



Investigation of Changes in the Expression of Proinflammatory Cytokines Caused by Extract *Silybum marianum* L. in In-vitro and In-vivo

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Abstract: In this paper, the effects of alcoholic extract of *Silybum marianum* L. (AESM) on inflammation and reduction of cartilage destruction in a rabbit model with monosodium iodoacetate (MIA) osteoarthritis were investigated. AESM was able to effectively and dose-dependently suppress the mRNA expression of proinflammatory cytokines, including iL-6, iL-1 α , iL-18, and TNF- α in LPS-stimulated synoviocytes. Furthermore, the expression of these genes in blood and plasma was significantly diminished. The effect of AESM was compared with competing for chemical drugs such as dexamethasone and ibuprofen among control and patient groups of rabbits with OA. The middle part of cartilage in rabbits was measured by hematoxylin and eosin (H&E) staining. It was found that AESM has caused the accumulation of indispensable proteoglycans of cartilage. Background: Researches indicate that silymarin is a compound that contains various properties like anti-inflammatory, hepatoprotective, antioxidant, heart-protective, hypocholesterolemic, anti-diabetic, anticancer, and cardioprotective activities. Clinical studies have been demonstrated that silymarin has very rare side effects at high doses (>1500 mg/day). Objective: The main aim of this study was to concentrate on the treatment of OA with the help of drugs with minimal side effects to decrease arthritis following the cessation of proinflammatory enzyme cascades. Methods: RNA extraction by TRIzol method (Carlsbad, Calif., USA), Convert RNA to cDNA (Malaysia - Selangor), evaluation of gene expression by RT-PCR, simulation of OA with the help of MIA, extraction with a rotary evaporator vacuum device, the MTT technique, isolation and culture of RFLS. Cartilage staining by method hematoxylin and eosin (H&E) (Bio-Optica, Italy). In addition (MIA, 4mg/50 μ l, Sigma-Aldrich, MO, USA). Results: AESM decreased the expression of iL-6, iL-1 α , iL-18 and TNF- α genes in RFLS cells and in cartilage and were confirmed the results by Real-Time PCR. The AESM almost caused a decrease in the percentage of cells stimulated by 50% which is a significant decrease compared to Dexamethasone and ibuprofen (NSAID). Therefore, it can be a worthy therapeutic purpose for OA patients in the future. Conclusions: AESM can compete meaningfully with drugs such as dexamethasone and ibuprofen in the treatment of OA. Our experiments indicated that consumption administration of AESM reduces the expression of TNF- α , iL-6, iL-1 α and iL-18 genes and can compete well with common drugs (Dexamethasone and Ibuprofen) in the treatment of OA. The effect of AESM intensified with increasing concentration and had no side effects at very high doses.

Keywords: *Silybum marianum* L., Monosodium Iodoacetate, Pro-inflammatory Cytokines, Osteoarthritis

1. Introduction

OA is a persistent, progressive infectious joint illness, that causes cartilage, and bone destruction. In addition to progressive destruction of articular cartilage, the disease

develops with symptoms such as calcium crystal deposition (calcification) on cartilage, subchondral bone thickening, osteophyte formation, synovial inflammation, and

chondrocyte hypertrophic differentiation of chondrocytes [1, 2]. However, the most frequent symptom and reason of disability is the pain in patients with osteoarthritis, inflammation can accelerate the process of cartilage and bone destruction. One of the factors that cause inflammation is cartilage fragmentation and their floating in the synovial fluid that stimulates the synovial membrane and eventually causes synovitis. This inflamed membrane subsequently releases other inflammatory mediators that further damage the cartilage [3].

Cytokines, along with growth factors, have been shown to play a key role in the pathophysiology of osteoarthritis. They are intimately related to functional changes in the synovium as well as cartilage, followed by subcutaneous bone. Cytokines, along with growth factors, are secreted mainly by synovial membrane cells and propagated through cartilage by synovial fluid. In the process, they inactivate cartilage, resulting in the secretion of secondary metabolites such as pro-inflammatory cytokines as well as proteases. The major cytokines (anti-inflammatory) and antagonists - involved in the pathophysiology of OA such as the Interleukin 1 family, especially $iL-1\alpha$, $iL-1\beta$ as well as interleukins include $iL-4$, $iL-6$, $iL-8$, $iL-10$, $iL-11$, $iL-13$, $iL-17$ besides $TNF-\alpha$ and $iL-1Ra$. Growth factors such as $TGF-\beta$, FGF, PDGF, and IGF are involved in OA, in which $TGF-\beta$ has both a synthetic and a catabolic effect that depends on several factors such as concentration, target cell, and related tissue [4]. It is right that pro-inflammatory cytokines, especially $iL-1\beta$ and $TNF-\alpha$, play a major function in the onset and progression of the OA so that $iL-1\beta$ causes cartilage destruction and $TNF-\alpha$ promoting the inflammatory process [5, 6].

Cartilage and synovial cells are stimulated by $iL-1$ beta and $TNF-\alpha$, and produce other cytokines such as LIF, $iL-6$ and $iL-8$, as well as prostaglandin E2 or PGE2 [7]. The cytokine $iL-1\beta$ plays a potential role in OA. It can independently induce catabolic effects and inflammatory response and combine with other intermediaries. It is one of 11 members of the IL-1 (IL-1F) family [8]. In patients with osteoarthritis, it is observed that the concentration of $iL-1\beta$ in the joint fluid and synovial membrane, as well as cartilage, is significantly increased.

In the course of OA, Tumor necrosis factor- α ($TNF-\alpha$), together with $iL-1\beta$ have been identified as two very important and fundamental factors occurring in the pathophysiological processes [9]. $TNF-\alpha$ is made and splashed by the similar joint cells produced $iL-1\beta$, and an increase in its concentration is also noticed in the similar parts, like SF, SM, cartilage, and SCB layer, where rose amounts of $iL-1\beta$ are also noted. Besides the FLS cells, tumor necrosis factor receptors 1 are rose [10].

Interleukin-6 is a complex distinguished by comprehensive interactions in actions taking place in human beings. It may be classified as anti-inflammatory interaction which powerfully operates the immune system and raises the inflammatory responses, even if several are considered of its results [11]. $iL-6$ production in damaged joint tissues is normally in reaction to Interleukin - 1β , $TNF-\alpha$ also is

mostly mediated by osteoblasts, chondrocytes, adipocytes, FLS, and macrophages [12]. An increase in the concentration of interleukin-6 in serum and synovial fluid has been observed and this increase is emphatically associated with the severity of wounds on X-ray body scans [13-15]. The effect that Interleukin -6 has on articular cartilage is no different from other cytokines, but it reduces the production of type 2 collagen and MMP enzymes [16, 17]. Interleukin-6 is known as the crucial cytokine, brings about variations in the subchondral bone layer [18, 19]. It is influenced by osteoclast structure or bone defect while manifesting its cooperation with Interleukin - 1β , and TNF [20].

Another corporator of IL-18 is the Interleukin -1F family [21]. At first, pro-interleukin-18 is like precursor form which has 192 amino acids but after activation of caspase-1 or proteinase-3, it reduces to 157 amino acids and switches to active forms. The rise of caspase-1 and articular cartilage or synovium cause to an active form of IL-18 and IL- 1β [11]. The creation of interleukin-18 is produced in osteoblasts, chondrocytes, macrophage, and FLS [22]. There is a significant relationship between increasing gene expression of $iL-18$ in synovium, synovial fluid, blood serum, and cartilage and the severity of the disease seen in radiographic images [23, 24].

One of the most widely used dietary supplements containing medicinal plants is the extract of milk thistle. This supplement is one of the most popular products in the United States [25]. *Silybum marianum L.*, also known as wild artichoke is a kind of herbal plant. Silymarin is an active ingredient in milk thistle [41]. Silymarin is a combination of flavonolignanes containing silibinin A, silibinin B, isosilibinin A, isosilibinin B, silydianin, silychristin, isosilychristin as well as taxifolin flavonoid [32, 64]. Silymarin consists of approximately 50 to 60% of Silibinin, 20% of Silicristin, 10% of Silydianin and 5% of Isosilybin and silibinin has the highest biological activity [26, 27]. Milk thistle is discovered throughout the milk thistle, nevertheless is more focused on its seeds and fruits. The seeds of milk thistle hold a relatively high level of oil, about 18-31 percent rich in unsaturated fatty acids, of which 42-54 percent is linoleic acid and 21-36 percent is oleic acid [37]. Silymarin is a compound contains various properties like anti-inflammatory hepatoprotective, antioxidant [28, 29], heart-protective [31, 32], hypocholesterolemic [32], anti-diabetic [33], anticancer [34, 35], and cardioprotective activities. Clinical studies have been demonstrated that silymarin has very rare side effects at high doses (>1500 mg/day), however it has quick metabolism and penurious absorption [36].

Although various treatment options are used to manage OA, these options do not affect on preventing the process of their degradation. Regularly used pharmacological agents include corticosteroids, nonsteroidal anti-inflammatory drugs (NSAIDs), and hyaluronan. Especially NSAIDs which are widely used and their long-term use is associated with serious side effects such as gastrointestinal lesions [42]. The use of effective therapies with fewer side effects has led patients with OA to think of a way to control pain and confirm their function and quality of life [37].

The intention of these experiments is to treat OA and acquiring a path to block the process of articular cartilage destruction. In this study, a new treatment method based on herbal medicines for the treatment and reduction of disease symptoms and pain was evaluated by using the AESM (Alcoholic Extract *Silybum marianum* L), furthermore to protect patients from the severe side effects of common chemical drugs. Dexamethasone and ibuprofen were used as steroids and nonsteroidal anti-inflammatory drugs (NSAIDs) in this experiment. Meanwhile, the rabbits became infected using monosodium iodoacetate (MIA) [38].

2. Material and Methods

All stages of this experiment were performed at the Rey's Payame Noor University biotechnology laboratory in Tehran.

2.1. Chemical Reagents

TAK (TAK-242) or the TLR4 signaling pathway suppressor as well as anti-TLR4 production of Sigma-Aldrich (St. Louis, MO, USA), alkaline phosphates conjugate, Secondary antibody consist of Goat Anti-Rabbit IgG (H+L) (Sigma-Aldrich, Germany), MTT or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, CD90, CD68, CD14, and Vimentin, dimethylsulfoxide (DMSO) (Sigma-Aldrich, MO, USA), hematoxylin and eosin staining (H&E) (Bio-Optica, Italy), Bovine serum albumin (BSA), Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium, and 0.25% trypsinethylendiaminetetraacetic acid (trypsin-EDTA). (Gibco, Life Technologies Corp, USA). TRIzol reagent (Carlsbad, Calif., USA). In addition (MIA, 4mg/50µl, Sigma-Aldrich, MO, USA) and RNA isolation kit (Cinna Pure RNA Kit Cat. No.: PR891620, IRAN Tehran).

2.2. Preparation of *Silybum marianum* L. Extract (ESM)

Silybum marianum L, was obtained from Iran's center of genetic resources. Then the plant was extracted under sterile conditions with a rotary evaporator vacuum device (Model 750, manufactured in West Germany). The ESM powder (1mg) needs to be soluble in DMSO and congested as aliquots (20 mM) at -20°C before reuse.

2.3. Ethics

In this research, the ethical ideology has been observed and acted by. We comply with international law on working

with laboratory organisms. This paper was accepted by the Ethics Committee of the University of Payame Noor Tehran/IRAN (IR.PNU.REC.1399.114).

2.4. Animals and Husbandry

Thirty healthy male New Zealand White rabbit sage 5 months, the average weight of 2.5 kg was used to investigate the role of AESM in osteoarthritis induced by MIA. The rabbits were maintained in an environment with precise temperature (25°C±1°C) and humidity settings (55%±15%), air conditioning, and regular light and dark cycles. All rabbits were retained in metal cages measuring (35×50×25 cm) and allowed to access standard food (Industrial Expansion Development Co Behparvar, Iran) and ad libitum water. at any time of the day or night.

2.4.1. Induction of OA

Monosodium and iodoacetate intra-articular injection (MIA, 4mg/50µl, Sigma Aldrich, ST. Louis, MO, USA) make the features at the beginning steps of OA disease and causes histopathological alternations and functional impairment same as the OA. MIA is a metabolic inhibitor that disrupts the Krebs cycle and prevents the activity of the enzyme GAPDH, leading to cell death. In joints, MIA can cause osteoporosis and articular cartilage degradation [39, 40], MIA sterile powder (LD50 80 mg/kg) (MIA-Sigma Aldrich, UK) was disbanded in physiological saline solution (Institute Pasture, Iran) to induce OA and 10 units of insulin syringe (equivalent to 1cc) were injected directly into the synovial space of knee joint of rabbits by a needle (a 31-gauge needle). Rabbits were anesthetized by intramuscular injection of a double compound containing ketamine hydrochloride (30 mg/kg) and Midazolam (1 mg/kg), The knee joint was disinfected with chlorhexidine. To the left knee, 0.2 ml of MIA Mg was injected intra-articularly. The same amount of physiological serum was injected into the right knee [43]. To check for the presence of dye in the joint capsule area, methylene blue solution was used and injected at different angles. According to Table 1, the rabbits were divided into 5 groups of 5. Rabbit ears were marked with indestructible ink and marked with numbers. During this study, regular water and food intake and pain at the joint injection site were measured. Blood sampling was gained before and at points 0, 7, 15, 21, 28, and 35 days after the intraarticular injection and was collected. all of the rabbits were induced with phenobarbital (LD 150mg/kg) after a month [44].

Table 1. Grouping Rabbits.

Rabbits	MIA	Dexamethasone	NSAIDs	AESM	Salin normal
Group 1	Negative	Negative	Negative	Negative	Negative
Group 2	Positive	Positive	Negative	Negative	Negative
Group 3	Positive	Negative	Positive	Negative	Negative
Group 4	Positive	Negative	Negative	Positive	Negative
Group 5	Positive	Negative	Negative	Negative	Positive

2.4.2. LD50 Silymarin and AESM, in Oral and Intra-articular Injection

Lethal dose value (LD50) estimated by the log-probit analysis method using GraphPad Prism software. The LD50 AESM was 300 mg/kg in articular injection and 1050mg/kg orally for the rabbits. The amount of LD 50 Silymarin after articular injection is 400mg/kg in mice, 385mg/kg in rats, 140 mg/kg in rabbits and dogs. LD 50 Silymarin is orally as follows: 1050mg/kg and 970mg/kg in male and female mice respectively, 825 mg/kg and 920 mg/kg in male and female rats respectively, 300mg/kg in rabbits and dogs.

2.5. Treatment Procedure for Osteoarthritis Induced by MIA in Rabbits

To reduce pain and swelling in groups 2, 3, and 4, respectively were used as Dexamethasone, Ibuprofen (NSAID) and, AESM (Alcoholic Extract *Silybum marianum L.*) inoculated intra-articularly at a dose of 0.5 mg/kg in the right knee joint. This amount was selected in accordance with previous studies [44, 45] as to entire suppress inflammation. The primitive dose of drugs was given one day after MIA ingestion, the rabbits were given a second dose of the drug 6 hours after the operation, afterward inoculation was given in the wake of 3 days till easy death at the end of a month [46]. diclofenac sodium intramuscularly injected for four successive days at a dose of 1.5mg/kg body weight (BW) twice daily until the time of euthanasia at 4 weeks [47].

2.5.1. Body Weight Measurements

Body weights were measured weekly from the inducing OA and after MIA injection At the beginning of the treatment process, also on the seventh, fourteenth, twenty-first, and twenty-eight days using an electronic balance. To reduce the impacts of feeding on the quantification, the rabbits were deprived of drinking water overnight.

2.5.2. Diameter Joint Size

The diameter joint was measured in three steps: before injection of MIA (WOMIA), after injection of MIA (WMIA) and after treating with medicines. In patients without treatment (Control group), rabbits number 1 to 5, the average of diameter joint in WOMIA stage was measures as 1612 mm and after injection of the MIA (WMIA), it was 2052 mm. As can be seen in this group, the inflammation of the joint is reduced by the time in spite of not using the chemical and anti-inflammatory medicines and pharmaceutical plant extract and reached the average 188.9 mm among 5 rabbits of this group. In patients treated with Ibuprofen, the average of diameter joint reached from 173.6 mm (in WOMIA stage) to 223.2 mm (in WMIA stage). After treating this group using the Ibuprofen, the average of diameter joint of rabbits reached to 195.2 mm and significant reduction in their diameter joint was seen. Dexamethasone had greater impact on the reduction of the average of diameter joint from 225 mm (in WMIA stage) to 184.2 mm than Ibuprofen after the treatment. Treatment using the ESM optimal effect on the

reduction of the Inflammation and the average of diameter joint and reached the average of diameter joint of the rabbits from 218 mm (in WMIA stage) to 187 mm after using AESM (Figure 1).

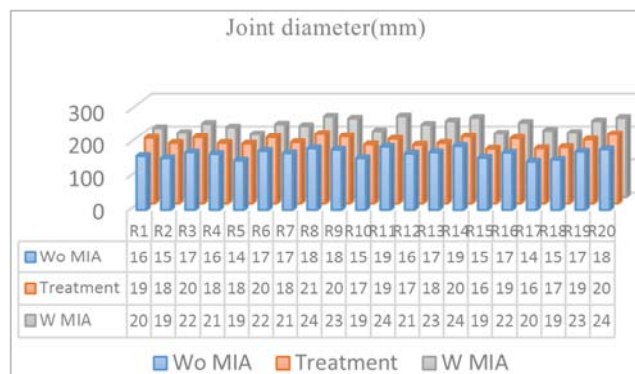


Figure 1. Joint diameter: The diameter joint was measured in three steps: before injection of MIA (WOMIA), after injection of MIA (WMIA) and after treating with medicines such as; Dexamethazone, Ibuprofen, AESM.

2.6. Cytotoxicity Assay

2.6.1. LDH Assay

As observed, in the lactate dehydrogenase method, abbreviated as LDH, the leakage of the LDH enzyme from the cytoplasm to the extracellular medium is calculated and the existence of the unique cytosolic enzyme called LDH, in the cell culture medium indicates cell membrane destruction. The cytotoxic effect of essential oil of *Fraxinus excelsior* (EOFE) was also examined using LDH assay as described by Linford [33]. With some modifications. Briefly, RFBL 1×10^4 cells were seeded per well in 24-well microtiter plates. Cells were exposed to increasing concentration (1×10^{-3} , 9×10^{-3} , 1×10^{-2} , 9×10^{-2} , 1×10^{-1} , 9×10^{-1} , 1, 9, 18, 36, 45, 54, 63, 72, 81, 90 and $100 \mu\text{g/ml}$) of AESM for 24-48 hours, the afloat were piled up from each pit. Cell monolayers were exposed to cell lysing solution at room temperature for half an hour and then they were gathered. Per the producer's orders for computing the activity of lactate dehydrogenase activity (Sigma-Aldrich, UK), LDH activity was estimated in two procedures and cell lysate (Figure 6). The sorption was regulated at 490 nanometers using a 96-well plate ELISA reader. Using the following formula, the percentage of LDH secreted from the cell was measured: $\text{LDH liberation} = (\text{sorption of the floating}) / (\text{sorption of the floating and cell cytolysis}) \times 100$.

2.6.2. MTT Assay

To evaluate the function of mitochondrial activity in living cells, cell number count or cell viability is used. The MTT technique is uncomplicated, correct, and surrenders repeatable outcomes. The main part is MTT or (3-[5-diphenyl tetrazolium bromide, 4,5- dimethylthiazol-2-yl]-2). Solutions of MTT are sandy in color, melted in medium, or balanced salt solutions without phenol red. In living cells, mitochondrial dehydrogenases split the tetrazolium ring, building purple formase crystals. These crystals are

unsoluble in aqueous solutions. At 570 nm, the amount of dye absorption is calculated [49]. Briefly, RFBL 1×10^4 cells were seeded per well in 24-well microtiter plates. Cells were exposed to increasing concentration (1×10^{-3} , 9×10^{-3} , 1×10^{-2} , 9×10^{-2} , 1×10^{-1} , 9×10^{-1} , 1, 9, 18, 27, 36, 45, 54, 63, 72, 81, 90 and 100 $\mu\text{g/ml}$) of AESM for 24 hours. There is no negative result after the reception with the highest concentration of $1 \times 10^{-1} \mu\text{g/ml}$. What leads to cell death and less survival is concentrations higher than 9 micrograms per milliliter (LC50 is 45 $\mu\text{g/ml}$ and its average 12.38 $\mu\text{g/ml}$) [50] (Figure 6).

2.7. Measurement of Serum Proinflammatory Cytokine Gene Expression

In this study, 10 ml of whole blood was collected from the vena cava, and after 10 minutes of centrifugation at 3000 rpm, the serum was isolated. Serum concentrations of Proinflammatory Cytokine were measured using RT-PCR.

2.8. Measurement of Synovial Proinflammatory Cytokine Gene Expression

In this study, synovial fluid was gathered from the knee and centrifuged for 4 minutes at 4°C at 12,500 rpm. Synovial concentrations of Proinflammatory Cytokine Gene expression were measured using RT-PCR.

2.9. Measurement of Proinflammatory Cytokine Gene Expression in RFLS

2.9.1. Isolation of Rabbit FLSs (RFLS)

In this study, after oblation, the knees were anatomized and the articulation cartilage of the femoral condyles and the menisci and the tibial plates were cut up and washed three times with PBS (one molar pH: 7.2).

2.9.2. Culture of Rabbit FLSs (RFLS)

RFLS cells were incubated with Collagenase for 3 hours at 37°C , digestion reaction was centrifuged at 7500RPM, and the sediment was transferred to a T-25 tissue flask containing, Dulbecco's Modified Eagle Medium: Nutrient Mix F-12 complemented with 10% fetal bovine serum, 50 $\mu\text{g/ml}$ ascorbic acid (Sigma-Aldrich, UK), Amphotericin B 0.25 $\mu\text{g/ml}$ (Cipla, Mumbai, INDIA), Penicillin 100 U/ml, 50 $\mu\text{g/ml}$ Gentamycin (Daropakhsh, IRAN), Streptomycin 100 $\mu\text{g/ml}$ (BIO IDEA, IRAN), Amphotericin B 0.25 $\mu\text{g/ml}$ (Cipla, Mumbai, INDIA). The flasks were put in an incubator at 37°C under a concentration of 5% CO_2 and 99% O_2 . By

using immunocytochemical analysis, the results showed the presence of positive surface marker vimentin and the absence of CD68 macrophage markers to recognize FLSs. The data manifested that multiplying FLSs are enhanced through three motions, they contain > 98% of cells [51].

2.10. RNA Isolation and RT-PCR

2.10.1. RNA Extraction Stages

RFLSs were plated at a density of 6×10^6 per flask. The cells were pretreated with the 12.38 $\mu\text{g/ml}$ of AESM for 72h and then incubated with 100 ng/ml LPS for 1h, then harvested the cells with Trypsin-EDTA 0.5%. After lysis of cells by using the TRIzol method (SinaClon, Iran), RNA was elicited with chloroform. In pursuit of severe turbulence and incubation for 3 min, all samples were centrifuged after that the aqueous phase comprising RNA was gathered. The tubes containing RNA were precipitated due to Isopropyl alcohol (Sigma-Aldrich, UK) then resuspended in RNase-free water (San Francisco, CA, USA). Spectrophotometry (Dayton, USA)(260nm-280 nm) was used to measure total RNA to assess RNA concentration and accuracy and to confirm the accuracy of the test. If the samples become infected with DNA, the DNase enzyme can be used to remove it.

2.10.2. Complementary DNA Synthesis

To perform the RT-PCR process, after extracting the RNA, we converted it to cDNA based on the relevant protocol using the Vivantis kit (Malaysia-Selangor). The samples were then amplified by PCR and reached a sufficient concentration.

2.10.3. Quantitative RT-PCR

Semi-quantitative RT-PCR was performed using specific primers, the characteristics of which are summarized in Table 2. Thermal cycling was performed on a MJ Research Tetrad Thermal Cycler (Bio-Rad, Hercules, CA, USA) using 2 μl cDNA template and reagents from the SuperTaqPlus Kit (Ambion, Austin, TX, USA). Each sample was analyzed in duplicate. Amplified PCR products were visualized under UV illumination on a 1.5% agarose gel. The band intensity of the PCR products was quantified using Adobe Photoshop C2 software and normalized to the GAPDH housekeeping gene. Three independent experiments ($n \pm 3$) were performed and the mean ± 1 standard deviation is shown in the figures. RT-PCR was performed using a TWO-STEP RT-PCR PreMix kit (Vivantis, USA), according to the manufacturer's instructions.

Table 2. Primer sequencing (TNF- α , iL-1 α , iL-6, iL-18).

Number	Enzyme	Primer sequencing	TM	Start	End	PCR Product size
01	FP TNF α	ACCACGTAGCCGTGTTTCAGG	62.43	1160	1179	483
02	RP TNF α	CTGACCAGTAGGGCGGTTAC	59.82	1642	1623	
03	FP IL-1 α	CATCTGGTGACATGGCAAG	59.83	787	806	946
04	RP IL-1 α	GTGGCCCATATCAGCTCAA	60.34	1734	1714	
05	FP IL-6	ACCTGCCTGCTGAGAATCAC	60.04	398	398	381
06	RP IL-6	GGATGGTGTGTCTGACCGT	59.97	759	740	
07	FP IL-18	TCATTAGGAATAAAGATGGC				
08	RP IL-18	CTAATTCTGTGTTTGGACACT				

2.10.4. AESM Inhibits LPS-stimulated TLR4 Activation in RFLSs Cells

In order to prove the anti-inflammatory affects of AESM, in addition to controlling proinflammatory cytokines in the blood and plasma, the activity of TLR4 receptors in LPS-induced RFLS cultured cells (50 ng / ml) was investigated. TLR4 has been determined to play a vital role in activating the NF- κ B pathway. AESM plays a negative regulatory role in the TLR4/MyD88/NF- κ B signalling pathway. The total RNA has been extracted by the protocol of TRIzol method (SinaClon, Iran) With the RNase free Dnase, the DNA was removed. The concentration of RNA by using

spectrophotometer with A260 and A280 was evaluated and the template of cDNA from RT-PCR was 2 μ . Primers of PCR has been noticed and revealed at the tables 2 and 3.

2.10.5. Statistical Discussion

All of the results were get hold by one-way analysis of variance (ANOVA), REST software version.20 then the Dunnett's post hoc test was performed with the help of the GraphPad Prism version 5 (GraphPad Software Inc, CA, USA). Facts were offered as mean \pm standard fault of the mean (SEM).

Table 3. Primer sequencing (TLR4, GAPDH).

Gene	Forward sequence (5' to 3')	Reverse sequence (3' to 5')
TLR4	GGGCCTAAACCCAGTCTGTTTG	AGCCCGGTAAGTCCATGCT
GAPDH	GAGAACGGGAAGCTCGTCAT	TTGATGGTACACAAGGCAGGG

Table 4. RFLS were incubated with AESM for 72 h and activated with LPS for 1 h. Normalized gene expression is shown as percent of activated control (Cell+LPS). *P<0.05; **P<0.001.

	R.TNF α	R.IL-18	R.IL-6	R.IL-1 α
Cell	22.57 \pm 5.68	21.84 \pm 6.12	23.89 \pm 5.37	22.15 \pm 4.3
Cell+ LPS	100	100	100	100
Cell+LPS+Dexamethazone	24.12 \pm 5.2**	23.45 \pm 3.98**	24.98 \pm 4.98**	24.78 \pm 4.1**
Cell+LPS+NSAIDs	23.56 \pm 5.12**	24.98 \pm 3.98**	25.04 \pm 5.24**	24.34 \pm 4.8**
Cell+LPS+Saline Normal	100	100	100	100
Cell+LPS+AESM	49.56 \pm 5.4*	45.23 \pm 3.9*	45.89 \pm 5.3*	48.21 \pm 4.5*
Cell+Saline Normal	22.84 \pm 5.1*	22.115 \pm 6.12*	24.45 \pm 5.37*	23.18 \pm 4.6*
Cell+LPS+Saline Normal	100	100	100	100

Table 5. Normalized gene expression is shown as percent of activated control. *P<0.05; **P<0.0016.

	R.TNF α	R.IL-1	R.IL-6	R.IL-18
Control	21.98 \pm 2.3	20.689 \pm 2.2	20.48 \pm 2.5	21.58 \pm 2.3
MIA	100	100	100	100
MIA+Dexamethazone**	26.08 \pm 2.6	24.98 \pm 2.5	26.35 \pm 2.3	25.14 \pm 2.4
MIA+NSAID**	28.45 \pm 2.5	26.34 \pm 2.8	28.98 \pm 2.6	27.14 \pm 2.5
MIA+Saline Normal	100	100	100	100
MIA+AESM*	50.56 \pm 2.4	29.85 \pm 2.3	50.45 \pm 2.5	49.48 \pm 2.8

Table 6. RFLS were incubated with AESM for 72h and activated with LPS for 1h. Normalized gene expression is shown as percent of activated control. *P<0.05; **P<0.001.

	R.TNF α	R.IL-1	R.IL-6	R.IL-18
Control	21.56 \pm 3.6	22.38 \pm 3.2	20.48 \pm 3.8	22.09 \pm 5.1
MIA	100	100	100	100
MIA+Dexamethazone**	23.98 \pm 3.9	24.59 \pm 3.8	23.94 \pm 4.1	24.08 \pm 4.9
MIA+NSAID**	26.09 \pm 3.2	26.98 \pm 3.6	27.89 \pm 3.9	27.24 \pm 4.8
MIA+Saline Normal	100	100	100	100
MIA+AESM*	53.18 \pm 3.5	55.09 \pm 4.5	52.48 \pm 4.2	53.49 \pm 5.3

Table 7. Effects of Treatment on Knee Thickness.

Rabbits	Before disease	After MIA	After Tretment
1	17	20	18
2	14	19	15
3	16	21	16
4	17	20	18
5	15	19	16
Average	15.8	19.8	16.6

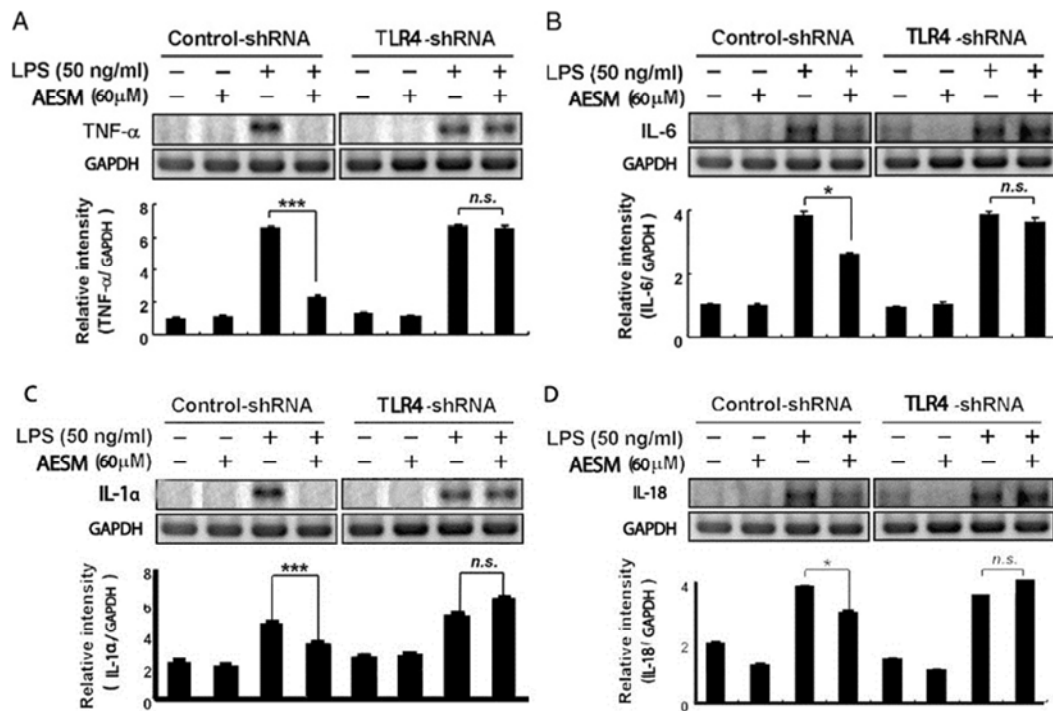


Figure 2. AESM inhibits LPS-stimulated TLR4 activation in RFLSs cells. After collecting of total RNA from Rabbits FLSS (RFLSs) cells. RFLSs cells were stimulated with LPS (50ng/ml) for 6h and then pretreated with 60μM AESM. A, gene expression of TNF-α and GAPDH were examined by RT-PCR. B, gene expression of iL-6 and GAPDH were examined by RT-PCR. C, gene expression of iL-1α and GAPDH were measured by RT-PCR. D, gene expression of iL-18 and GAPDH were examined by RT-PCR. All information were expressed as the mean±SD (n=3). T-student, $P<0.05^*$, $P<0.01$ and $P<0.001^*$.

3. Results

3.1. Effects of Treatment on Body Weight

At 35 days after surgery, the average weight of rabbits in each group was 2.39 ± 0.15 , respectively. No significant difference in body weight was observed between the groups.

3.2. Effects of Treatment on Knee Thickness

The diameter of the rabbit joints was measured accurately before OA and then one week after OA as well as after treatment. The mean joint diameter of rabbits was measured before the onset of the disease at 15.8 mm and this value increased to an average of 19.8 mm after MIA. Finally, after treatment, the rabbit joint diameter, although generally reduced, did not reach the initial mean and the average was recorded at 16.6 mm (Table 7).

3.3. Cytotoxicity Assays

Although cell cultures were served 24 hour with AESM, it did not bring about any changes in cell numbers at the concentrations of (1×10^{-3} , 9×10^{-3} , 1×10^{-2} , 9×10^{-2} , 1×10^{-1} , 9×10^{-1} , 1, 9, 18, 36, 45, 54, 63, 72, 81, 90 and $100\mu\text{g/ml}$). There is no sign of side effects higher than $1\times10^{-10}\mu\text{g/ml}$. Enhancement of concentration $9\mu\text{g/ml}$ leads to a particular diminished in the cell viability (LC_{50} was calculated $45\mu\text{g/ml}$ and its average was $12.38\mu\text{g/ml}$) [50] (Figure 6).

3.4. The Result of AESM in RFLS

RFLS cells cultured for 3 days in the medium alone, AESM leads to decrease the level of genes such as iL-1, iL-6, iL-18, and TNF-α regarding to LPS-induced chondrocytes (Table 4 and Figure 3). Operated of cells by 20 ng/mL of LPS caused a remarkable rise in the articulation of the proinflammatory cytokines. As expected, dexamethasone and NSAID had a significant effect on reducing the expression of cytokines in stimulated cells, but DMSO did not affect. The AESM almost caused a decrease in the percentage of cells stimulated by 50% which is a significant decrease compared to Dexamethasone and NSAID (Tables 4, 6 and Figure 3).

3.5. The Effect of AESM on Cytokine Gene Expression in Cartilages

The plant extract was able to compete with chemical drugs and also had a significant effect on reducing inflammation and decreasing the process of joint cartilage destruction. The highest impact effect of AESM after the iL-1 at 29.85 is related to iL-18 at 49.48 (Table 5 and Figure 4). Similar results were obtained in the articulation of the Interleukin-6 and Tumor Necrosis Factor-α genes which was acceptable.

3.6. The Change in Serum Level of Proinflammatory Cytokines

Examination of blood samples and analysis of its data indicated that consumption of AESM not only significantly

reduced the expression of pro-inflammatory genes in the blood, especially iL-6 but also proved its anti-inflammatory properties and confirmed the results of former tests. Therefore, it can be a worthy therapeutic purpose for OA

patients in the future. It is certainly predicted that the effects of AESM on reducing the expression of proinflammatory genes will be more severe with increasing concentration. (Figure 5).

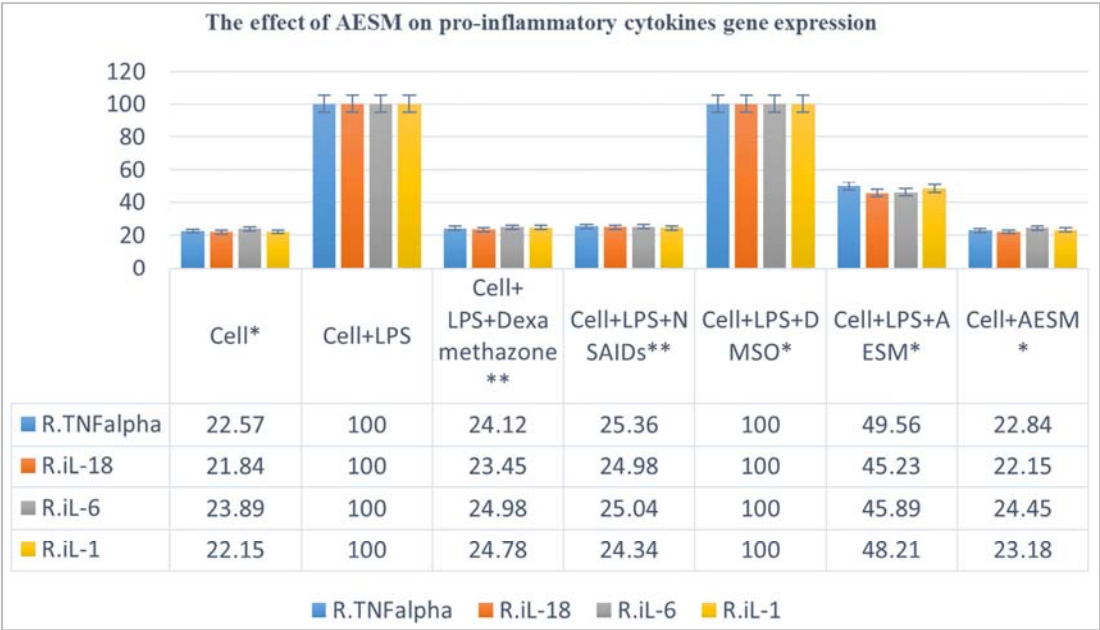


Figure 3. The effects of AESM on pro-inflammatory gene expression in RFLS using Real-Time PCR. RFLS were incubated with AESM for 72h and activated with LPS for 24h. Quantification of normalized iL-1, iL-6, iL-18 and TNF- α expression are shown. Statistical significances between cell and cell+AESM and other groups were analyzed using the student Newman Keuls test (mean \pm 1 SD, n=3).

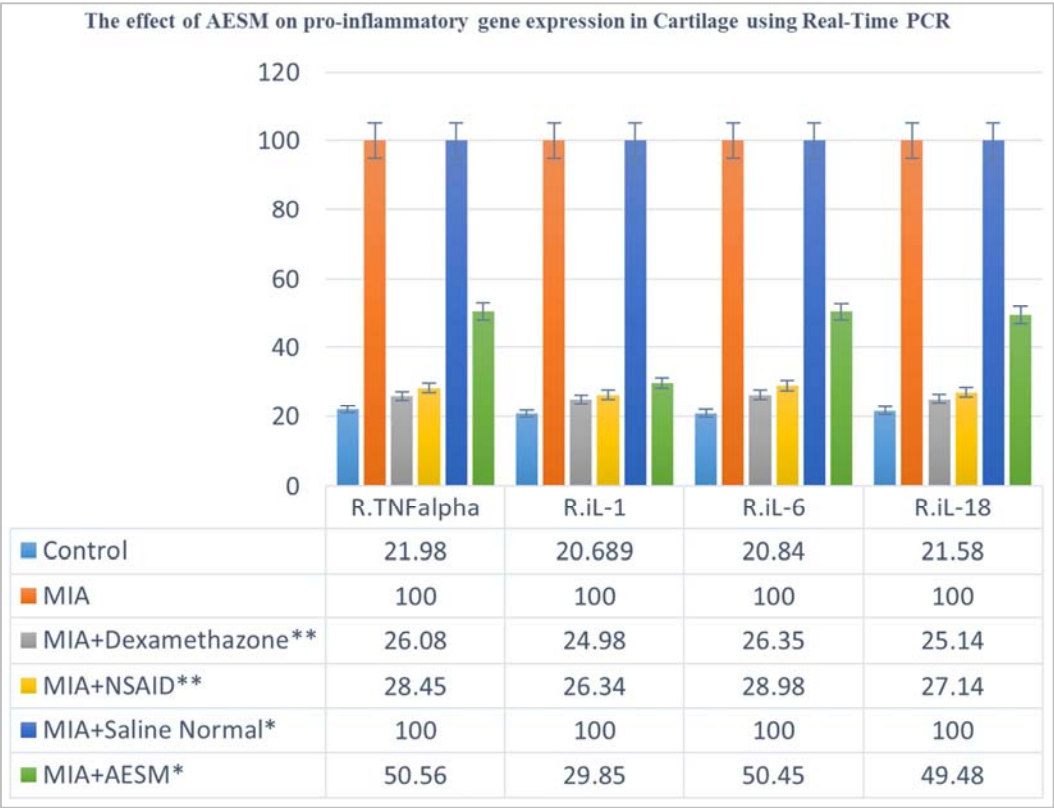


Figure 4. The effects of AESM on pro-inflammatory gene expression in Cartilage using Real-Time PCR. Statistical significances between activated control and other groups were analyzed using the t.student Newman Keuls test (mean \pm 1 SD, n=3).

3.7. Rabbits Histological Evaluation

Thickening of the middle part of cartilage in rabbits was done by hematoxylin and eosin staining (H&E) (Bio-Optica, Italy) and the results are shown on Figure 7. The concentration of proteoglycans and elastin fibers in the matrix of cartilage in the group of rabbits with OA treated with AESM was obviously increased compared to the control group ($P<0.05$). According to the results, it can be mentioned that the plant extract has increased the thickness of the

middle part of cartilage in rabbits with OA. Due to the high amount of essential fatty acids, AESM has caused the accumulation of indispensable proteoglycans of cartilage. Increasing cartilage thickness and accelerating the healing process of cartilage wounds and accumulation of proteoglycans in the group treated with AESM indicate that AESM has been able to improve articular cartilage and help treat OA (Figure 7).

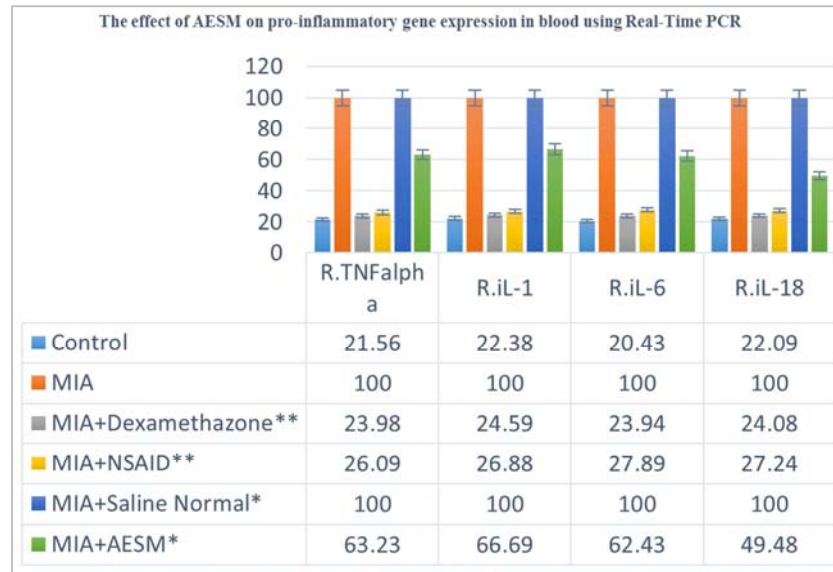


Figure 5. The effects of AESM on pro-inflammatory gene expression in RFLS using Real-Time PCR were incubated with AESM for 72h and activated with LPS for 24h. Qualification of Normalized TNF- α , iL-1, iL-6 and iL-18 expression are shown. Statistical significances between activated control and other groups were analyzed using the t-student Newman Keuls test (mean \pm SD, $n=3$).

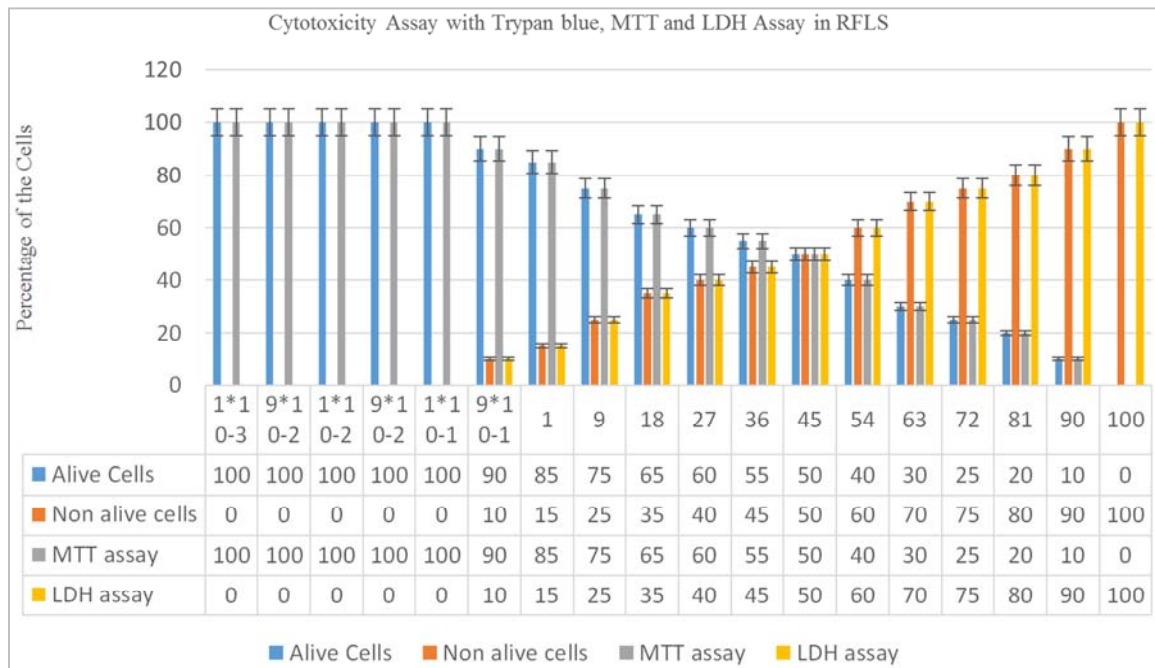


Figure 6. Determination of the cytotoxicity by Trypan blue, MTT and LDH Assay for AESM. 1×10^4 RFLS was seeded per well in 24-well microtiter plates. Cells were exposed to increasing concentration (1×10^{-3} , 9×10^{-2} , 1×10^{-2} , 9×10^{-2} , 1×10^{-1} , 9×10^{-1} , 1, 9, 18, 27, 36, 45, 54, 63, 72, 81, 90 and $100 \mu\text{g/mL}$) of AESM for 24h. In the concentration of $45 \mu\text{g/mL}$ dead cells and living cells are equal. Data are present the mean \pm S.E. $M.n=6$. * $P<0.05$.

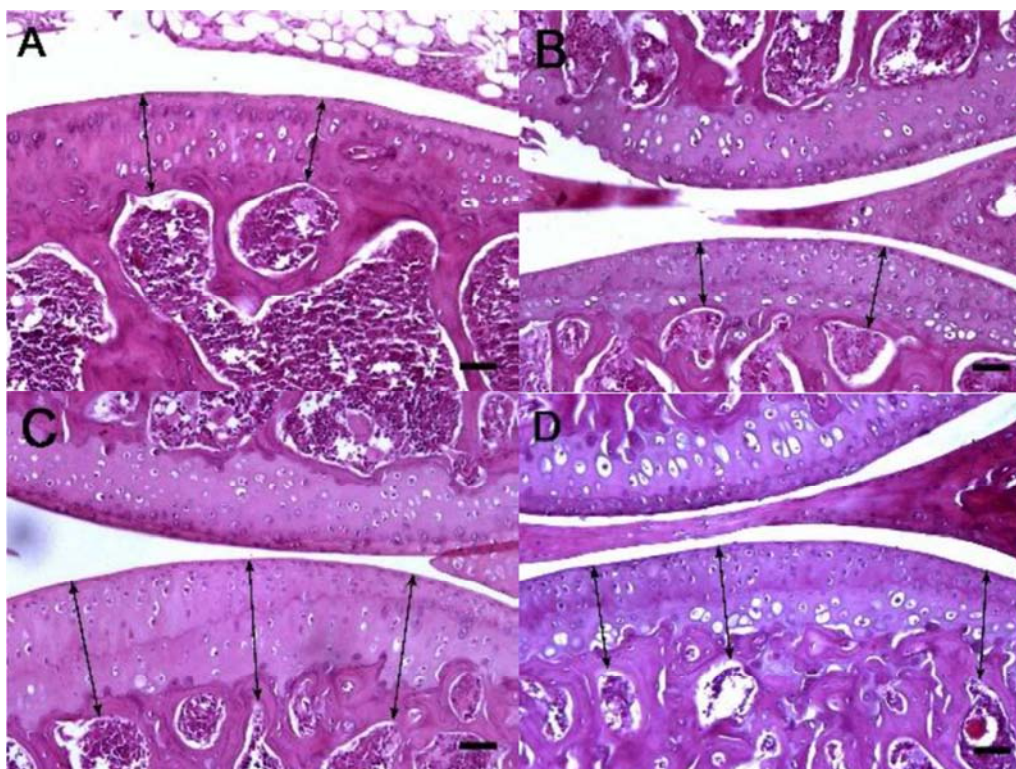


Figure 7. Rabbits histological evaluation. Cartilage cells were stained with hematoxylin-eosin (H&E). Nucleic acids were stained the purplish-blus meantime Extracellular matrix and Cytoplasm turned to pink. A, Cartilage cells in the group of rabbits with OA have no treatment (Control group). B, Cartilage cells of rabbits with OA treated with Ibuprofen (NSAIDs group). C, Cartilage cells of rabbits with OA treated with Dexamethazone. D, Cartilage cells of rabbits with OA treated with AESM ($P < 0.05$). In section D, there is an increase in cartilage thickness and accumulation of proteoglycans, elastin fiber and a diminish in inflammation.

4. Discussion

OA is known as a joint illness distinguished by inflammation of the synovial membrane and progressive loss of extracellular matrix proteins and gradual destruction of articular cartilage and is the best source of physical disease [52, 53]. The biochemical cause of OA is still unknown, but the onset of the disease usually begins with the formation of abnormal structures in the joint or abnormal pressure on the joint surface [54]. Nowadays, no satisfactory and definitive treatment has been developed for OA [55]. Common treatments for OA include pharmacotherapy, non-pharmacotherapy, supplementation, and surgery [56]. Non-specific cyclooxygenase inhibitors (COX-II) Non-steroidal anti-inflammatory drugs (NSAIDs) and oral analgesics such as acetaminophen [57], antidepressants, and glucosamine are used in combination with intra-articular injections of steroids and hyaluronic acid derivatives [58, 59]. Unfortunately, the use of these chemical drugs has many side effects such as severe gastrointestinal bleeding and digestive disorders, and has severely limited the use of this treatment by patients [60]. Therefore, this study was performed to find a suitable treatment method to reduce the symptoms of the disease and pain with the least side effects in patients. Our method is a complementary, and alternative treatment method. *Silybum marianum L.* (AESM) was used to treat sick rabbits and the effect of AESM injection was evaluated in comparison with

NSAIDs and steroid drugs in vitro or in vivo. Accurate identification of the components of AESM is possible using HPLC-UV [61, 62]. Milk thistle seeds contain 4 to 6% silymarin and its extract contains 65 to 80% silymarin [63] and about 25 to 35% of the oil full of fatty acids. The highest concentration of fatty acids is 48.88% unsaturated fatty acid Linoleic acid [64]. The compounds are divided into two main groups, flavonolignans and non-flavonolignans, and Non-flavonolignans group itself is divided into two smaller groups, polyphenolic compounds, and flavonoids. Finally, the flavonoids group can be divided into taxifolin and quercetin. Silibinin (or Silybin) has the highest concentration and maximum biological properties among other components of silymarin [63]. In OA, immune cells potentiate the inflammatory process in the joint by secreting proinflammatory cytokines such as Interleukin -6, Interleukin-1 α , Tumor Necrosis Factor- α and Interleukin -1 β [65]. Interleukin -1 β is not expressed by tissues under normal and physiological conditions of the body [66] and is first synthesized as pro-iL-1 β , which is biologically inactive and converted to active iL-1 β after caspase-1-dependent proteolysis [67, 68]. In addition, interleukin-1 β is activated by neutrophils-derived elastase and cathepsin-G and mast cell-derived proteolysis [69, 70]. Unlike iL-1 β , iL-1 α does not require primary processing for biological activity and activation. Activated iL-1 β along with iL-1 α both persuade many cytokines and play an important role in the body's irritant response [71]. Interleukin -6 is known as a

proinflammatory cytokine required in many incurable inflammatory illnesses secreted by joint tissue and, after binding to its soluble receptor (iL-6R), transsignals and activates the immune system by calling mononuclear cells (such as monocytes) to the inflamed area of the joint [72]. TLR4 activates the MyD88 pathway in the plasma membrane. Then it enters the cytoplasm through CD14 dependent endocytosis and initiates the TRIF-dependent cascade. Also, TRIF activates TRAF3, TRIF3 which causes the release of type 1 interferons and CCL5. TRAF6 activates three important signaling pathways after TAK1 including AP-1, CREB and NF- κ B. They increase the expression of proinflammatory cytokine genes such as iL-6, TNF- α , pro-iL-1 β and pro-iL18. Our researches revealed that AESM has an ability to suppress the TLR4 signaling pathway and also the reduction of genes expression such as iL-1 α , iL-6, iL-18 and TNF- α on plasma and after the induction of RFLS cells induced by MIA and AESM. Staining of cartilage cells by H&E method showed that AESM can also inhibiting inflammation accelerates of the healing process of articular cartilage [73].

5. Conclusion

As we know, during OA disease, the immune system is activated by increasing the expression of proinflammatory cytokine genes such as iL-1 α , iL-18, TNF- α , and iL-6 by immune cells and cartilage cells at the joint surface, the destruction of articular cartilage begins, intensifies progressively and eventually leading to cell death. Today, the drugs used to treat OA are not very effective and also have dangerous side effects. Therefore, patients are less inclined to take these drugs. This study aimed to find and propose a drug to reduce the symptoms of OA and treat it with minimal side effects and increase the community's hope for definitive treatment of osteoarthritis. In this article, the effectiveness of AESM on rabbits with OA was investigated. The rabbits became ill by injecting MIA into the wrist joint. After 60 days of treatment of rabbits with AESM, The gene expression of proinflammatory cytokines including TNF- α , iL-6, iL-1 α and iL-18 was measured. Also, the effect of AESM with the chemical drugs such as dexamethasone and ibuprofen (NSAID) on the expression of previously related genes was compared. Our experiments indicated that consumption administration of AESM reduces the expression of TNF- α , iL-6, iL-1 α and iL-18 genes and can compete well with common drugs in the treatment of OA. Reduction of expression of these cytokines in articular cartilage cells as well as immune cells (such as monocytes and macrophages) cause reduce joint inflammation and symptoms such as pain, swelling, and inflammation gradually disappear. It is suggested that with further research on the medicinal plant milk thistle and its seeds extract, this plant can be introduced as a very effective and efficient drug to reduce joint inflammation, pain, and decrease the expression of proinflammatory cytokine genes and a new treatment for OA with minimal side effects.

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