International Journal of Biomedical Science and Engineering
2022; 10(1): 19-25
http://www.sciencepublishinggroup.com/j/ijbse
doi: 10.11648/j.ijbse.20221001.13
ISSN: 2376-7227 (Print); ISSN: 2376-7235 (Online)

The Effect of Alcoholic Essence Satureja Sahandica Bornm L. on the Expression of Metalloproteinases 1, 2, 9 and 13 in a Model Similar to Osteoarthritis

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To cite this article:

Hossein Maghsoudi, Amir Akbarnezhad Eshkalak, Mahsa Khosrogardi, Enayat Allah Yazdan Panah. The Effect of Alcoholic Essence Satureja Sahandica Bornm L. on the Expression of Metalloproteinases 1, 2, 9 and 13 in a Model Similar to Osteoarthritis. *International Journal of Biomedical Science and Engineering*. Vol. 10, No. 1, 2022, pp. 19-25. doi: 10.11648/j.ijbse.20221001.13

Received: January 17, 2022; **Accepted**: February 5, 2022; **Published**: February 16, 2022

Abstract: Loss of the extracellular matrix of cartilage is one of the most important and prominent features of osteoarthritis (OA). The ECM has two important components, including the proteoglycan aggrecan and type II collagen, which are the main targets of MMPs and ADAM TSs enzymes. In this paper, the effect of ASSB Compared with dexamethasone and ibuprofen as representatives of steroidal and nonsteroidal anti-inflammatory drugs. One way to prevent irreversible joint damage in OA is to inhibit MMPs. ASSB can improve the condition of patients with osteoarthritis by reducing the expression of the MMPs 9 and 13 gene. *Background*: OA is a progressive joint disease characterized by cartilage degeneration, cartilage bone regeneration, and synovial membrane inflammation and is exacerbated with age. Given the side effects of conventional OA treatments, including nonsteroidal anti-inflammatory drugs and corticosteroids, it is important to consider new treatments for this disorder. Recently, the effect of matrix metalloproteinases on the pathogenesis of OA has been attracted attention. *Objective*: The aim of this paper was to evaluate the impact of Alcoholic Satureja Sahandica Bornem L. (ASSB) essence on inhibiting the expression of metalloproteinase gene 1, 2, 9 and 13 in bovine fibroblast-like synoviocytes stimulated by lipopolysaccharide as a model of osteoarthritis. *Methods*: Synovial cells were isolated from the articular cartilage of the radiocarpal joint of an 8-month-old Holstein cow. After determining the degree of toxicity by MTT Assay, the cells were exposed to stimulation of LPS without or in the presence of dexamethasone, ibuprofen and alcoholic Satureja Sahandica Bornem essence (ASSB). Metalloproteinase gene expression 1, 2, 9 and 13 were measured by RT-PCR and ELISA. The effect of ASSB on migration and cell invasion was investigated by Transwell chambers. *Results*: The results showed that Satureja Sahandica Bornm (SSB) essential oil significantly reduced the expression of metalloproteinases 9 and 12 in bovine fibroblast-like synovocytes stimulated by lipopolysaccharide. It also suppressed migration and invasion of these cells. However, Satureja Sahandica Bornm L. had no significant impact on the expression of MMPs 1 and 2. *Conclusion*: Based on our results, ASSB can significantly reduce the activity and inflammatory effect of MMPs 9 and 12 in OA, its potential role as a complement to NSAIDs and common corticosteroids was confirmed. However, cellular modeling does not confirm the beneficial effect of OA in patients.

Keywords: Osteoarthritis, Metalloproteinase, Bovine Fibroblast-like Synoviocytes, Satureja Sahandica Bornm (SSB) Essential Oil, Migration and Cell Invasion

1. Introduction

Osteoarthritis is a type of arthritis that presents with progressive demolition of articular cartilage. It is one of the most important causes of disability that depends on age, gender, obesity and genetics in different populations.

The onset of chronic diseases such as inflammatory arthritis is closely related to the overexpression of

inflammatory mediators and uncontrolled inflammatory responses [1]. Unfortunately, there is no definitive cure for OA and the use of common drugs has serious side effects that their long-term use can cause serious harm to patients. In addition, excessive drug use has a harmful and debilitating effect on patients' mood. Today, OA is treated with corticosteroids and NSAIDs, or surgical procedures, which are the most invasive. The use of a drug with minimal side effects in the treatment of OA is felt among researchers. Medicinal plants that have a long history can be a new and effective method of treatment. According to the World Health Organization (WHO), more than 80% of the human population is dependent on the use of medicinal plants [2].

The extracellular matrix of cartilage called ECM has a great effect on the development of osteoarthritis if it is destroyed. ECMs are not only synchronized by ADAMs TSs but also by MMPs [3, 6]. Degradation of type 2 collagen and proteoglycan aggrecan causes ECM to disappear and symptoms of osteoarthritis to develop. Satureja Sahandica Bornem L. is a perennial plant of the Lamiaceae family that is widely used in pharmacy and perfumes and cosmetics (Figure 1). Satureja Sahandica Bornem L. has around 40 compounds, which mainly include: thymol (19.6–41.7%), γ-terpinene (1.0–12.8%) as well as p-cymene (32.5–54.9%).

The plant extract has been shown to reduce the expression of the MMPs gene, especially MMP-9 and MMP-13. In this way, alcoholic essence Satureja Sahandica Bornem L. can prevent the progressive destruction of cartilage by preventing the demolition of the extracellular matrix of cartilage (ECM).

Figure 1. *Satureja Sahendica Bornum L. (SSB)***,** *Collected in the central plateau of Iran.*

2. Procedures and Chemicals

Cell culture media RPMI-1640, DMEM-F12, L-glutamine, fetal bovine serum (FBS), sodium bicarbonate, glucose-4- (2-hydroxyl) piperazine-1-ethanesulfonic acid (HEPES), MTT Assay kit, grease reagent, Lipopolysaccharide (Escherichia coli serotype O127: B8), sodium pyruvate, trypan blue, and beta mercaptoethanel (Sigma, UK), gentamicin, penicillin and streptomycin (Idea Bio Recombinant Company, Iran) and RNA extraction kit, RT-PCR and primers (Sinagen Company, Iran). Amphotericin-B (Sipla Company, India).

2.1. Preparation of Essences

Satureja Sahandica Bornm (SSB) essences was obtained by gas chromatography by the National Center for Genetic Resources of Iran in completely sterile conditions.

2.2. Culture of Synoviocyte-like Fibroblast Cells

Metacarpal joint of healthy eight-month-old calves was used to isolate synoviocytes from synovial cells. After draining the joint fluid into a flask containing DMEM-F12 medium enriched with 10% fetal bovine serum (FBS), 50 µg/mL ascorbic acid and a combination of antibiotics including: 100 U/mL penicillin, streptomycin 100 µg/ml, gentamicin 50 µg/ml, amphotericin 25 µg/mL, was stored in 25 cm^2 flasks in an incubator with 5% CO_2, 37°C and 95% humidity. After reaching a cell density of more than 80%, the cells were counted with trypan blue solution at 0.4% and the cell viability percentage was determined and cells with 95% viability were selected for the next culture.

2.3. Division of Study Groups and Cell Treatment

The study groups were divided into eight groups and Table 1 demonstrate how they are grouped. Cultivate 10×10^6 synoviocytes in 75 cm^2 flasks in eight groups and store for 1-2 hours in an incubator and then inject ASSB at 16.22 µg/mL into all flasks for 5 minutes on a shaker. They were rotated at 50 rpm/m and kept in the incubator for 72 hours. To induce osteoarthritis and increase the expression of proinflammatory cytokine genes, LPS of 100 ng / ml was used. Sodium dexamethasone 4 mg/mL, ibuprofen (non-steroidal anti-inflammatory drug) (10 mg/mL) as positive control and their effect with essential oil, DMSO as negative control were used in the study groups. In previous studies, this group of synoviocytes was confirmed by the vimentin antibody [4].

2.4. Cytotoxicity Test by MTT Assay Method

The colourimetric method was used to evaluate the cytotoxicity of Sahandi safflower essential oil and to determine LC50. MTT powder is a water-soluble tetrazolium salt that dissolves in a culture medium without phenol red or PBS buffer to form a yellowish compound. The basis of this test is the breakdown of MTT salt by the enzyme live mitochondrial succinate dehydrogenase. The result of this state is the formation of insoluble crystals of purple colour, which are dissolved by DMSO. The more active the cells and the more numbers, the more colour is created.

According to the instructions [5], 105 synoviocytes were cultured in 24 well plates and kept in an incubator for 1-2 hours and then ASSB was added to all wells with the desired concentrations 1-2 hours were incubated in cell culture. LPS injection at the rate of 100 ng/ml was then added to each well and placed in a cell culture incubator again for 24 hours. In the next step, 20 µl of MTT solution was adjusted to the wells and placed in a 37°C cell culture incubator again for 4 hours. At the end of this period, all cell culture medium was removed from the wells, and 100 µl of isobutyl alcohol was added to each well. Until the matter is dissolved. The adsorption rate of the solution of each well at 570 nm was read using a spectrophotometer. With increasing the amount of Sahandi safflower essential oil, the survival rate gradually decreases, so that at a concentration of 50 µg/ml, this percentage decreases by 50% in synoviocyte cells. For cell treatment, an average of 16.22 µg/ml is used.

2.5. Assessment of Migration and Cell Invasion in Vitro

Cell migration was measured in vitro by 6.5 mm thick transulse chambers and 8 mm pore diameter (Costar NY14831, USA). 1×105 synoviocytes after treatment with ASSB. The transwell chamber plate was cultured on top of the two-layer filter for 24 h, then 100 ng/ml serum-free medium with or without LPS (E coli, Sigma) was added to the bottom of the plate. It was kept in an incubator at 37°C and 5% CO_2 for 24 hours to allow the cells to migrate downstream, then the non-migrated cells upstairs were collected by sterile swap and stained with crystal violet dye. The field was counted under a microscope with a magnification of 100. To determine the rate of invasion according to the protocol, the pores at the top of the bilayer filter were first covered with matrigel and the culture medium was added to the lower part of the chamber. Then 5×104 cells were transferred to culture medium mixed with 2% FBS and added to Transwell. invasion and migration of cells was allowed for 24 h. The upper cells were then swabbed and the lower cells were counted.

2.6. Real-time Polymerase Chain Reaction

2.6.1. RNA Isolation

For lysis of cells from the Trizol reagent (Sinagen, Iran) and extraction of RNA with chloroform was performed. After intense stirring and incubation for 3 minutes at room temperature, After centrifugation, the formed aqueous phase containing RNA was gathered. The RNA was then precipitated with isopropyl alcohol and dissolved again in water without RNase. Finally, spectrophotometry was utilized to distinguish the RNA concentration. Samples were purified by the enzyme DNase if they were DNA contaminated.2.6.2. cDNA Synthesis.

According to the two-step RT-PCR instructions, 1 µg of RNA was converted to cDNA for each sample under optimal conditions (temperature: 42°C for one hour and temperature: 94° for 5 minutes). Semiquantitative PCR was performed for each of the studied cytokines using specific primers (Table 2) and GAPDH gene was used as the housekeeping gene. PCR product was controlled in 1.5% agarose gel.

2.6.2. Real-time Quantification of Polymerase Chain Reaction

Real-time PCR reaction was performed with the help of special primers in Table 2 and the GAPDH gene was used as the Housekeeping gene. Also, the mastermix of this process was evergreen. The obtained results of CTs (Threshold Cycle) were evaluated by two standard curve methods and Pfaffi Method. In principle, CTs are numerical numbers and their significance was first determined by ANOVA and in the second stage by Student-Newman software Keuls (SNK), REST-2000 were evaluated. Finally, the Pfaffi Method (ct) was used to determine the expression of the method gene.

Table 1. *Classification of study groups.*

	Cells $6*10^6$	**LPS 100 ng/ml**	**Alcoholic essence Of Satureja Sahendica Bornm**	**Dexamethazone**	**NSAIDs**	**DMSO**		
1	Seeded	Untreated	Untreated	Untreated	Untreated	Untreated	Negative Control (Untreated Cells)	Group 1
2	Seeded	Untreated	Treated	Untreated	Untreated	Untreated	The effectiveness Essential Oil Of Satureja Sahendica Bornm (Un-treated Cells)	Group 2
3	Seeded	Treated	Untreated	Untreated	Untreated	Untreated	Positive Control (Treated Cells)	Group 3
4	Seeded	Treated	Treated	Untreated	Treated	Untreated	The effectiveness Essential Oil Of Satureja Sahendica Bornm on LPS treated Cells	Group 4
5	Seeded	Treated	Untreated	Treated	Untreated	Untreated	Comparison of the effectiveness of Dexamethazone with Essential Oil Of Satureja Sahendica Bornm on LPS treated Cells	Group 5
6	Seeded	Treated	Untreated	Untreated	Treated	Untreated	Comparison of the effectiveness of NSAIDs with Essential Oil Of Satureja Sahendica Bornm on LPS treated Cells	Group 6
7	Seeded	Treated	Untreated	Untreated	Untreated	Treated	Comparison of the effectiveness of DMSO in LPS treated cells	Group 7
8	Seeded	Untreated	Untreated	Untreated	Untreated	Treated	Comparison of the effectiveness of DMSO in Un-treated cells	Group 8

Table 2. *Sequence of primers used.*

European Nucleotide Archive (ENA)	Gene	Forward primer	Reverse primer	Product length (bp)
XM024975704.1	B.MMP-1	CTGGAGCAATGTCACACCCT	GGGATTTTGGGAAGGTCCGT	379
NM_174745.2	B.MMP-2	TCCTGAGCTCTGCAAACAGG	ATTACCAGGTGGGGGTGAGA	726
BC142430.1	B.MMP-9	GACCAAGTGCTATCCTCACCA	CGCCCTCAAAGGTCTGGAAT	357
NM_174389.2	B.MMP-13	GTTGCTGCCCATGAGTTTGG	TACGGTTGGGAAGTTCTGGC	3436
NM_001034.2	B.GAPDH	GGTCACCAGGGCTGCTTTTA	CCAGCATCACCCCACTTGAT	222

2.7. ELIZA

Cultivate synoviocytes 10×10^6 cells in each flask for eight groups and keep in incubator for 2-3 hours and then treat the cells with dexamethasone, ibuprofen, ASSB 16.22 µg/ml and DMSO and keep in incubator for 72 hours followed by 100 ng/ml LPS injection for all groups except the first group and re-storage in the incubator for 24 hours.

In the next step, 100 µL of standard solutions and 100 µL of supernatant were added to the wells, respectively, and the plates were kept in the dark for 2.5 hours at laboratory temperature. After removing the surface of the wells, wash all the wells twice with the washing buffer and after removing the last drop of buffer from each well, add 100 µL of Biotinylated antibody to each well and then the plate for one hour on the shaker with circular motions. It was placed at a gentle temperature at laboratory temperature, then the solution was poured on and all wells were washed 3 times with the washing solution (300 µL). Invert the plate to remove the last drops of detergent solution. After removing the surface of the wells, wash all the wells twice with the washing buffer and remove the last drop of buffer from each well, add 100 µL of HRP-Streptavidin solution to each well and plate for 45 minutes at laboratory temperature. It was placed on the shaker with gentle rotational movements. The surface solution of each well was discarded again and then the wells were washed 3 times with the rinsing solution. Invert the plate to remove the last drops of detergent solution. 100 µL of ELISA Colorimetric TMB solution was added to each well and covered with black nylon and the plate was placed on a shaker for 30 minutes at laboratory temperature with gentle rotational movements. Then 50 µL of Stop Solution to each The well was added and the optical density was measured by Microplate Reader at OD 450 nm.

2.8. Statistical Analysis

All data was carried out using ANOVA, Student – Newman – Keuls, REST-2000 and used for Cts analysis. all data was demonstrated as mean ± SD. In this paper, $P < 0.05$ is thought-out statistically significant.

3. Results

3.1. The Inhibitory Effect of ASSB on the Ability of Cells to Migrate and Invade

In order to investigate the inhibitory impact of ASSB on the ability of LPS-stimulated synoviocyte cells to migrate and invade, transol chamber was used. As shown in Figure 1, the in vitro cell migration capacity of synoviocyte cells increased up to 3.5 times that of LPS-stimulated cells. Similarly, the data gained from the cell invasion assay showed that stimulation with LPS increased the invasive power of synoviocytes up to 7.02 times compared with the control group. However, suppression of cell migration and invasion ability in LPS-stimulated synoviocytes is dose-dependent. The results show that non-toxic concentrations of alcoholic Satureja Sahandica Bornem essence (ASSB) from 20 to 100 µg/ml in vitro can reduce the ability of cell invasion and migration in LPS-stimulated synoviocytes.

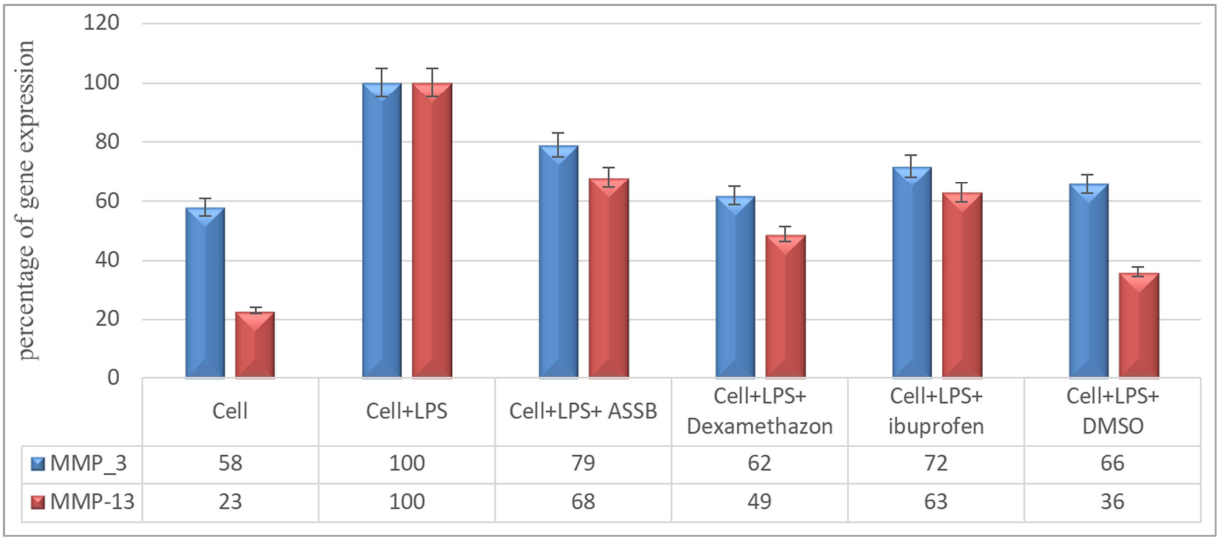

Figure 2. *Comparison the effect of ASSB, dexamethasone, ibuprofen on the production of metalloproteinases 3 and 13 in synoviocytes in six groups. Statistical significances between groups were analyzed using the student-Newman- Keuls test (mean ± 1 SD, n+3).*

3.2. The Effect of ASSB on the Production of Metalloproteinases 1, 2, 9 and 13 in Synovitis

In order to compare the effect of ASSB, dexamethasone, ibuprofen on the production of metalloproteinases 3 and 13 in synoviocytes in six groups (Figure 2) and to compare the quantitative analysis of RT-PCR results showed that the addition of LPS resulted in a significant increase. Increased production of metalloproteinases 3 and 13 in synoviocytes Treatment with dexamethasone (group 4) and ibuprofen (group 5) in LPS-stimulated cells significantly ($P<0.001$) reduced the production of metalloproteinases 3 and 13 (90%). In the case of treatment with ASSB (group 3), this decrease in expression level (50%) was determined for all studied proteins ($P<0.001$). The expression of the studied proteins in the cells treated with ASSB (group 3) was almost similar to the cells of the first group and DMSO had no change in the expression of the studied proteins in the cells stimulated with LPS (group 6) and the unstimulated cells (group 1) did not show stimulation (Figure 2). In order to express the accuracy or reproducibility of immunoassay test results, two variables of Inter CV and Intrera CV variability should usually be considered. Coefficient of Variability (CV) that is determined as the standard deviation of a set of measurements divided by the average of the set, CV is a dimensionless number. Intrera CV results < 15% and Inter CV results < 10% are acceptable ELISA results.

4. Discussion

The matrix family of metalloproteinases includes more than 26 endopeptidases that have homologous protein sequences [7]. Matrix metalloproteinases are proteolytic enzymes that affect the destruction of extracellular matrix and basement membrane. In addition, cysteine proteinases, aspartic proteinases and serine proteinases play a similar role. [8]. Extracellular matrix in both physiological conditions such as fetal development and pathological conditions such as inflammatory diseases [9] and skin aging caused by UVB rays. UVB is involved [10]. Overproduction of metalloproteinase-13 has been reported in several pathological cases and the most important of them are: In cases of tissue destruction: such as cancer cell invasion and metastasis, rheumatoid arthritis, OA, bedsores, stomach ulcers, corneal ulcers, periodontal disease, Brain injury and in inflammatory neurological diseases and fibrosis, such as liver cirrhosis, pulmonary fibrotic disease, atherosclerosis, matrix weakness, such as cardiomyopathy and aortic aneurysm [11]. Under normal conditions, articular chondrocytes maintain a dynamic balance by maintaining a balance between the synthesis and degradation of extracellular matrix components by type II collagen, aggrecan, and proteoglycan in articular cartilage [12]. In OA, what causes the progressive destruction of cartilage is the dysfunction of the extracellular matrix, which causes clonal proliferation of cartilage cells in the affected areas. By continuing this process, oxidative states are induced and eventually lead to cell death [13]. Progression of the disease usually results in increased degradation and synthesis of extracellular matrix molecules in the joint, and with increased chondrocyte metabolism due to overproduction of inflammatory cytokines and matrix-degrading enzymes, along with improper regulation of anabolic signaling and ultimately leading to Degradation of the extracellular matrix and subsequent destruction of cartilage [14]. LPS activates the innate immune system such as macrophages and neutrophils. It shows its activity by binding to the TLR4 complex. In this study, LPS was used to simulate inflammation and increase MMPs gene expression in cell culture [15]. MMP-13 plays an effective role in the degradation of extracellular matrix proteins including fibrillar collagen, fibronectin, experimental non-confirmation (TNC) and (Aggrecan) Aggrecan. MMP-13 destroys type I, II and type III collagens, called triple helical collagens, and is more active with type II collagen. In addition, it can demolish type IV, XIV and type X collagens. MMP-13 may activate or degrade key regulatory proteins, including TGFB1 and CCN2. This metalloproteinase is highly expressed in connective tissue, especially in growing cartilage and bone. Overexpression of MMP-13 is quite evident in the cartilage tissue of OA patients and enhances the effect of high expression of this gene in the incidence of OA. [16]. The activated form of MMP-13 contains a catalytic domain and a hemopexin-like domain. Importantly, the hemopexin-like domain dominates the degradation properties of MMP-13. Although the catalytic domain of MMP-13 alone can destroy collagen, it is not as efficient as the hemopexin-like domain [17]. The results of the present study show that there was no clear change in the expression of MMP-1, MMP-2, and MMP-3 in different concentrations of ASSB in comparison with the samples that were exposed only to LPS, and as a result alcoholic Satureja Sahandica Bornem L. (ASSB) does not affect these target genes. On the other hand, there was a significant decrease in MMP9, 13 gene expression as well as a decrease in migration and cell invasion compared to dexamethasone and ibuprofen. Probably due to the greater ability of metalloproteinase-9 to degrade fibronectin tissue and induce cell invasion compared to metalloproteinase-2. Also, this effect may be related to the binding of TIMPs to the PEX MMP-2 domain, which prevents the activity of this MMP or the presence of the OG domain in MMP-9. The effect of metalloprotein 13 may be related to the binding of TIMPs to the PEX MMP-2 domain, which inhibits the activity of this MMP or binds to the catalytic domain or the hemopexin-like domain. However, more studies are needed to clarify this content. According to Guoqing's study of Celastrol, treatment with anti-TLR4 antibody and TLR4 inhibitor reduced LPS-induced expression and enzyme activity of MMP-9. In addition, suppression of TLR4 activity has been shown to reduce LPS-induced NF-κB transcriptional activity. TLR4 is the major NF-κB upstream signal transducer activated by TLR ligands and cytokines.

NF-κB is secreted into the cytoplasm by binding to IκB family molecules and activated by IκBα phosphorylation, the subsequent degradation of which in the proteosome into NF-κB subunits, including p65 and p50, to enter the nucleus and genes. Activate the goal [18]. MMP-13 is overexpressed in osteoarthritis and is partly responsible for the breakdown of collagen and proteoglycans in cartilage and bone, leading to impaired joint function and a very important role in osteoarthritis [19]. Inhibition of MMP-13 activity is an attractive therapeutic target for pharmacological research against OA. However, many of the broad-spectrum MMP inhibitors that have been discovered to date have had painful side effects. MMPs were other than MMP-13 play an important role in the regeneration and repair of normal tissue and inhibition of these functions is thought to cause joint stiffening side effects [20].

5. Conclusion

It is suggested that suppression of MMPs gene expression could be considered as a new and acceptable therapeutic strategy for the treatment of OA and prevent the progressive destruction of cartilage. This is a goal that could lead to the development of drugs to reduce inflammation and pain in OA patients. MMP-9 can activate two major pathways through protein kinases, including NFκB and mitogen [20]. It has been shown that alcoholic Satureja Sahandica Bornem L. (ASSB) can reduce the expression of MMP-9, 13 genes, the results of this study should be interpreted according to certain limitations. The effects of ASSB on the treatment of OA in a cellular model do not significantly guarantee its beneficial effect in patients with OA, so further studies should use clinical trials to determine the effect of ASSB in patients with OA.

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