reverse : 67/9°C, PCR product size : 100 bp

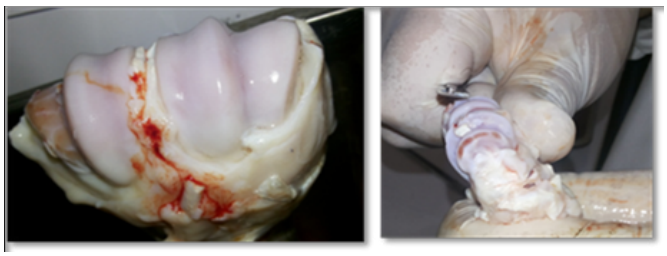


Figure 1: Samples are taking from the radiocarpal joint cartilage Payam noor shar-e- Rey Lab May-2105

Isolation, culture and proliferation of chondrocyte cells

Samples were taken from the *radiocarpal joint cartilage* of *an 8-month-old Holstein* cow. In the laboratory, in sterile conditions, the cartilages were taken from the internal and external *condyle* of the lower surface of metacarpal joint, according to what is shown in the (Figure 1).

A layer with the thickness of about 1 mm was isolated and washed out three times by 1M PBS buffer (PH = 7.4) containing a combination of antibiotics, including 50 µg/ml gentamicin and 100 unit/ml penicillin, 100 µg/ml streptomycin (PHARMATEX ITALIA) and 0.25 µg/ml Amphotripcin B (KimiaDaru-Tehran). Then, it was incubated in 220 µl/ml collagenase at 37°C for 24h. After 18h, the content of test tube was mixed for 5 min and filtered through 1mm Wire Strainer Screen, which was sterilized , and the wastes resulted from the impact of the collagenase type II were isolated from chondrocyte cells. The tube was centrifuged for 3 min, the supernatant was discarded and the pellet cells were washed four times with HBSS. The supernatant was removed with a pipette and finally deposited cells were incubated in the medium containing DMEM-F12 supplemented with FBS, 50 µg/ml ascorbic acid,100 u/ml penicillin and 0.25 µg/ml streptomycin, with a density of 5×10^5 cell in the 22.2 cm plates at a temperature of 37 °C, the humidity of 90% and 5% CO_2 to reach cell density of 80-85%.

Culture and proliferation of monocytes/macrophages (Human THP-1)

A flask containing 60 million monocytes and 60 ml enriched cell culture medium was prepared from Pasteur Institute and incubated at 37°C and 5% CO_2 for 4 to 5 days to reach cell density above 80%. The cells were resuspended slowly over 3 to 4 times in RPMI-1640 medium supplemented with L-gutamin, 10 mM HEPES , 0.1 mM sodium pyruvate , 0.05 mM B-mercaptoethanol, and a series of antibiotics including 50 µg/ml gentamicin,100 U/ml penicillin, 100 µg/ml streptomycin , 0.25 µg/ml amphotripcin B. The LC50 for chondrocyte cells was 50 µg/ml, and the mean was calculated 25 µg/ml. The LC50 for monocyte cells was 15 µg/ml.

cDNA Production: A strand of mRNA was synthesized as template using reverse transcriptase and DNA polymerase enzyme, in the following, cDNA was amplified using RT-PCR standard method [7].

Real-Time PCR: Real-Time PCR method quantitatively measures the copy number of templates such as DNA orc DNA. Therefore, it is able to eliminate the defect of PCR standard method which is End point method.

Study of the gene expression in chondrocyte cells was done by REST 2009 V2.0.13 software.

Data analysis and interpretation of results (Tables 1-4, Graph1,2)

Result	P(H1)	95% CI	Standard error	Expression	Reaction coefficient	Type	Gene
P(H0=H1)	0.990	0.139- 7.307	0.276- 3.629	1.000	0. 6973	Target	GADPH
P(H0=H1)	0.990	0.238- 4.021	0.388- 2.578	1.000	0. 6973	Target	COX-2 in chondrocyte
P(H0=H1)	0.980	8.586- 0.116	0.219- 4.564	1.000	0. 6973	Target	TNF-α in chondrocyte
P(H0=H1)	0.993	0.517- 1.938	0.691- 1.448	1.000	0. 6973	Target	IL-1B in chondrocyte
P(H0=H1)	0.965	0.116- 8.612	0.219- 4.564	1.000	0. 6973	Target	i NOS in chondrocyte
P(H0=H1)	0.989	0.012- 88.842	0.206- 4.865	1.000	0. 6973	Target	TNF-α in THP-1
P(H0=H1)	0.990	0.238- 4.201	0.388- 2.578	1.000	0. 6973	Target	GADPH in THP-1
P(H0=H1)	0.989	0.198- 5.044	0.407- 2.458	1.000	0. 6973	Target	IL-1B in THP-1

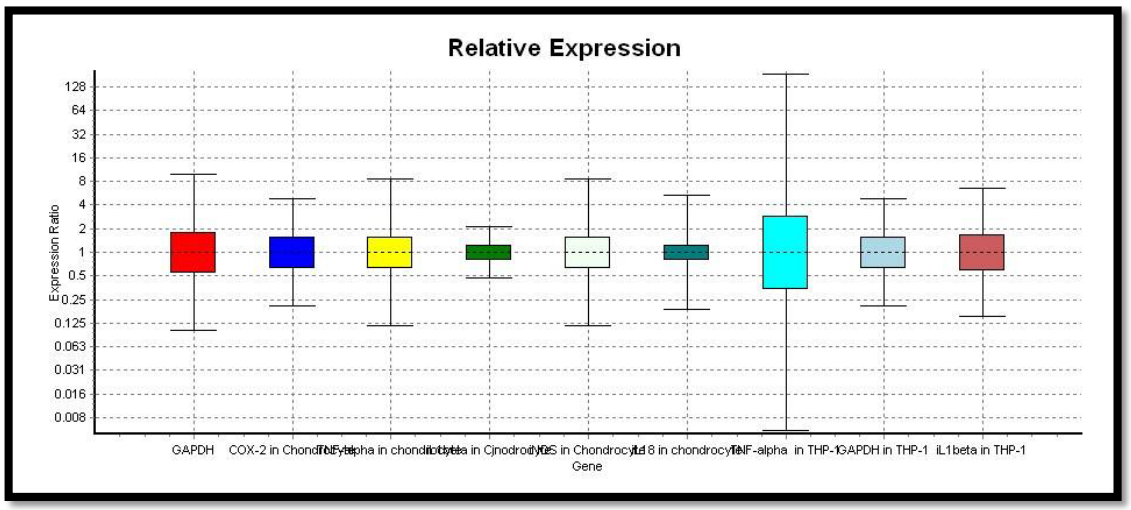


Chart1

Table 1: Study of the gene expression in chondrocyte cells with the plant extract.

result	P(H1)	CI 95%	Standard error	Expression	Reaction coefficient	Type	Gene
P(H0=H1)	0.430	0.258- 2.074	0.409- 1.685	0.827	0. 6973	Target	GADPH
P(H0=H1)	0.430	0.258- 2.074	0.409- 1.685	0.827	0. 6973	Target	COX-2 in chondrocyte
P(H0=H1)	0.463	0.150- 1.939	0.185- 1.560	0.785	0. 6973	Target	TNF-α in chondrocyte
P(H0=H1)	0.186	0.560- 3.187	0.818- 2.118	1.281	0. 6973	Target	IL-1B in chondrocyte
P(H0=H1)	0.475	0.164- 1.944	0.185- 1.568	0.807	0. 6973	Target	i NOS in chondrocyte
P(H0=H1)	0.701	0.003- 99.902	0.172- 4.612	0.751	0. 6973	Target	TNF-α in THP-1
P(H0=H1)	0.430	0.0258- 2.074	0.409- 1.685	0.827	0. 6973	Target	GADPH in THP-1
P(H0=H1)	0.265	0.308- 14.654	0.560- 5.435	1.617	0. 6973	Target	IL-1B in THP-1

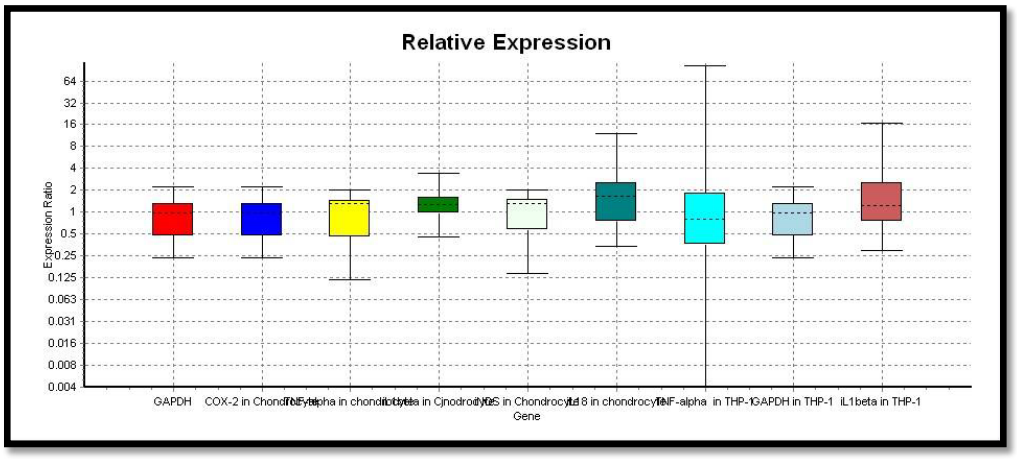


Chart2

Table 2: Study of the gene expression in chondrocyte cells with the plant extract.

result	P(H1)	CI 95%	Standard error	Expression	Reaction coefficient	Type	Gene
UP	000	45.141- 823.344	79.862- 479.974	212.941	0. 6973	Target	GADPH
UP	000	15.870- 619.758	34.079-299.127	95.666	0. 6973	Target	COX-2 in chondrocyte
UP	000	84.831- 1834.505	198.395- 969.694	446.300	0. 6973	Target	TNF-α in chondrocyte
UP	0.001	158.978- 2852.357	318.019-2188.912	828.777	0. 6973	Target	IL-1B in chondrocyte
UP	000	94.078- 3428.845	197.366- 2188.912	518.584	0. 6973	Target	i NOS in chondrocyte
UP	000	17.106- 1566.223	57.913-764.403	518.584	0. 6973	Target	TNF-α in THP-1
UP	0.001	1.124- 265.957	7.013-104.047	25.056	0. 6973	Target	GADPH in THP-1
UP	0.001	67.866- 1734.518	112.128-968.383	398.318	0. 6973	Target	IL-1B in THP-1

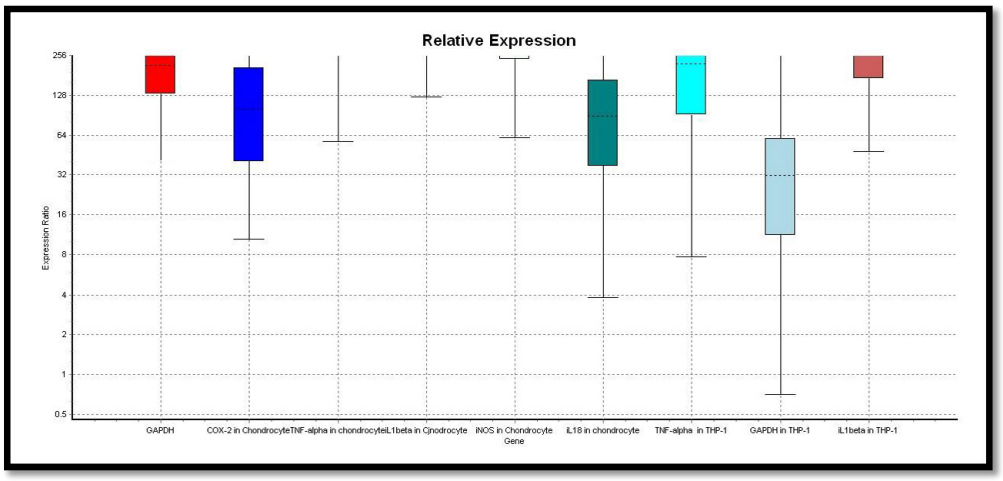


Chart 3

Table 3: Study of the gene expression in chondrocyte cells with LPS.

result	P(H1)	CI 95%	Standard error	Expression	Reaction coefficient	Type	Gene
UP	0.00	22.454- 788.686	38.296- 381.573	93.725	0. 6973	Target	GADPH
DOWN	0.001	0.002- 1.129	0.147- 0.694	0.232	0. 6973	Target	COX-2 in chondrocyte
DOWN	0.001	0.002- 1.144	0.145- 0.701	0.216	0. 6973	Target	TNF-α in chondrocyte
	0.701	0.003- 99.902	0.172- 4.612	0.751	0. 6973	Target	IL-1B in chondrocyte
DOWN	0.003	0.087- 1.689	0.162- 0.701	0.325	0. 6973	Target	i NOS in chondrocyte
DOWN	0.001	0.002- 1.102	0.147- 0.665	0.231	0. 6973	Target	TNF-α in THP-1
UP	0.001	1.148- 617.856	2.902- 356.241	32.235	0. 6973	Target	GADPH in THP-1
	0.579	0.061- 8.436	0.333- 1.978	90.760	0. 6973	Target	IL-1B in THP-1

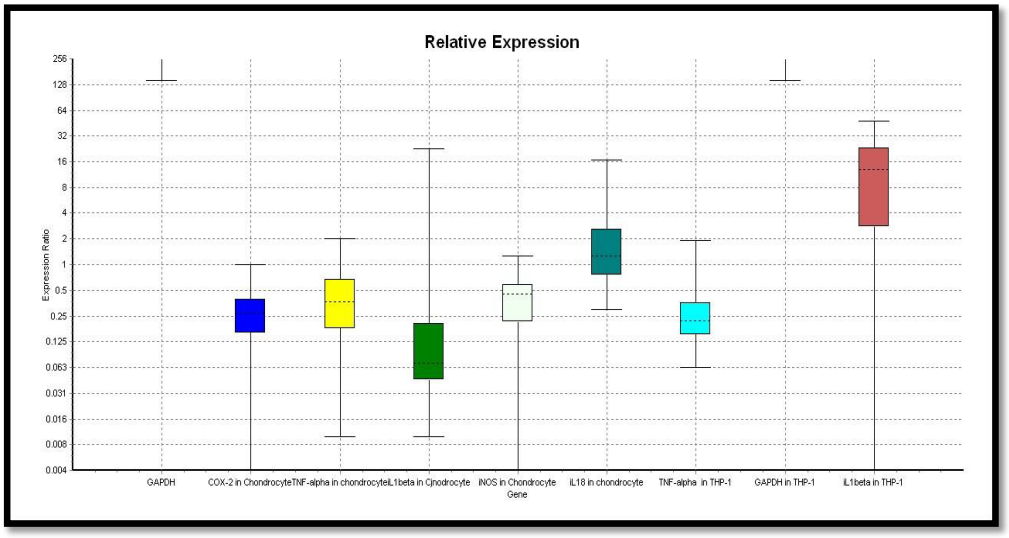
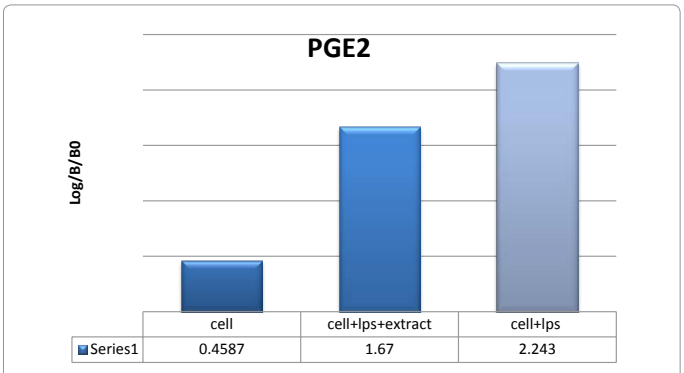
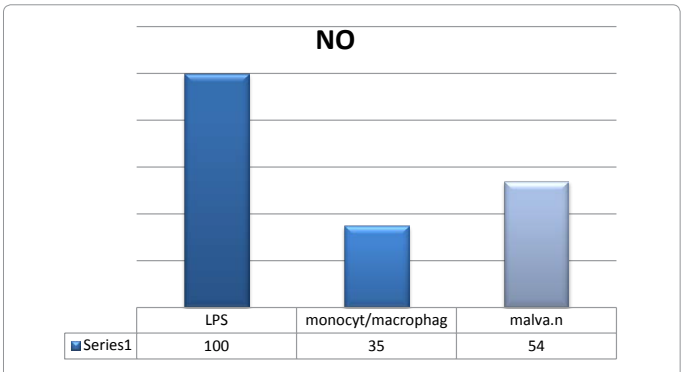


Chart 4

Table 4: Study of the gene expression in chondrocyte cells with the plant extract and LPS.



Graph 1: The effect of aqueous extract of Malva on the production of prostaglandins in monocyte cells.



Graph 2: The effect of aqueous extract of Malva on the production of nitric oxide in monocyte cells.

Conclusion

Osteoarthritis is a common joint disease in old ages. It has very advanced destructive activities and is identified by the metabolic changes and the changes on the structure of cartilage, bone and joint surfaces [8]. Management of the osteoarthritis takes place with both pharmacologic and non-pharmacologic treatments. It has been approved that the treatment methods are often ineffective in some patients [9]. Physiology of the osteoarthritis shows that the lower levels of growth factors such as IL-1 , IL-10, IL -13 and IL -8 increases the levels of cytokines such as nitrite oxide . All these series of events leads to inflammation with swelling, cartilage cell death and cartilage destruction. Some researchers believe that IL-1B is the primary factor in the destruction of cartilage matrix [10]. In the present study, we have shown that the anti-inflammatory effects of Malva extract are not limited to the chondrocytes and fibroblasts, and the effects are extended to their placement cells of monocyte/macrophage tissue. We proved that the aqueous extract of Malva, in the similar model of monocytes/macrophages which is stimulated with LPS, effectively suppresses the expression of pro-inflammatory cytokine genes. As a result, according to the role of these substances in the disease progression , and given that steroid drugs also reduce the expression of genes involved in this matter, the Malva plant can also be offered as an alternative [7]. As, as well as the expression of cytokine TNF-α gene in chondrocyte cells. If the cells be treated with LPS and the aqueous extract of Malva, measured 95.04%, considering that the steroidal and nonsteroidal drugs reduce the expression of this gene, therefore, the Malva plant can also be effective [11]. The expression of cytokine IL-1B gene decreased to 73.81% in chondrocyte cells in the presence of LPS and the aqueous extract of Malva. Since the mentioned cytokine is involved in the progression of disease, so the

application of the aqueous extract of Malva to treat osteoarthritis seems promising. The expression of COX-2 gene in the presence of the aqueous extracts of Malva and LPS decreased to 93.79%. The steroidal and nonsteroidal drugs also reduce the expression of this gene, therefore, Malva plant can also be offered as an alternative [12]. The expression of pro-inflammatory cytokines TNF-α in THP-1 cells in the presence of the aqueous extract of Malva was measured 84.76%. Furthermore, we confirmed previous findings indicating that Awakado suppresses the expression of PGE2 and NO in chondrocyte cells grown in a single layer [13]. This finding suggests that Malva affects the different cell types involved in inflammation. In this study it was shown that the Malva extract can act as a potent mediator to decrease the inflammation.

However, IL-1B is physiologically more effective than TNF-α, animal studies have shown that these two cytokines are active in a parallel manner to stimulate the weakening of cartilage that the damage increases with one of the cytokines alone [14]. Malva extract reduces the expression of both TNF-α and IL-1B cytokines that may slow the process of cartilage weakening. The matrix-degrading enzymes also induce the synthesis of pro-inflammatory factors (TNF-α, i NOS, IL-1B, COX-2). The induction of the secytokines is resulted from the high levels of nitric oxide and prostaglandin, which leads the cartilage weakening, the inhibition of matrix production, and chondrocyte cell death . In contrast, the reduction in the production of prostaglandin and nitric oxide is reported in the pain relief and inflammation [14,15]. Our results confirmed that the Malva extract reduces the expression of inflammatory genes. For the first time, our research indicated that the Malva extract as a potent inhibitor of the cytokine gene expression suppresses the production of prostaglandin and nitrite in both chondrocyte and monocyte-like/macrophage cell lines. In this study, it was shown that the Malva reduces the expression and the production of inflammatory mediators in multiple cell types in the in vitro conditions. By evaluating the effects of Malva on both cell types we proved that the anti-inflammatory effects are not limited to chondrocyte cells and the effects are extended to the monocytes- like/macrophage related to the synovial membrane. When the Malva's ability to reduce the pro-inflammatory factors was examined in various tissue cell types, its potential role as an alternative and supplement method to traditional NSAIDs (non-steroidal anti-inflammatory drug) used in the treatment of osteoarthritis was confirmed.

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