Investigation of Punicic Acid Effects on Matrix Metalloproteinase Genes Expression in Bovine Fibroblast like-Synoviocytes as a Model of Osteoarthritis

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Abstract

Background: **Osteoarthritis (OA) is a progressive, age-associated disease that is characterized with cartilage destruction, subchondral bone remodeling and inflammation of the synovial membrane. Considering the complications of common treatments of OA, including non-steroidal anti-inflammatory drug (NSAIDs) and corticosteroids, investigating new treatments for this disorder is crucial. Recently, the role of matrix metalloproteinases (MMPs) expression in pathogenesis of OA has attracted attention.**

Objective: **This study aimed to explore the effect of punicic acid (PA) in inhibition of MMPs gene expression in LPS-stimulated Bovine Fibroblast-like synoviocytes (BFLS) as a model of OA.**

Methods: **In the first stage, the toxicity of PA was measured using MTT assay on BFLS cells. Afterward, the cells were stimulated by LPS (Lipopolysaccharide) and MMPs (Matrix Metalloproteinase) expression level in the BFLS cells were investigated using Real-Time PCR, in vitro Migration and Gelatin Zymography, Western Blot Analysis, ELISA Assay and Invasion Assay.**

Results: **The results showed that PA significantly decreased MMP-9 expression levels in LPS-stimulated BFLS cells; also, it suppressed migration and invasion of the mentioned cells. However, PA had no significant effect on MMP-1-2-3.**

Conclusion: **Based on our results PA could significantly reduce the activity and inflammatory effect of MMP-9 in OA, its potential role as a supplementary agent to common NSAIDs and corticosteroids was confirmed. Nonetheless, cellular modeling does not significantly confirm the beneficial effect of OA in patients.**

Keywords: **Bovine Fibroblast-like synoviocyte, Lipopolysaccharide, Metalloproteinase, Osteoarthritis, Punicic acid**

Introduction

OA is associated with cartilage destruction, subchondral bone remodeling and inflammation of the synovial membrane [1]. OA is the most common disease of the joints among elderly and the knee OA is the largest cause of disability in the most developed countries [2]. Although the pathogenesis of the OA is not fully understood, Fibroblast-like synoviocytes (FLS) invasion in synovium appears to be one of the main pathogenesis of the disease [3], which plays an essential role as effector cells in joint destruction through the production of MMPs [4]. In addition to cartilage, all associated tissues such as synovium and subchondral bone are also involved in OA. Some of the most effective treatments for OA, includes isotonic exercise, weight control, joint protection, physical rehabilitations and physiotherapy. Also., pharmacotherapy such as prescription of non-steroidal anti-inflammatory medications, topical analgesics, intra-articular injection of glucocorticoids and hyaluronic acid, muscle relaxants, and joint replacement is use to overcome OA disabilities [5]. Non-steroidal anti-inflammatory medications cause gastrointestinal, cardiovascular, renal, liver and hematological problems depended on the dose, duration of use and age. So, it is recommended to use topical medications to reduce these side effects [6]. Natural alternative and supplementary medicine can also reduce the need for synthetic analgesics according to less side effects and some proved effectiveness [7]. Today according to side effects of chemical drugs which are common in treatment of inflammatory diseases, topical ointments containing herbs with proven efficacy in the treatment of OA are used to treat and relieve pain [8]. Some plants or herbs such as *Nigella sativa* L and which their oral or topical medications have been beneficial in the treatment of OA [9]. In vitro study by N. Akhtar demonstrated that Pomegranate (*Punica granatum* L.) could prevent collagen degradation and may inhibit joint destruction in OA patients [10]. In the traditional Chinese medicine, the peels were considered as a powerful astringent and anti-inflammatory agent and were used in the treatment of traumatic hemorrhage, ulcers, infections and disorders of the digestive tract such as diarrhea and dysentery [11]. During the past two decades, it has been revealed experimentally or clinically that the therapeutic effects of pomegranate products encompass but are not limited to attenuating the chemotherapy-induced nephrotoxicity and hepatotoxicity [12], alleviating allergic symptoms [13], protecting the cardiovascular system [14], and improving skin wound healing [15]. Various types of proteinases have been implicated in extra cellular matrix (ECM) degradation, but a major enzyme group involved is that of the MMPs, also called the matrixins. Most recent studies have shown that MMPs have other roles in the regulation of the cellular environment and modulating many bioactive molecules at the cell surface and behavior [16]. Degeneration of the ECM of cartilage, which is a feature of arthritic diseases, is orchestrated by both MMPs and ADAMTSs which degrade two major structural components of cartilage extracellular

matrix, the proteoglycan aggrecan and type II collagen [17]. The human genome has 24 matrixin genes which include a duplicated Mmp23 gene. Thus, there are 23 different MMPs. The collagenolytic MMPs (MMP 1, MMP 2, MMP 8, MMP13, and MMP 14) are all produced by chondrocytes as well as cells in the synovium, but their expression in arthritic tissues seems to vary enormously in models of human disease. MMPs play a role in osteoclastic resorption in pathological conditions, including MMP 1, MMP 2, MMP 3, MMP 9, MMP 12, MMP 13, and MMP 14 [7]. The use of biological reagents to block inflammatory cytokines also reduces MMP expression in many cases. The tetracyclines, which are rather weak inhibitors of MMP catalytic activity, also influence on their synthesis and have been successfully trialled in rheumatoid arthritis [18]. n-3 Polyunsaturated fatty acids reduce expression and activity of collagenases and aggrecanases, as well as a number of pro-inflammatory mediators in osteoarthritic cartilage and have been strongly suggested as a dietary supplement [18]. However, no study has investigated the potential effects of PA on MMPs in OA. Hence, as one of the first efforts, the present study aimed to determine the effect of PA on inflammation through its effect on MMPs gene expression in an induced model of osteoarthritis.

Materials and Methods

Chemicals and Reagents

PA is the bioactive phytochemical constituent of the seed oil of *P. grantum* L. The powder was dissolved in dimethyl sulfoxide (DMSO) and were purchased from Sigma Chemical Co. (Sigma-Aldrich, UK, Product Number: 274666).

The chemicals and antibodies including 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), alkaline phosphates conjugate, Vimentin, Secondary antibody included goat anti mouse IgG, anti-mouse, anti-rabbit and anti-goat were purchased from Sigma-Aldrich, UK.

Also the applied kits include RNA Isolation kit, 2-step RT-PCR kit and ELISA kit were prepared from Sigma-Aldrich, UK.

Specific antibodies for MMP-1, MMP-2, MMP-3, MMP-9 were purchased from Santa Cruz Biotechnology, USA.

Migration and invasion kit were purchased from Costar NY14831, USA. All other chemicals were of reagent grade.

Cell Culture

BFLS cells were separated from carpometacarpal (CMC) joint of a healthy eight-month-old calves. Briefly, synovial fluid was collected with a sterile syringe and then cells were washed three times with phosphate-buffered saline (PBS). BFLS were minced and digested with 220 U/mL type II collagenase 0.15% (Sigma-Aldrich, UK) for 6 h at 37 °C for 24 h. After centrifugation at 1000 rpm for 8 min, the pellet was suspended in DMEM-F12 (Gibco®, life Techonolgy, UK) medium containing 10% fetal bovine serum (FBS) (Sigma-Aldich, UK), 100 units/ml of penicillin (bioidea, IR), and 100 mg/ml of streptomycin (bioidea, IR), 50 mg/mL of gentamicin (Darou Pakhsh, IR), 0.25 mg/mL of amphotericin B (kimia darou IR) and 50 mg/mL of Ascorbic acid (Sigma-Aldrich, UK). The BFLS cells

were cultured in a 5% CO_2 atmosphere, 21% O_2 and 75% N_2 at 37ºC. Cells between passages 1 and 3 were used in this study [19].

Confirmation of BFLS cells

BFLSs were verified by Vimentin which is a specific cell marker for mesenchymal cells (19). 5 × 10^5 cells were prepared and lysised by lysis buffer. Whole cell lysates from BFLSs were generated using a Total Protein Extraction Kit (sigma-Aldrich, UK) according to the manufacturer's instructions. Protein concentrations were determined using a Bradford method (sigma-Aldrich, UK). Equal amounts of protein (20 µg) were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to enhanced chemiluminescence (ECL) nitrocellulose membranes (Roche, USA). After blocking with 5% BSA for 2 h, blots were probed with primary antibodies, including vimentin (1:200) (sigma-Aldrich, UK), at 4ºC for 12 h and then incubated with secondary antibodies, including goat anti mouse IgG, alkaline phosphates conjugate (1:5000) (sigma-Aldrich, UK), for 2 h. Membranes were then incubated with appropriate secondary antibodies for 2 h at room temperature. ECL reagent (GE Healthcare) was used for protein detection. The relative expression of each protein was determined by densitometry and normalized to the control. Each blot shown is representative of at least three similar independent experiments.

Cell viability assays

MTT assay

The cytotoxicity of PA on BFLS cells was assessed by the MTT assay. In brief, BFLS cells were seeded in a 96-well plate at a density of 2 × 10^5 cells/ml. Then the cells were treated with different concentrations of PA for 24 h. The medium was removed and 20 µl MTT (5 mg/ml) was added to each well for 4 h. After that, the supernatant was removed, and the formazan crystals were dissolved using 150 µl of DMSO. Absorbance at 570 nm was measured with a micro-plate reader (MTT assay kit, Sigma-Aldrich, UK).

Treating cells with Lipopolysaccharides (LPS)

In order to provide conditions similar to disease and also to increase MMPs expression, 20 ng/ml of LPS were used. First 6 × 10^6 cells were cultured. After 72 hours 100 ng LPS was added to the medium in order to express MMP-1, MMP-2, MMP-3, MMP-9, The first set of plates were incubated in CO_2 incubator for 24h. The second series were incubated for 1h in order to check MMPs expression's change [19].

Total RNA Isolation

Total cellular RNA was harvested with trypsin-EDTA 0.5 %, then cellular RNA was isolated by lysing the cells with TRIzol® reagent and extracted with chloroform. Following vigorous agitation and 3 min incubation at room temperature, the samples were centrifuged and the aqueous phase containing RNA was collected. The RNA was precipitated with isopropyl alcohol and resuspended in RNase-free water. (RNA Isolation kit, sigma-Aldrich, UK).

cDNA synthesis

For each sample, 1 µg of total RNA was converted to complementary DNA (cDNA)

using Moloney-Murine Leukemia Virus reverse transcriptase from the Advantage 2-step RT-PCR kit (sigma-Aldrich, UK). RT was carried out at 42°C for 60 min followed by heating at 94°C for 5 min to stop the cDNA synthesis reaction and to destroy any DNase activity.

Semi quantitative PCR

Each one of MMPs were studied using specific primers (table 1).

The GAPDH gene was used as a housekeeping gene. PCR was performed under standard conditions then PCR products were checked in agarose gel 1.5 % and with ethidium bromide 0.5 µg/ml [20].

Quantitative Real-time Polymerase Chain Reaction (qRTPCR)

Total RNA was extracted using Trizol according to the manufacturer's protocol. qRT-PCR was performed using the ABI PRISM 7900HT cycler (Applied Biosystems) and Super- ScriptTM III PlatinumH SYBRH Green one-step qRT-PCR kit (Invitrogen, Carlsbad, CA, USA). The reaction was started at 94°C for 2 min, amplified by 35 cycles of denaturing at 94°C for 20 s, 20 s of annealing at 50°C, and ended with a 45s extension at 72°C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for all analyses. The forward and reverse primers were designed using Primer Express software (version 2.0-PE Applied Biosystems). Primer sequences are shown in Table 1. Primer specificity was assessed from monophasic dissociation curves, and all had a similar efficiency (data not shown). The threshold cycle (C_t) for the endogenous control GAPDH mRNA and target signals were determined, and relative RNA quantification was calculated using the comparative $2^{-\Delta\Delta Ct}$ method where

$$\Delta\Delta C_t = (C_t^{Target} - C_t^{GAPDH}) - (C_t^{Control} - C^{tGAPDH}).$$

All reactions were performed in duplicate [20].

In Vitro Migration and Invasion Assay

Cell migration was determined using 6.5 mm transwell chambers with 8 mm pores (Corning, NY, USA). PA treated BFLSs (1×10^5 cells) were plated in the upper chambers in duplicate filters. Serum-free culture medium with or without 1 mg/mL LPS (Escherichia coli, strain 0128:B12, Sigma) was added to the lower part of the chambers. Cells were allowed to migrate for 24 h. After a 24 h incubation period at 37°C, the non-migrating cells were removed from the upper surface by cotton swabs and the filters

Table 1- List of Primer

Primer Name	Forward 5\` to 3\`	Reverse 5\` to 3\`
B.MMP-1	3-´ACACCCCAGACCTGTCAAGA-5´	3´- CGACTGAGCGACTAACACGA-5´
B.MMP-2	3´-CGAATAGAATAGCCCCTCGG-5´	3´- AGGCTCGGTGCGTGTG- 5´
B.MMP-3	3´-GAGGAGGCGACTCCACTTAC-5´	3´- GGCTGATGCATGTGACCAGA-5´
B.MMP-9	3´- CGCTATGGCTACACTCCTGG-5´	3´- TTGTCCTGGTCGTAGTTGGC-5´
B.GAPDH	3´-GAGAACGGGAAGCTCGTCAT-5´	3´-TTGATGGTACACAAGGCAGGG-5´

were stained with crystal violet. Cells that migrated through the membrane to the lower surface were counted in five representative microscopic fields (×100 magnification) and photographed. Cell invasion ability was determined using Matrigel invasion chambers (BD Biosciences, Tokyo, Japan) according to the manufacturer's instructions. The upper chambers were freshly coated with Matrigel and medium was added to the lower chamber as described above. RA-BFLSs (5×10^4 cells) were suspended in medium containing 2% FBS and seeded into Matrigel pre-coated transwell chambers. Cell invasion was allowed to occur for 24 h and the gel and cells on the top membrane surface were removed with cotton swabs. Cells that had penetrated to the bottom were counted (Costar NY14831, USA) [21].

Western Blot Analysis

10^6 BFLS cells were cultured, then PA and LPS were added and then it was incubated for 24 h in 37°C. After experimental treatment, whole cell lysates from BFLSs were generated using a Total Protein Extraction Kit (sigma-Aldrich, UK) according to the manufacturer's instructions. Protein concentrations were determined using a Bradford assay (sigma-Aldrich, UK). Equal amounts of protein (20 µg) were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to enhanced chemiluminescence (ECL) nitrocellulose membranes (Roche, USA). After blocking with 5% BSA for 2 h, blots were probed with primary antibodies at 4ºC for 12 h, primary antibodies MMP-1 (1:200), MMP-2 (1:200), MMP-3 (1:200), MMP-9 (1:200) (Santa Crus, USA) and then incubated with secondary anti-mouse antibody (1:5000), anti-rabbit (1:5000), anti-goat (1:5000) (sigma-Aldrich, UK) for 2 h. Membranes were then incubated with appropriate secondary antibodies for 2 h at room temperature. ECL reagent (GE Healthcare) was used for protein detection. The relative expression of each protein was determined by densitometry and normalized to the control. Each blot shown is representative of at least three similar independent experiments [22].

Gelatin Zymography

The enzymatic activities of MMP-2 and MMP-9 were determined by gelatin zymography. Briefly, cells were seeded and allowed to grow to confluence and then incubated in serum free medium for 24 h. The supernatants were collected 24 h after stimulation, mixed with non-reducing sample buffer, and separated by 10% SDS-PAGE containing 1% gelatin. After electrophoresis, gels were renatured by washing in 2.5% Triton X-100 solution twice for 30 min to remove all SDS. The gels were then incubated in 50 mM Tris-HCl (pH 7.5), 5 mM CaCl2, and 1 uM ZnCl2 at 37°C overnight. Gels were then stained with 0.25% Coomassie brilliant blue R-250 for 30 min and then destined in distilled water [23].

Enzyme-linked Immunosorbent Assay (ELISA)

Serum-free conditioned media samples were collected and centrifuged at 10000 g for 5 min to remove particulates. The concentrations of MMP-9 were measured using an ELISA kit (Sigma-Aldrich, UK) according to the

manufacturer's instructions.

Results

The result of Confirmation of BFLS cells by Vimentin Antibody

To confirm the BFLS cells, Vimentin Antibody which is specialized marker for mesenchymal cells, was used. The result is shown in Figure 1.

Effects of PA on Cell Viability of BFLS Cells

The potential cytotoxicity of PA on BFLS cells was evaluated by MTT assay. The results showed that PA did not significantly affect cell viability at concentrations ≤0.1 µg/mL. In the concentration of 1µg / mL dead cells and living cells are equal (LC50) Thus, we chose PA (1 µg/mL) in the subsequent experiments (Figure 2).

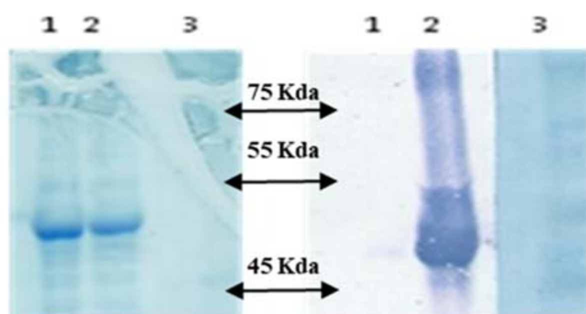

Figure 1- SDS PAGE (A) and Western blot (B). Line 1 in both images is related to the lysis of BFLS cells without antibodies. Line 2 in both images is related to the lysis of BFLS cells plus the Vimentin antibody. One sample was gotten ready for colouring with Trypan blue and another sample for Western blot. The presence of a molecular weight of 55 KDa represents a specific reaction of Vimentin with BFLS cells and a lack of reaction to macrophage cells [24].

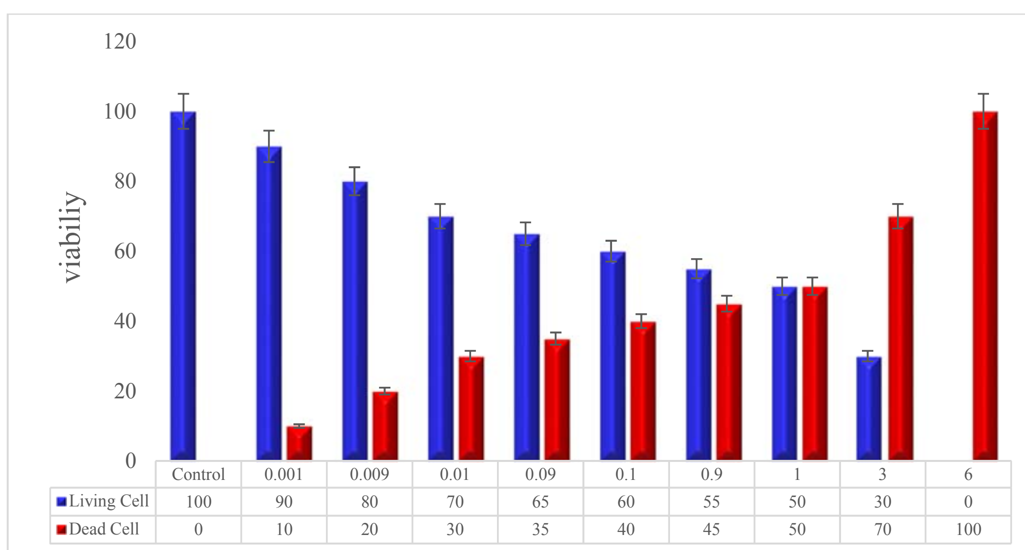

Figure 2- Determination of the cytotoxicity by PA. Comparison of percent viability (vertical axis) and dead synoviocytes using different concentrations of PA (horizontal axis; µg/mL). In the concentration of 1µg/mL dead cells and living cells are equal.

Effect of PA on MMP-1, MMP-2, MMP-3, MMP-9 expression in Real-time PCR

Real-time PCR results analysis was done by Pffafi or ΔΔC method.

$2^{\Delta\Delta C}$= Expression Ratio ct

CT= ΔC Calibrater-ΔC main cytokine

CT= (CT main cytocine- CT GADPH)-(CT sample positive–Ct GADPH) ΔΔ

The results showed that PA significantly decreases the MMP-9 gene expression compared to Cell+LPS group (Table 2 and Figure 3). The highest effect of PA in reducing MMP-9 was observed when Median LC50 PA was administrated. PA, in different doses, did not show a significant effect on reducing MMP-1, MMP-2 or MMP-3 gene expression in OA.

Migration and Invasion Assay results

While PA concentrations increases, synoviocyte cell migration decreases compared to cells which did not migrate. This is due to cell loss in high concentration of PA. Therefore, Experiments shows that the best dose for this test is Median LC50. (Fig 4.A). Cell Invasion has been reduced by increasing PA concentrations (Fig 4.B).

Table 2- Effect of PA on synoviocyte cells stimulated with LPS via Real-Time PCR

MMPs	Cell	Cell + LPS	Cell + LPS + Median LC50 PA	Cell + LPS + LC50 PA	Cell + LPS + Lethal Dose PA
MMP-1	18.68	21.86	21.47	22.22	21.69
MMP-2	18.68	40.85	43.23	45.32	44.06
MMP-3	18.68	20.46	21.17	23.21	24.62
MMP-9	18.68	68.15	9.64	28.78	42.08

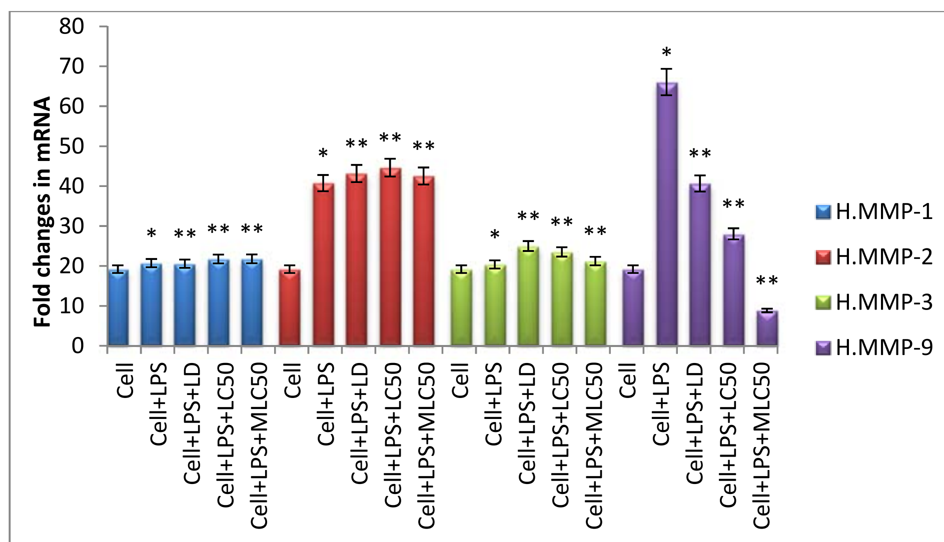

Figure 3- Effect of PA on expression of MMP-1, MMP-2, MMP-3, MMP-9 in BFLS cells by Real –Time PCR. **Gene expression (vertical axis) in various groups (horizontal axis) is measured and compared with the group of Cell + LPS. Statistically significant differences between the groups Cell + LPS and other groups were measured by t-student and ANOVA. The tests are repeated for three times. (mean ± SD ،n=3) **P < 0.001; *P <0.05. LD50: Lethal Dose 50%, LC50: Lethal Concentration 50%, MLC50: Median LC50.**

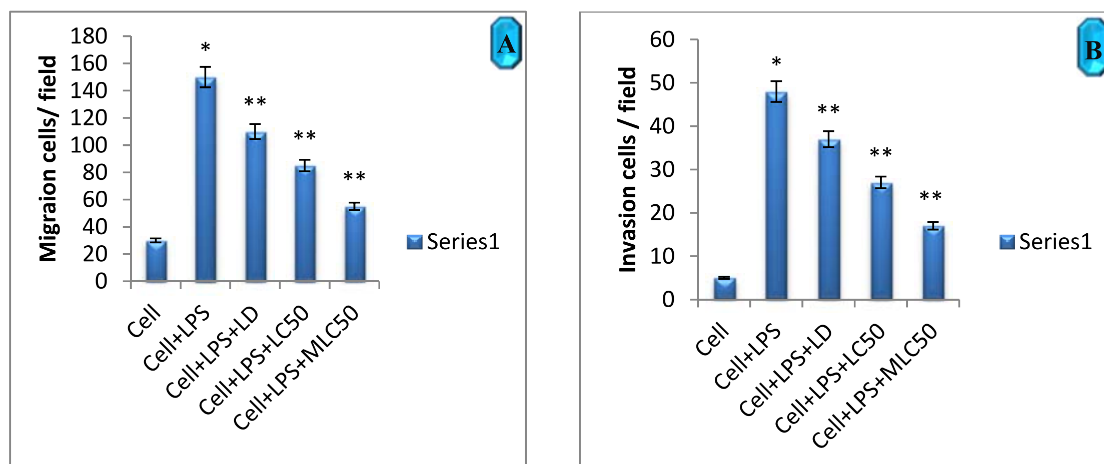

Figure 4- PA effect on the migration and invasion of BFLS cells stimulated with LPS.
The number of migratory cells (Figure A) and invasive cells (Figure B). (mean ± SD ‹n=3) **P < 0.001; *P <0.05. LD50: Lethal Dose 50%, LC50: Lethal Concentration 50%, MLC50: Median LC50.

The effect of the PA on MMP-1, MMP-2, MMP-3, MMP-9 protein expression investigated by Western blot

Although protein expression of MMP-1, MMP-2, MMP-3, was not observed in BFLS cells (except in positive control) there was significant MMP-9s protein expression. This matter was confirmed by the fact that PA does not affect MMP-1, MMP-2, MMP-3s protein expression, but there was a meaningful effect on MMP-9s expression (Fig. 5).

The effect of PA on the expression of MMP-2, MMP-9 determined by gelatin zymography

Zymography in this study is used to confirm PA's inhibitory effect on MMP-2, MMP-9s activity in BFLS cells stimulated with LPS. PA caused reduced MMP-9 activity in BFLs cells stimulated by LPS however no effects on MMP-2 were observed (Fig. 6).

PA effect on MMP-9s expression investigated by ELISA

ELISA is done for confirming PA's inhibitory effect on MMP-9 activity in BFLS cells stimulated with LPS. With increasing concentrations of PA, MMP- 9 activity was reduced (Fig. 7), but as it was mentioned before, the best concentration is median LC50, because despite a further reduction in the MMP-9 expression in high concentrations of PA, it has a toxic effect on cells and kills the cells (Fig. 7).

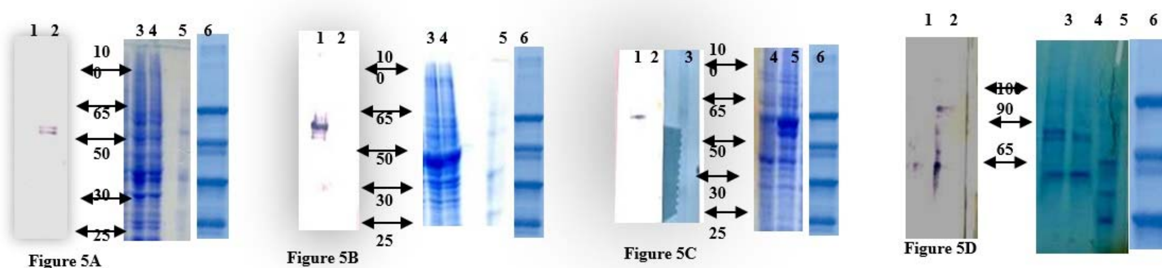

Figure 5- Western blot gel image. MMP-1 (A). Line 1 on the membrane: Cell+LPS+PA / L2 on the membrane. Cell+ LPS/ L3 on the SDS PAGE: Cell+LPS+PA/ L4 on the SDS PAGE: Cell+LPS, L5, 6: Protein MM on the SDS PAGE. MMP-2 (B). Line 1 on the membrane: Cell+LPS / L2 on the membrane. Cell+ LPS+PA/ L3 on the membrane: protein marker/ L4 on the SDS PAGE: Cell+LPS+PA/ L5, 6: Protein MM on the SDS PAGE.
MMP-3 (C). Line 1 on the membrane: Cell+LPS / L2 on the membrane. Cell+ LPS+PA / L3 on the membrane protein MM/ L4 on the SDS-PAGE: Cell+LPS/ L5 on the SDS PAGE: Cell+LPS+PA, L 6: Protein MM on the SDS PAGE.
MMP-9 (D). Line 1 on the membrane: Cell+LPS+PA / L2 on the membrane. Cell+ LPS/ L3 on the SDS PAGE: Cell+LPS+PA/ L4 on the SDS PAGE: Cell+LPS, L5, 6: Protein MM on the SDS PAGE.

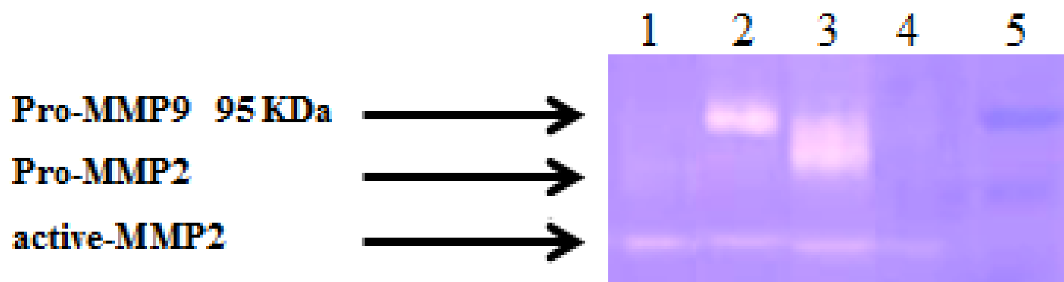

Figure 6- Image gelatin zymography. Well 1: Cell + LPS + PA. Well 2: Cell+LPS. Well 3: Cell+LPS+Vehicle. Well 4: -ve control. Well 5: MW Marker.

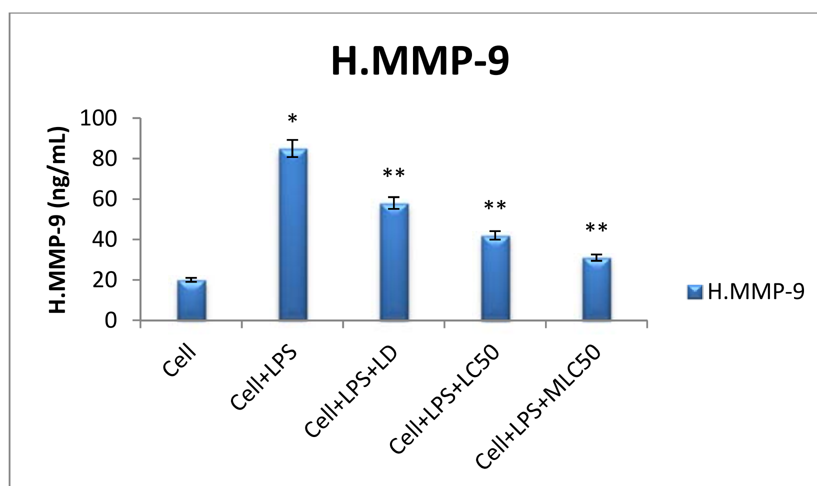

Figure 7- Investigation of PA effect on MMP-9s expression using ELISA. Vertical axis shows MMP-9 activity while horizontal axis shows different concentration of PA. By increasing concentrations of PA, MMP- 9 activity was reduced significantly. (mean ± SD) **P < 0.001; *P <0.05. LD50: Lethal Dose 50%, LC50: Lethal Concentration 50%, MLC50: Median LC50.

Discussion

MMPs are a large family of proteolytic enzymes which are related to breaking down of various extracellular matrix compounds [25]. It is well-known that MMPs play a major role in osteoarthritis's pathogenesis by the degradation of extracellular matrix chondrocytes [26]. Various MMPs, including MMP 1, 2, 3, 8, 9, 13, 14 are expressed in various diseases of the joint [27]. MMP expression level is lower in healthy joints compared to abnormal joints. For instance, in arthritis and interstitial collagen degradation, MMPs expression levels increase [28]. MMP-1 is able to break down collagen type II while MMP-3 is damaged extracellular matrix components like laminin and fibronectin [29]. MMP-2 is able to break collagenase type IV and it is commonly released in early wound healing [28]. MMP-9 plays a significant role in angiogenesis and it is considered as well with elastase as a regulatory factor in neutrophil migration to the base of the membrane [30]. MMP-9 is the main enzyme in ECM destruction and its overexpression is important in cell invasion [31]. Invasion and migration of fibroblast cells and macrophages play an important role in the osteoarthritis pathogenesis. It seems the reason is the existence of a PEX domain. The MMP-9 PEX domain specifically binds to TIMP-1 and the MMP-2 PEX domain binds to TIMP-2 while other MMPs are activated by catalytic domain bound to TIMP. It has been proved recently that PEX domain plays an intermediary role in protein–protein interactions and increasing cell migration [32]. O-glycosylated (OG) domain that is only found in MMP-9 has an effect on the binding of TIMP-1 to PEX domain [33].

The expression of MMPs in Synoviocytes can be induced using LPS. LPS is a huge molecule known as endotoxin, and is found in the outer membrane of gram-negative bacteria. LPS is an activator of innate immune system such as macrophages and neutrophils by binding to TLR4 complex. So, we used of LPS for simulation of inflammation and increasing of MMPs gene expression in cell culture [34].

Results of the current study indicate no obvious change in the MMP-1 and MMP-2 and MMP-3s expressions in different concentrations of PA compared to the samples which were only exposure to LPS, implying that PA has no effect on these target genes. On the other hand, the results of MMP-9 gene expression highlighted that different concentrations of PA have significant impact on reducing MMP-9s gene expression and so decreases LPS-induced invasion of BFLS cells. The optimum concentration of PA is 0.26 μg/ml, which is equal to Median LC50. More than LC50 the number of living cells were decreased, thus were not suitable for the experiment. The effect of PA on reducing expression of MMP-9, but not MMP-2, is probably due to the higher ability of MMP-9 in destruction of fibronectin tissue and inducing cell invasion compared to MMP-2. Also, this effect may be related to the binding of TIMPs to PEX domain of MMP-2 that inhibits the activity of this MMP or the existence of OG domain on MMP-9; however, further studies are needed to elucidate this content. According to study on the Celastrol by Guoqing, treatment with anti-TLR4 antibody and TLR4 inhibitor was reduced the LPS-induced expression and enzyme activity of MMP-9. Additionally,

TLR4 inhibition reduces the LPS-induced transcriptional activity of NF-κB. Since NF-κB regulatory effect on multiple inflammatory cytokines transcriptional activation is widely known, it is expected that celastrol might target NF-κB to suppress MMP-9 transcription by LPS. TLR4 is a principal upstream signal transducer of NF-κB, that is activated by TLR ligands and cytokines. NF-κB is sequestered in the cytoplasm by binding to IκB family molecules and is activated by IκBα phosphorylation, whose subsequent degradation in the proteasome allows the NF-κB subunits, including p65 and p50, to enter the nucleus and activate target genes [35]. We propose that inhibiting MMPs at the level of gene expression serves as a viable therapeutic strategy to prevent the irreversible destruction of joints and may ultimately lead to the development of therapeutics aimed at suppressing joint destruction in OA. The two main pathways which are activated by MMP-9 are the NFκB and mitogen-activated protein kinase pathways [36]. It is found that PA could decrease MMP-9 gene expression, we recommend to compare PA whit pomegranate seed oil to identify that other components have the same effect or not.

The results of this study should be interpreted in the light of a certain limitations. The effects of PA on the treatment of OA in a cellular model does not significantly guarantee its beneficial effect in patients with OA and so further studies should use clinical trials to determine the effect of PA in patients with OA.

Conflict of Interest: The authors have no conflict of interest to declare.

Author contributions: all authors participated in the design, interpretation and review of the manuscript, and conducted the experiments.

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