Down regulation of COX-2, IL-1β, TNF-α in cynoviocyte by essential oil of Fraxinus excelsior

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Abstract:
Osteoarthritis (arthritis) is biomechanical, biochemical and cellular phenomenon, and is not known as a degenerative disease. Arthritis is one of the common chronic diseases and the most important reason of physical disability in the world. According to its side effect such as peptic ulcers, gastrointestinal bleeding, liver toxicity and renal complications due of prescribing current treatment contain corticosteroid and non-steroidal, we decided to evaluate possible effect of anti-inflammatory Esential oil of Fraxinus excelsior (EOFE) on biomarkers involved in disease. E.O.F.E were prepared of genetic resources center. Bovine articular cartilage derived from the metacarpophalangeal joints of 14–18-month-old animals (without any sign of inflammation and bleeding) sent to laboratory in sterile bags at 4ºC. Cells were cultured in appropriate condition and counted by hemocytometer, viability assessed by trypan blue. After LPS treatment, cytokine levels were assayed. Cells cultures again and were kept in 37ºC, 90% humidity in CO2 incubator and after RNA extraction, RT-PCR and PCR done. Also by Real-time PCR, gene expression was evaluated. E.E.F.E level cause down regulation of COX-2, IL-1β, TNF-α in LPS-stimulated cells.

Key words: E.O.F.E, Osteoarthritis, Cytokines, Cynoviocyte

1. Introduction:
Osteoarthritis is a common joint disease all around the world, eighty percent of 75 years old people affected. It is more predominant in elderly people. By attention to life expectancy in developing country until the 2020, about 71% of population older than 65 may involve and osteoarthritis lead to adverse effects on health and economy [1, 2]. Osteoarthritis represent incompetence of limb diarthrodial joints and have been known as a degenerative disease falsely. Osteoarthritis is result of several mechanical and biological factors which onset process associated in joint destruction [3]. Mesenchymal stem cells (MSCs) has potential capacity for cartilage regeneration. Cartilage destruction through inflammation in the synovium is mediated with synovial fluid (SF) fibroblasts. SF fibroblasts have been proven to be closely related in phenotype to
bone marrow (BM) MSCs \[4\]. This suggests that normal SF has a resident MSC population that increases during OA \[5\]. The synovial membrane (SM) is a specialized mesenchymal tissue lining the spaces of diarthrodial joints, bursae, and tendon sheaths \[6\]. SM includes two layers: the intima inner layer, composed of one or two sheets of macrophages or fibroblast-like synoviocytes, and the subintima outer layer, composed of two to three layers of synoviocytes lying over loose connective tissue rich in fibroblasts, secreting collagen, and other extracellular matrix proteins. The subintima layer has few macrophages and lymphocytes, fat cells, and blood vessels, which provide nutrients to the SM and the adjacent avascular cartilage \[6\]. Cells from the SM intimal layer secrete the SF, which provides articular cartilage lubrication, chondrocyte activity, and nutrition. The synovial intima is composed of two different cell types: type A and B synoviocytes \[7\]. Type A and B synoviocytes present cell surface markers that identify them as coming from macrophage and fibroblast lineages, respectively \[8\]. Nowadays, osteoarthritis didn’t know as degenerative disease, but it is due to biomechanical, biochemical and cellular active phenomenon \[1\]. Therapy aids decreasing pain, inflation and prevention of joint destruction progress. In the most cases, current treatment, is not effective, also have side effects such as peptic ulcers and gastrointestinal bleeding. Non-steroidal drugs like Ibuprofen, Sodium Diclofenac, naproxen is prescribed. Also Steroidal drugs, prescribed intramuscular and intra-cartilage. High dose of acetaminophen in chronic condition cause liver toxicity and renal complications. So the importance of research is totally clear \[9\]. According to high incidence of knee arthritis, life expectancy, the increasing number of elderly, over-consumption of synthetic drugs and severe side effects lead to high costs in National health budgets \[10\]. So in recent years and especially after clarifying the role of inflammation in pathogenesis of osteoarthritis, researcher attempt to highlight anti-inflammatory effect of several plant species \[11\], with the aim of providing pain-killer and anti-inflammatory drugs. In this study, we evaluate anti-inflammatory role of Fraxinus excelsior via TNF-α, IL-1β, COX2, iNOS expression in synoviocyte isolated of carpometacarpal (CMC) Holstein healthy eight-month old calf. Fraxinus excelsior, one of the lamiales plant species is a tree with the height of 15-20 meters. Trees with many branches grow in the Northern Forests of Iran \[12\]. It has citrus leaf with the length 15-20 centimeter, with 4-5 toothed leaflets and a terminal leaflet. It’s red-brown Flowers is uncovered which usually appear before leaves. The leaves are picked after full development in july and june. The leaves and bark contain glycoside named fragyzazide or fragzine plus manite-inosite-quercetin-dextrose-malic acid-gum-tannins and essence. Its bark is bitter, mucus, astringent and febrifuge. Also it used as febrifuge Before chinchona appearance. Leaves have anti-inflammatory, anti-oxidant, anti-microbial effects \[13, 14\], supposed as proper treatment for gout and rheumatism. According to previous studies, Fraxinus excelsior inhibit inflammatory process and prescribed for kidney stone, relieve muscle spasms and infertility \[15\]. Fruit juices contain Nuzhenid and G13 two phenolic that reduce plasma glucose concentrations in non-diabetic after meal \[16\]. A study is done on obese mice with high blood sugar, juice prescribing in short time, lower blood sugar \[15, 16\]. Also in another study, it has been proved that this plant inhibits T regulatory cells and arachidonic acid \[17\]. Fraxinus excelsiorhas been proven used for treatment of variety of diseases such as: Osteoarthritis, arthritis \[18\], antihypertensive effect \[19\]. Fraxin showed free radical scavenging effect at high concentration (0.5 mM) and cell protective effect against H2O2-mediated oxidative stress. Fraxin recovered viability of HUVECs damaged by H2O2- treatment and reduced the lipid peroxidation and the internal reactive oxygen species level elevated by H2O2 treatment \[20\]. A wide range of chemical components including coumarins, secoiridoids, phenylethanoids, flavonoids, and lignans has been isolated from Fraxinus species. Extracts and metabolites have been found to possess antiinflammatory, immunomodulatory, antimicrobial, antioxidative, skin regenerating, photodynamic damage prevention, liverprotecting, diuretic and antiallergic activities. Some species find application in contemporary medicine \[21\]. This plants is a member of Oleaceae family \[22-24\]. This plant present two phenoliccompounds mainly iridoids and a natural extract of this plant is glucevia. This extract has coumarins.
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(fraxin, fraxetin, esculin, esculetin, cichoriin, scopolin and fraxidin glucoside) [25]. The leaves present tannins (8%); iridoids; coumarins; flavonoids; mannitol (16% to 28%) and mucilages (10% to 20%) 48. This part of plants can be used as vasoprotective and venotonic [26]. In this study, we proved Fraxinus excelsior’s extract suppress pre-inflammatory cytokines stimulated by LPS. As a result, according to inflammatory role of cytokines in disease progression which reduce by steroidal and anti-steroidal drugs, E.O.F.E also may have prescribed as an alternative therapy.

2. Methods and Materials:
2.1. Sample preparation plants:
Fraxinus excelsior leaves prepared from East Azarbaijan Province which located in terms of geographical location in the range of 45 degrees 7 minutes, 20 minutes East longitude and 36 degrees 45-minute North latitude to 39 degree and 26 minutes. Samples approved by Iran genetic resources center, then ethanolic extract was prepared. The -hexane, DCM and MeOH extracts (0.025g) were dissolved in 5 mL DMSO (or MeOH) to obtain stock solutions of 5 mg/mL concentration.

2.2. Cell culture:
2.2.1. Bovine chondrocyte:
Articular surfaces of bovine fetlock joints are covered by hyaline Cartilage. Chondrocytes reside singly or in pairs in the lacunae of a vast extracellular matrix (Fig. 2). Rough endoplasmic reticulum and the Golgi complex zones are prominent, indicative of active matrix synthesis. Each chondrocyte is surrounded by a distinct rim of territorial matrix (lacunar matrix). This matrix consists of a dense network of fine collagen fibrils whose crossbanding pattern is partly obscured by RR-positive material. The extraterritorial matrix contains a prominent network of thick collagenous fibrils with diameters ranging from 40 to 80 nm. These fibers have a characteristic cross banding with a periodicity of 64 nm and are partially coated by RR-positive material [27]. Chondrocyte cultures were prepared from bovine articular cartilage. The metacarpophalangeal joints (fetlock joints) from thoracic limbs of 8-old bovines were opened under aseptic conditions (Fig. 1). Shavings of hyaline cartilage (4 x 4 x I mm) were removed from the outer two-thirds of the articular cartilage, such that contamination with bone cells or other connective tissue cells could be avoided. Cartilage slices (15-20 g) were collected in Ham's F-12 medium, which was enriched with 50 µg/ml gentamycin, 100IU/mL Penicillin, 100 µg/mL Stereptomycin and 5 µg/mL Amphotericin B. Cartilage slices were digested in 1% (wt/vol) promise in 100 nil of F-12 medium containing 10% fetal bovine serum (90 min, 37°C), followed by .4% (w/v) bacterial collagenase in the same medium (180 min, 37°C). The final digest was centrifuged at 900 rpm for 10 min. The cell pellet was washed three times in serum-free medium. Cell suspensions were passed through Nitex nylon filter (mesh width 90 pin) and resuspended in serum-containing medium. Cell viability was determined by trypan blue dye exclusion [28].

2.2.2. Human THP-1 monocyte/macrophage like cells (Pasteur Institute of Irancells defrost in laboratory (were propagated in control media containing:
RPMI-1640 medium ( GibCO®, Grand Island, NY, USA) supplemented with 2mM L-glutamine (Sigma-Aldrich,St. Louis, MO, USA), 10% FBS (Sigma-Aldrich, St. Louis, MO, USA), 1.5 g/l sodium bicarbonate (Sigma-Aldrich), 4.5 g/L glucose (Sigma-Aldrich,St. Louis, MO,USA), 10mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES, Sigma-Aldrich,St. Louis, MO, USA), 1.0mM sodium pyruvate (Sigma-Aldrich,St. Louis, MO,USA) and 0.05mM β-mercaptoethanol (Sigma-Aldrich,St. Louis, MO, USA), and 50 mg/ml Gentamycin (Daropakhsh, Tehran, IRAN), Penicillin 100 U/ml, Streptomycin 100 µ/ml (BIO IDEA, Tehran, IRAN), Amphotericin B 0.25 µg/ml (Cipla, Mumbai, INDIA) Cells were pelleted via centrifugation and assessed for viability using the Trypan-blue exclusion method. Viable cells were plated in six-well plates at a density of 5×105 cells/well and treated the same day [29].
2.3. Characterization of Bovine Fibroblast-like Synoviocyte:

Fibroblast-like synoviocytes were characterized by immune staining with vimentin, a specific cellular marker for mesenchymal cells [30]. Whole cell lysates were prepared from about 2×10^5 cells by homogenization in the lysis buffer and subsequent centrifugation at 14,000 rpm for 15 min. The protein concentration in the supernatant was determined using the Bradford method (Bio-Rad, Hercules, CA, USA). Protein samples were separated on 12% SDS-PAGE and then transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). The membranes were incubated with primary antibody to the cytoplasmic enzyme LDH into the supernatants were collected from each well. Cell monolayers were then treated with 1X SDS loading buffer added to the lysis sample and boiled for 10 minutes and then loaded on 12% SDS-PAGE. The proteins were transferred to the nitrocellulose membrane and the surface of the membrane was blocked overnight in blocking buffer (PBS, PH:7.3, 0.5% BSA, 0.5% Tween). The filter was washed thrice 20 minutes each for the wash in washing buffer (PBS, pH:7.3, 0.05% BSA, 0.05% Tween-20). After washing it was incubated with primary mouse antibody specific for the vimentin at 1:200 dilution for 1 h at 37°C. After incubation, the filter was washed three times for 10 minutes each in wash buffer. It was followed by incubation with secondary antibody (goat anti mouse IgG, alkaline phosphates conjugate) (Sigma, USA) 1:5000 dilutions in wash buffer for 2 hours at room temperature. After incubation filter was rinsed twice with alkaline phosphate buffer (Tris-HCL, pH=9.5 100mM, NaCl 100mM, and MgCl2 50mM), and then incubated in the substrate solution (added 66μl of 50 mg/ml of nitro blue tetrazolium [NBT], and 33μl of 50mg/ml of bromochloroindiyil phosphate [BCIP], to 10 ml of alkaline phosphate buffer), room temperature with gentle agitation a dark place, until band appear [31].

To confirm that fibroblast-like synoviocytes were not contaminated by macrophages, CD14 expression was evaluated by two methods. The synovial membrane is normally a thin membrane consisting of a lining and a sublining layer of cells. The lining consists of 1-3 layers of fibroblast-like synoviocytes (FLS) and macrophage-like synoviocytes (MLS) that overlay the sublining [32]. In the healthy synovium, the FLS express Cadherin-11 (Cad-11) as the only cell type, whereas MLS express CD14 and CD68 [33, 34]. First one is the detection of CD14 expression as a marker for macrophage [35], and second one the expression of CD14 with the reverse transcription polymerase chain reaction (RT-PCR). Total RNA extraction was performed using a commercial kit (SinaClon kit IRAN). RNA conversion to cDNA was performed using the commercial kit Superscript First-strand synthesis System for RT-PCR (2 step RT-PCR Vivantis) and real-time PCR was performed with (SinaClon) Two microliters of cDNA were amplified by specific oligonucleotide primers. Two sets of human-CD14-specific primers were used. The first one consisted of sense 5′ GCT GGA CGA TGA AGA TTT CC 3′ and antisense 5′ ATT GTC AGA CAG GTC TAG GC 3′ primers with expected product sizes of 535 bp. Primer sequences were selected to amplify both human and bovine genome and were from separate exons exclude a possibly genomic DNA contamination of the RNA samples. PCR reactions were performed in a total volume of 25 ml containing 1U Taq DNA polymerase (SinaClon, Iran), 25 pmol of each primer, 200mM deoxynucleotide triphosphates (dNTPs) in 1x PCR buffer (10mM Trise, pH 8.3, 50mM KCL, 1.5 mM MgCl2). Cycling parameters were as follows: 1min at 94°C, 1 min at 55°C, 1 min at 72°C for CD14. The size of the amplified sequence was 403 bp. mRNA from human macrophages was used as a positive control for CD14 expression. PCR products were analyzed on 1.5% agarose gel, stained with ethidium bromide.

2.4. Determination the toxicity effect on synoviocyte with MTT, LDH, SDH:

2.4.1. LDH assay:

In the lactate dehydrogenase (LDH) assay, the leakage of the cytoplasm-located enzyme LDH into the extracellular medium is measured. The presence of the exclusively cytosolic enzyme, LDH, in the cell culture medium was indicative of cell membrane damage [36]. The cytotoxic effect of essential oil of *Fraxinus excelsior* (EOFE) was also examined using LDH assay as described by Linford [37]. With some modifications. Briefly, 5×10^3 THP-1 was seeded per well in 96-well microtiter plates. Twenty-four hours after cell seeding, cells were exposed to varying concentrations of alcoholic extract of *Stachys lavandulifolia* L. After 24 hours of treatment, the supernatants were collected from each well. Cell monolayers were then

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Treated with a cell lysis solution for 30 minutes at room temperature. The cells and the lysate were collected. LDH activity was measured in both the supernatants and the cell lysate fractions using Lactate Dehydrogenase Activity Assay Kit (Sigma-Aldrich, St.Louis, MO, USA), in accordance with the manufacturer instruction. The absorbance was determined at 490 nm using 96-well plate ELISA reader. The percent of LDH released from the cells was determined using the formula: LDH release = (absorbance of the supernatant)/(absorbance of the supernatant and cell lysate) × 100.

2.5. 2. SDH assay:

SDH activity assay Succinate dehydrogenase (SDH; EC 1.3.5.1) is a mitochondrial enzyme that catalyzes the oxidation of succinate to fumarate and carries electrons from FADH to CoQ in eukaryotes and bacteria [38]. It has a central function in the maintenance of cellular energy metabolism via the Krebs (tricarboxylic acid) cycle and the electron transport chain [39, 40]. Mutations in SDH cause hereditary paraganglioma / phaeochromocytoma syndrome and a neurodegenerative disorder known as Leigh syndrome. SDH activity is determined by generating a product with absorbance at 600 nm proportional to the enzymatic activity present. One unit of SDH is the amount of enzyme that generates 1.0 µmole of DCIP per minute at pH 7.2 at 25°C [41].

2.4.3. MTT cell proliferation assay:

The metabolic activity can be evaluated by measuring the activity of the mitochondrial enzyme succinate dehydrogenase using the MTT test. This test is widely used in the in vitro evaluation of the toxicity of plant extract [42]. We applied the MTT test to evaluate the safety of extract from ECE in cells from the human monocyte cell line THP-1. Cells were exposed to increasing concentration (0.001, 0.009, 0.01, 0.09, 0.1, 0.9, 1, 9, 18, 0.9, 1, 9, 18, 36, 45, 54, 63, 72, 81, 90 and 100 µg/ml) of EOFE for 24 hours. There is no sign of negative effect after treatment with concentration up to 9µg/ml. Concentrations higher than 15µg/ml caused a significant reduction in the cell viability (LC50 is 45µg/ml and its medium 12.38 µg/ml).

2.5. Treatment procedure:

One mg of EOFE was dissolved in 10 ml of DMSO and diluted with cell media to achieve the required final concentration. The optimal experimental concentration of AESL was first determined by incubation bovine chondrocyte and THP-1 (5x105 cells/well) for 72 hours with control media supplemented with 10% FBS or EOFE at the concentration of 0.001, 0.009, 0.01, 0.09, 0.1, 0.9, 1, 9, 18, 0.9, 1, 9, 18, 36, 45, 54, 63, 72, 81, 90 and 100 µg/ml. Cells were activated with lipopolysaccharide (LPS, 20 ng/ml: Sigma-Aldrich), Dexamethasone sodium sulphate (390.3 μM) [43], buprofen (IC50 = 11.20 ± 1.90 μg/mL) both used as appositive control to compare the effect of E.O.F.E with dexamethasone and NSAID [44]. For 24 hours and the cellular supernatant was analyzed for secreted PGE2 and nitrite concentrations. There was no significant effect on PGE2 and nitrite levels at 10µg/ml. There was a slight suppression of PGE2 and nitrite levels at 15µg/ml. The optimal level of suppression was found at 7.5µg/ml.

2.5.1. LC50 determination by with EOFE:

LC50 is defined as amount of compound to induce death in 50% of cell population. 5x10^5 cells incubated in 12 wells with 1 ml of DMEMF-12 media enriched 10% FBS, 50µg/ml ascobic acid, 100 units 50µg/ml, 100µg streptomycin and 0.25µg/ml amphotericin. Plates rotated 2-3 minutes then kept in incubator 37C, 5% CO2, 90% humidity about 20-30 minutes. E.O.F.E in 0.01, 0.09, 0.1, 0.9, 1, 9, 18, 36, 45, 54, 63, 72, 81, 90, 100 µg/ml prepared and added to 12 wells plates, followed by keeping in incubator 37C, 5% CO2, 90% humidity. After 24 hours, plates check out for LC50 determination. Accumulation of cell mass is sign of Cell lysis. by E.O.F.E injection to media nearly 50% of cell population died. In order to avoid error, median LC50 estimated 16.41µg/ml [45].

2.5.2. LPS treatment:

In order to stimulate illness circumstance and elevate pre-inflammatory cytokines, 1µg/ml LPS [46], added.
At first, 6x10^6 cells cultured followed by 1000 ng LPS treatment after 72 hours. Plates kept 24 hours in incubator CO2 to evaluate pre-inflammatory cytokines like COX-2, TNF-α, iNOS.

2.6. RNA extraction steps of cynoviocyte:

We used Trizol reagent in order to RNA extraction (SINACLON BIOSCIENCE CO, Tehran, Iran) then by UV-2100 spectrophotometer, RNA concentration determined, followed by DNase to remove DNA. We used 2 step RT-PCR kit for changing cDNA to RNA according to protocol (SINACLON BIOSCIENCE CO, Tehran, Iran)

2.6.1. DNA production from cynoviocyte:

cDNA synthesis was performed using cDNA synthesis kit (CinnaGen). For each sample, 1 μg RNA with 2 μl buffer was added to 1X M- MuLV Reverse Transcriptase (100 unit), followed by adding 10 μl nuclease free water and 6 - 10 μl cDNA synthesis mixture. The experiment was performed at 42 °C for 60 min followed by incubation at 94 °C for 5 min to stop the reaction. The samples were transferred in tubes in order to cool on ice and long term storage.

2.6.2. Semiquantitative:

Semiquantitative RT-PCR was performed using specific primers: 1-Specific primers for bovine – COX2:
Forward : 3´-CTC TTC CTC CTG TGC CTG AT-5´ Reverse : 3´- TG AGT ATC TTT GAC TGT GGG A -5´
Tm forward:52/9°C, Tm reverse: 52°C, PCR product size: 100 bp 2- Specific primers for bovine TNF – α:
Forward : 3´-TAA CAA GCC GGT AGC CCA CG-5´ Reverse : 3´-GCA AGG GCT TTT GAT GGC AGA-5´
Tm forward: 61°C, Tm reverse: 59/4 °C, PCR product size: 100 bp 3- Specific primers for bovine IL-1β :
Forward : 3´-TTC TCT CCA GCC AAC CTT CTA T -5´ Reverse : 3´-ATC TGC AGC TGG ATG TTT CCA T -5´
Tm forward: 56/5°C, Tm reverse: 57/2°C, PCR product size: 100 bp 4- Specific primers for bovine Glyceraldehyde 3- phosphate (GAPDH) : Forward :3´-ATT CCA CCC ACC GAC ATG T -5´ Reverse: 3´-CGC TTC TGG AAG ATG GTG ATG -5´
Tm forward: 56/3°C, Tm reverse: 56/, PCR product size: 100 bp 2..6.3. Real-time PCR Quantative methods: RT-PCR by mentioned primers done, we used evergreen as mastermix (sinacolon, Iran-Tehran). Threshold cycle by standard curve and Pfaffi method assessed.

3. Statistical analysis:

Data were presented as mean ± standard error of the mean (SEM). All experiments were performed in triplicate, and their results were analyzed by one-way analysis of variance (ANOVA), REST software version.20 followed by Dunnett's post hoc test using GraphPad Prism version 5 for Windows (GraphPad Software Inc., San Diego, CA, USA). The statistically significantly value was set at p < 0.05.

4. Results:

4.1. Cytotoxicity:

Results of MTT, LDH and SDH assay showed at diagram 1, IC50% value for BFLS by Trypan Blue, MTT, LDH and SDH assay respectively are 36-45 μg/mL, 45-54 μg/mL, 45, 45-54 μg/mL, and median IC%)% respectively is 8.13 μg/mL, 12.23 μg/mL, 12.23 μg/mL and for last assay is 16.

4.2. The effect of EOFE on TNF-α and IL-18 gene expression

Bovine sinoviocytes cultured for 72 h with control media alone and EOFE alone expressed low levels of TNF-α and IL-1β relative to LPS-activated chondrocytes (Table 1, Fig.1). Sinoviocytes activated for 1 h with 100 ng/ml LPS expressed increased levels of TNF-α and IL-18. EOFE suppressed TNF-α and IL-18 expression by approximately 45% in activated chondrocytes when compared to the activated control. TNF-α and IL-18 expression were reduced by approximately 40% when compared to activated control. IL-18 and TNF-α expression levels in LPS-stimulated cells and treated with dexamethasone and NSAID...
Dr Maghsoudi, Hossein et al. Down regulation of COX-2, IL-1β, TNF-α in synoviocyte by essential oil of Fraxinus excelsior respectively were 27.34% and 29.89%, which reduced expression by about 70% and 70% respectively. Due to the fact that the DMSO has an anti-inflammatory effect, in this study was used as a control. As a result, DMSO treatment has no effect on the reduction of expression in stimulated cells as well as in the increasing expression in unstimulated cells.

4.3. The effect of EOFE on COX-2 geneexpression and PGE_2 production in bovine Sinoviocytes:
Bovine sinoviocytes incubated with control media alone and E.O.F.E alone expressed low levels of COX-2 relative to activated sinoviocytes (Table 1, Fig.1). Sinoviocytes also secreted low levels of PGE_2 in the cellular supernatant (Fig.2). Sinoviocytes activated with 100 ng/ml LPS expressed high levels of COX-2 and secreted a significant increase in PGE_2 in the cellular supernatant. AENS downregulated COX-2 expression by greater than 35% when compared to activated control levels (Table 1, Fig.1). Pretreatment with EOFE in activated sinoviocytes reduced PGE_2 levels by 40% when compared to activated control (Fig.2). COX-2 expression and PGE_2 production in EOFE -treated activated sinoviocytes was reduced to levels similar to non-activated control levels (Table 1, Fig1.1).

4.4. The effect of EOFE on iNOS gene expression and nitrite production in bovine Sinoviocytes:
Bovine chondrocytes incubated with control media alone and E.O.F.E alone displayed low levels of iNOS expression (Table1, Fig.1), and nitrite production (Fig.3), compared to activated chondrocytes. Activated chondrocytes expressed high levels of iNOS expression. Nitrite levels increased three-fold in activated chondrocytes compared to nonactivated cells. Activated chondrocytes pretreated with EOFE showed significant downregulation of iNOS expression by greater than 40% (Table1, Fig.1). Pretreatment with EOFE suppressed nitrite secretion by 30% in activated chondrocytes relative to activated control levels (Fig.3).

4.5. The effect of EOFE on cytokine gene expression in human THP-1 cells:
Human THP-1 cells incubated for 72 h with control media alone and E.O.F.E alone expressed low levels of TNF-α and IL-1β relative to LPS-activated cells (TABLE2, Fig.4). Cells activated with 100 ng/ml LPS showed a significant upregulation of TNF-α and IL-1β expression. In activated THP-1 cells pretreated with EOFE, TNF-α was reduced by 35% when compared to activated control cells. Pretreatment with EOFE suppressed IL-1β expression by approximately 35%. EOFE suppressed TNF-α and IL-1β expression to levels similar to nonactivated control levels (Table2, Fig4).

5. Discussion:
The source of many drug compounds such as penicillin, Digitoxin and warfarin from plants. However, many physicians doubt the use of natural compounds. This doubt is based on the fact that in these cases, the patient himself diagnoses and treats and assesses the result himself, and there are no scientific methods to prove the validity of these claims. However, a new group of natural compounds called nutritional supplements has been shown to have efficacy and pharmacological properties. The regulation of these substances causes problems in quality control of the products. However, patients are confronted with the flood of these products and they respond to many of these natural and organic compounds. Osteoarthritis is a degenerative joint disease that slowly involves a joint or several joints. Primary form of this condition develops in the middle age and older women and less commonly in men, but secondary osteoarthritis could occur in any age group. The most commonly involved joints include knee, spine and small joints of hands (distal interphalangeal and proximal interphalangeal joints) and hips. It characterized by joint stiffness and pain, sometimes joint swelling and finally joint limitation of motion and deformity, primary osteoarthritis essentially is an age related condition that over the years of life. Mechanical stresses gradually lead to cartilage breakdown. Pathologic features include cartilage destruction, subchondral bone sclerosis and cysts, and new bone formation at the joint margin makes osteophytes. Loss of cartilage in medial and retro patellar compartments of knee is an epidemic condition in millions of women around the world. Superimposition of inflammatory changes in the synovium are usually occur that accelerates cartilage degradation and breakdown. Increased level of proinflammatory cytokines (TNFα, IL1β), nitric oxide, the product of
prostaglandin pathway (PGE2) and overexpression of cox2 has been shown in osteoarthritic joints. Aging process, chronic repeated mechanical stresses and superimposed synovial inflammation are three main causes of degenerative joint disease in human. Any treatment modalities that decrease mechanical shearing forces and proinflammatory mediators in the joint could prevent degradative process of cartilage and save millions of people, especially women, from chronic pain and disability. In the present study, we show for the first time that the suppression of pro-inflammatory cytokine gene expression by E.O.F.E. Chondrocytes, fibroblasts and monocyte/macrophage are involved in these suppression. We demonstrate that E.O.F.E suppresses the gene expression of potent proinflammatory cytokines, TNF-α, IL-1β, COX-2 and iNOS in cultured chondrocytes. Fraxinus excelsior L. (Family: Oleaceae), commonly known as ‘ash’ or ‘European ash’, is an anemophilous tree native to the countries of temperate Asia and Europe, including Scotland [48, 49]. To date, various classes of compounds including benzoquinones, coumarins, flavonoids, phenylethanoids, secoiridoid glucosides, indole derivatives and simple phenolics have been reported from F. excelsior [50, 51]. The barks of F. excelsior have long been used as antipyretic [46]. From the beginning of the 20th century, the leaves of this plant have been recommended in prescriptions for the treatment of fever or rheumatism [52]. The alcoholic extract of F. excelsior barks possesses an anti-inflammatory property similar to diclofenac [52, 53]. The leaf tea is popular in Europe as a mild purgative and is often used for rheumatism, while the bark is effective against intestinal worms [52]. Other medicinal uses of this plant include its use in the treatment of arteriosclerosis, hypercholesterolemia, jaundice kidney problems [53]. Aqueous-ethanolic extracts from Fraxinus excelsior, Populus tremula and Solidago virgaurea inhibit biochemical model reactions representing inflammatory situations to various extents. These model reactions include xanthine oxidase, diaphorase in the presence of the autoxidizable quinone juglone, lipoxygenase and photodynamic reactions driven by riboflavin or rose bengal. The tested extracts are the components of the phytomedicine Phytodolor N (abbreviated as PD) which possesses antipyretic, analgesic, antiinflammatory and antirheumatic activity. Since several reactive oxygen species produced by the mentioned model systems are also involved in inflammatory processes, the beneficial activities of the complete drug may at least in part be due to the reported antioxidative functions of the individual components [54].
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Figure 4: In order to count live and dead cells, at 24-hour intervals, the cells were first trypsinized and carefully separated from the plate completely. Then, for each plate cavity, 100 µl of cell suspension (4x10^5) was added to 12 well plate mixed with 100 µl of E.O.F.E with these concentration respectively [0.001, 0.009, 0.09, 0.1, 0.9, 1, 9, 18, 36, 45, 54, 63, 72, 81, 90 1nd 180 µg/ml], then 800 µl of supplemented DMEMF-12 was added, kept at 37°C, 5% CO₂, humidity 95% for 24 hours. After 1 day cytotoxicity was performed with macroscopic and microscopic methods. In The macroscopic method cytotoxicity effect has symptom like lysis cell, detached cells, plug and debris, for microscopic method used hemacytometer for count the cells. After removing the used media trypsinized the cells, centrifuge, washing with PBS pH 7.2, 1 molar, through supernatant out, mix he pellete with 10 µl media and 10 µ trypan blue 0.4 %, then counting the cells. The number of colored cells (dead cells) and uncolored cells (living cells) determined by the use of a hemocytometer lam (neobar lam). Viability percentage was calculate using follow Equation: Viability percentage= (survival of cells in the presence of the doses of fesitin/ total cells)* 100

Figure 5: The effect of E.O.F.E on proinflammatory gene expression in BFLS using Real=Time PCR. 12x10^6 cells were incubated for 72 hours, with 12.38 mcg/mL O.F.E, then activated with 1 mcg/mL of LPS for 1 hour. Quantitative of normalized B.COX-2, B.TNF-alpha, B.IL-1beta, B.IL-18 and B.INOS expression were shown. Statistical significance between activated control group (Cell+LPS) and other groups were analysed using Student test(mean±1 Sd, n=3).
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Table 1 The effect of E.O.F.E on proinflammatory gene expression in chondrocytes using semiquantitative RT - PCR analysis . Bovine chondrocytes were incubated with E.O.F.E for 72 h and activated with LPS for 1 h. Normalized gene expression is shown as percent of activated control (C+LPS). *P < 0.05, **P < 0.001

|          | Cell | Cell+LP S | Cell+LPS+Dexamethasone | CellLPS+NSAID | CELL+LPS+DMSO | Cell+LPS+E.O.F.E | Cell+DMSO |
|----------|------|-----------|------------------------|---------------|---------------|-----------------|-----------|
| B.COX-2  | 21.83±4.2 | 100       | 26.9±5.1               | 34.9±12.23    | 100           | 52.9±9.3       | 21.8±4.2 |
| B.TNF Alpha | 22.45±3.9 | 100       | 28.12±4.8              | 35.67±12.56   | 100           | 54.28±8.4      | 22.45±3.9 |
| B.IL-18  | 23.12±3.8  | 100       | 27.34±4.1              | 36.98±13.45   | 100           | 55.79±7.9      | 23.12±3.8 |
| B.INOS   | 21.23±4.0  | 100       | 29.89±7.8              | 37.24±16.12   | 100           | 56.1±9.18      | 21.23±4.0 |

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