

***Crataegus sinaica* defatted methanolic extract ameliorated monosodium iodoacetate-induced oxidative stress and inhibited inflammation in a rat model of osteoarthritis**

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Abstract

Background and purpose: Osteoarthritis is a degenerative joint disease without definite treatment. It is characterized by intra-articular inflammation, cartilage degeneration, subchondral bone remodeling, and joint pain. The objective of the current study was to assess the anti-osteoarthritic effect and the possible underlying mechanism of action of *Crataegus sinaica* extract (CSE).

Experimental approach: Intra-articular injection of monosodium iodoacetate in the right knee joint of all rats was done except for the sham group. One week later, the anti-inflammatory efficacy of CSE (100, 200, 300 mg/kg, daily p.o) for 4 successive weeks versus ibuprofen (40 mg/kg, p.o) was assessed. Serum inflammatory cytokines; as well as weekly assessment of knee joint swelling, joint mobility, and motor coordination were done. At the end of the experiment, a histopathological investigation of the affected knee joints and an x-ray investigation were also executed.

Findings / Results: CSE significantly decreased joint swelling, pain behaviors, and serum levels of TNF- α , IL6, hyaluronic acid, and CTX-II. The radiographic findings revealed almost normal joint space with normal radiodensity and diameter in CSE-treated rats. As well, the histopathological and immunohistochemical investigations of the knee joints in CSE-treated groups retained the cartilage structure of knee joints. A significant reduction in the percentage of caspase-3-stained chondrocytes and a decrease in TGF- β 1 immuno-positive areas in the synovial lining and sub lining were recorded in CSE-treated rats, compared to the osteoarthritis control group.

Conclusion and implications: This study approved the chondroprotective effects of CSE, and its ability to inhibit the pain associated with osteoarthritis.

Keywords: *Crataegus sinaica*; Inflammation; Monosodium iodoacetate; Osteoarthritis; Pain; Rats.

INTRODUCTION

Osteoarthritis is one of the leading causes of chronic disability and a major cause of pain, especially in the aged population all over the world. Osteoarthritis is a degenerative joint disease without definite treatment, it results in massive clinical symptoms, including joint pain, physical disability, and joint stiffness (1,2). On the global level, about 80% of

osteoarthritis patients are suffering from limitations in movement, and about 25% are struggling in performing their major daily activities (3).

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In osteoarthritis, inflammation along with the abnormal production of reactive oxygen species, chemokines, and cytokines by chondrocytes consequently activates degradation of the components of the extracellular matrix and disrupts the metabolism of chondrocytes leading to oxidative stress and cartilage damage (4,5).

The degradation of cartilage is triggered by different inflammatory mediators, such as interleukin (IL)-6, IL-1 β , and tumor necrosis factor-alpha (TNF- α). This process occurs along with structural changes of the knee joint as well as articular cartilage erosion, joint ankylosis, synovitis, progressive motor disturbance, and remodeling of subchondral bone (6). The pathologic changes seen in osteoarthritis joints are characterized by degradation of the articular cartilage, degradation of collagen type II, thickening of the subchondral bone, osteophyte formation, synovial inflammation in varying degrees, and hypertrophy of the joint capsule (7).

In osteoarthritis, pain is the hallmark symptom resulting from the progressive loss of articular cartilage which is mostly accompanied by the new bone formation and synovial proliferation that culminated in pain (8). Different pharmacologic and non-pharmacologic therapies are aiming at alleviation of pain and inflammation to improve the function of the joint and the quality of osteoarthritis patients' life. Other treatments are directed to the reduction of symptoms in addition to deliberating the progression of the disease (9). Many of the prescribed medicaments have serious adverse effects, such as renal toxicity, hepatotoxicity, gastrointestinal toxicity, etc. Hence, traditional herbal therapies could offer minor or no side effects, acting as adjuvant therapy to control pain and inflammation and also improve joint function (10).

According to the treatment guidelines stated by both the American College of Rheumatology and the Arthritis Foundation, oral and topical administrations of non-steroidal anti-inflammatory drugs (NSAIDs) and intra-articular corticosteroids are highly recommended, besides exercise and weight loss. Furthermore, because of the adverse

effects of different medicaments, the guidelines recommended against the use of glucosamine and chondroitin, hyaluronic acid (HA) injections, methotrexate, and hydroxychloroquine, stem cell injections, platelet-rich plasma injections, IL-1 receptor antagonists, TNF inhibitors, and bisphosphonates (4).

Crataegus sinaica, a thorny shrub, belongs to *Rosaceae* family. *C. sinaica* grows wildly in the Saint Catherine mountains, South Sinai of Egypt, and is named Za'rur or Za'rur al-awdiyah. It is a short tree that can grow up to 7 m high (11). *Crataegus* species are broadly used in folk medicine in the form of infusion or decoction for management of respiratory manifestations, nervous system disorders such as insomnia, migraines, and memory loss, and for improving the circulatory system (12). Moreover, *Crataegus* species, which is commonly known as hawthorn or hawberry, are rich in flavonoids, procyanidins, tannins, organic acids, and triterpene derivatives that have been reported to have antioxidant, analgesic/anti-nociceptive, anxiolytic, and sedative effect (13).

Animal models of OA using monosodium iodoacetate (MIA) in rats, mimic human degenerative OA evidenced by the histological changes in the articular cartilage and pain-related behavior (9). Since the inhibitory effects of *C. sinaica* methanolic extract (CSE) on articular cartilage degradation have not been reported yet. Thus, this work was deliberated to investigate the effect of CSE in MIA-induced knee joint OA in rats and the probable implied mechanisms for pain prevention and management in motor disturbance accompanied by chondrocytes disorders.

MATERIALS AND METHODS

Plant material

C. sinaica fruits were collected from Southern Sinai, Egypt in Saint Catherine Mountains, in September 2017. A voucher specimen (No. 2017.10.05) was placed at the herbarium of the Pharmacognosy Department at the Faculty of Pharmacy-Cairo University. The fruits were then air-dried in shade and their size was reduced to mesh No. 36 powder,

and the powdered fruits were reserved in tightly closed jars until extraction.

Extraction and phytochemical analysis

Three kg of the powdered fruits were extracted three times by applying cold maceration in 5 L methanol with sonication for 20 min every time. Under reduced pressure, the solvent was evaporated at 40 °C to obtain a gummy mass (~250 g) of CSE. CSE was further defatted by liquid-liquid fractionation using CHCl₃. Finally, the dried extract (220 g, about 7.3% yield) was kept at 4 °C in a tightly closed container.

In our previous work on the defatted methanolic extract, CSE was subjected to UPLC/PDA/ESI-MS analysis for metabolomic profiling to identify its main phytochemical constituents (13).

Acute toxicity test

An acute toxicity study of CSE was carried out in adult male albino rats. Rats were divided into 2 groups (6 each) and fasted overnight. Rats received CSE in a dose of 3000 mg/kg (5 mL/kg) by the oral route. Another group of rats (control) were orally administered the vehicle (distilled water) and kept under the same conditions. Each animal was observed for signs of toxicity and/or mortalities during the first hour and after 24 h, and then daily for two weeks.

Animals and induction of osteoarthritis

In the current study, mature male Wistar rats weighing 150-200 g were housed under standard conditions 12/12-h light/dark cycle and well-ventilated rooms. Animals were kept in hygienic cages and accessed to clean standard pellet diet food and water *ad libitum*. All rats were left to be adapted to the laboratory environment, a week before conducting experiments. All procedures for the handling, use, and euthanasia of the animals were in accordance with the Institutional Animal Care and Use Committee at Cairo University (approval No. CU-II-F-14-18).

Induction of OA was done according to the study of Yassin *et al.* (14), *via* a single intra-articular injection of MIA (50 µL, 40 mg/mL) at the beginning of the experimentation in the

right knee joints of all animals except rats in the sham group. Saline was injected into rats of the sham group instead of MIA, then 1 mL of distilled water was orally administered daily for four successive weeks, a week after injection with saline into the knee joint. One week after induction of OA (baseline), 1 mL of vehicle (distilled water) was orally administered to rats of the osteoarthritis control group, daily for 4 successive weeks. The anti-inflammatory effect of oral ibuprofen (BRUFEN[®], Abbott, IL, USA) (40 mg/kg daily for 4 successive weeks) versus CSE (100, 200, 300 mg/kg) was assessed.

The anti-inflammatory potential of CSE was evaluated in all treated rat groups by measuring the inflammatory cytokines in serum including TNF- α , IL-6, and IL-8 using commercial ELISA kits (FineTest[®] ELISA kits, Wuhan Fine Biotech Co., Ltd., Wuhan, China). In addition to evaluating C-telopeptide of type II collagen (CTX-II) and HA levels (FineTest[®] ELISA kits, Wuhan Fine Biotech Co., Ltd., Wuhan, China), together with the measurement of knee joint diameter as an indication for knee joint swelling weekly *via* vernier digital caliper. Motor coordination and joint mobility were evaluated as well, once weekly, by the accelerating rotarod apparatus (Ugo Basile 47600, Milan, Italy), and the cut-off time was set at 300 s to assess osteoarthritic-related pain, according to the study of Yassin *et al.* (14). At the end of the experimentation period, rats were euthanized *via* cervical dislocation under anesthesia. Histopathological investigation of the affected right knee joints and x-ray examination was also achieved.

Radiological examination

The rats were anesthetized by intramuscular injection of xylazine HCl 2% (5-10 mg/kg body weight) and ketamine 5% (30-60 mg/kg body weight). The rats' right knee joints were scanned radiologically by ventrodorsal and mediolateral views using Fisher[®] X-ray generating tube (Fisher R183, Emerald Tube 125, Australia) with radiographic factors included in the imaging system (40 KV), exposure level (10 mAs), and 80-cm film-focus distance (15). The radiographic examination was performed at the radiography unit of

surgery, Anaesthesiology & Radiology Department, Faculty of Vet. Medicine, Cairo University, as previously described (4). The severity of joint lesions (n = 4 for each group) was evaluated using the Kellgren-Lawrence scoring system (16).

Histopathology of knee joints

For histopathological examination and osteoarthritis scoring, five knee joints from the different experimental groups were collected and fixed in 10% neutral buffered formalin for 24-48 h. The fixed joints were decalcified in 20% ethylenediaminetetraacetic acid (Sigma-Aldrich, MA, USA) for 4-6 weeks. The joints were excised sagittally, then processed for obtaining 3-4 μm paraffin-embedded sections. The sections were stained with hematoxylin and eosin (H&E) stain for cells and surface integrity evaluation. The toluidine blue stain was utilized to detect proteoglycan content in the articular cartilage (17).

The five tissue sections were examined per group under the microscope. Osteoarthritis scoring was performed according to the osteoarthritis cartilage histopathology assessment system (OARSI score) which was recognized by Pritzker *et al.* (18). This system depends on the assessment of grade and stage of arthritis with subsequent calculation of osteoarthritis score. The OA score ranged from 0-to 24 depending on the OA severity and the extent of cartilage involvement. The synovial reaction was also scored according to the method established by Gerwin *et al.* (17).

Inflammation of the synovial membrane was categorized from 0 to 4, in which (0) indicates no changes, (1) increased number of lining cell layers more than 3-4 layers or mild proliferation of subsynovial tissue, (2) increased number of lining cell layers more than 3-4 layers and or proliferation of subsynovial tissue, (3) increased number of lining cell layers more than 4 layers and or proliferation of subsynovial tissue with few inflammatory cells infiltration, (4) increased number of lining cell layers more than 4 layers and or proliferation of subsynovial tissue with a large number of inflammatory cells infiltration.

Specifying the numbers 0-4 depends on the increased number of synovial lining cell, the

proliferation of sub-synovial tissue, and inflammatory cell infiltration as follow (18).

Immunohistochemistry of TGF- β 1 and caspase-3 protein expression

Immunohistochemistry of transforming growth factor-beta (TGF- β 1) and caspase-3 was done following the methods described by Ogaly *et al.* (19). The tissue sections of knee joints of different experimental groups were deparaffinized in xylene and rehydrated in alcohol, then the antigenic retrieval was performed using citrate buffer at pH 6.0 as described by (20) Ogaly *et al.* (20). Rabbit anti-TGF- β 1 polyclonal antibody (ab92486; Abcam, Cambridge, UK) at 20 $\mu\text{g}/\text{mL}$ and rabbit anti-caspase-3 polyclonal antibody (ab13847; Abcam, Cambridge, UK) at 1:50 were added to the tissue section then placed in a humidified chamber for overnight. Three times washing with tris buffer saline were done. Endogenous peroxidase activity was blocked using drops of hydrogen peroxide blocking solution. The knee sections were incubated with secondary antibody goat anti-rabbit IgG H&L (ab205718; Abcam, Cambridge, UK) for 1 h. Three times washing with tris buffer saline were performed to remove excess unconjugated antibodies. 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, MA, USA) was added to the tissue section in order to visualize the immune-positive reaction, then the sections were counterstained with Mayer's hematoxylin and mounted. In the articular cartilage, the percentage of active caspase-3 immune-positive stained chondrocytes (%) was calculated in both superficial zone and deep zone. In the synovium (synovial lining, sub lining, and periosteum), the brown color intensity of the immune-positive stained area was analyzed. The image analysis was performed on 5 knees per group and in each group, three microscopical fields were examined by high magnification power ($\times 400$), then analyzed by the Image J program.

Statistical analysis

Before proceeding with the statistical analysis, data values were checked for normality using the Shapiro test and if the data did not pass the normality test non-parametric

test was performed. The data are presented as mean \pm SEM. Data were processed by one-way or two-way ANOVA followed by the Tukey-Kramer post-hoc test except for the histopathological scoring, which was analyzed by nonparametric K-independent samples Kruskal-Wallis H-test, followed by two independent samples Mann-Whitney U test. GraphPad Prism software (version 8; GraphPad Software, Inc., San Diego, CA, USA) was employed to perform the statistical analysis and establish the represented graphs.

RESULT

Acute toxicity test

CSE was non-lethal even at the maximum single oral dose of 3000 mg/kg after 2 weeks of administration. No observed signs of toxicity were reported in rats, such as abnormal motor activity, or gross behavioral changes over a period of 24 h. Consequently, the oral LD₅₀ of CSE was considered to be above 3000 mg/kg body weight.

Effect on knee joint diameter

Intra-articular injection of MIA revealed a significant elevation in the knee joint

diameter (Table 1). Oral administration of CSE (100 mg, 200, and 300 mg/kg) to rats, significantly diminished the joint diameter, one week post-administration, and till the end of the experimental period when compared to the osteoarthritis control group.

Effect on motor activity using rotarod

Intra-articular injection of MIA resulted in impairment of joint motion and augmented pain behavior, which was demonstrated by a decreased duration elapsed on rotarod apparatus (Table 2). A significant decrease in the rotarod latency was recorded all over the 4-weeks experimental period in the osteoarthritis group compared to the sham control group. CSE at 300 mg/kg exhibited a significant elevation in the time elapsed on the rotarod after 2 weeks of treatment, whereas ibuprofen and CSE at 200 mg/kg administered to rats exhibited a significant rise in the rotarod latency after 3 weeks of administration, compared to the osteoarthritis control group. Oral administration of rats with CSE at 100 mg/kg improved joint mobility by the elevation of rotarod latency after 4 weeks of treatment.

Table 1. Effect of CSE on knee joint diameter. Data represent the mean \pm SEM of eight animals.

Groups	Δ Knee joint diameter (%)				
	Basal	1 st week post-treatment	2 nd week post-treatment	3 rd week post-treatment	4 th week post-treatment
Sham	1.6 ^b \pm 0.24	1.7 ^b \pm 0.25	1.6 ^b \pm 0.27	2.0 ^b \pm 0.18	1.7 ^b \pm 0.3
Osteoarthritis control	22.3 ^a \pm 0.62	22.0 ^a \pm 0.85	21.3 ^a \pm 0.95	20.6 ^a \pm 0.79	20.3 ^a \pm 0.68
Ibuprofen 40 mg/kg	20.9 ^a \pm 0.88	10.0 ^{ab} \pm 0.72	9.6 ^{ab} \pm 0.66	8.7 ^{ab} \pm 0.27	8.3 ^{ab} \pm 0.63
CSE 100 mg/kg	21.4 ^a \pm 0.64	10.9 ^{ab} \pm 0.45	8.1 ^{ab} \pm 0.43	7.9 ^{ab} \pm 0.77	7.5 ^{ab} \pm 0.46
CSE 200 mg/kg	20.9 ^a \pm 0.81	8.2 ^{ab} \pm 0.74	8.0 ^{ab} \pm 0.61	6.6 ^{ab} \pm 0.49	6.1 ^{ab} \pm 0.31
CSE 300 mg/kg	20.4 ^a \pm 1.00	6.5 ^{ab} \pm 0.54	4.8 ^b \pm 0.30	4.5 ^b \pm 0.30	4.2 ^b \pm 0.61

^a $P \leq 0.05$ indicates significant differences in comparison with the sham group; ^b $P \leq 0.05$ versus osteoarthritis control group; CSE, *Crataegus sinaica* methanolic extract.

Table 2. Effect of CSE on motor activity. Data represent the mean \pm SEM of eight animals.

Groups	Rotarod latency (s)				
	Baseline	1 st week post-treatment	2 nd week post-treatment	3 rd week post-treatment	4 th week post-treatment
Sham	278.9 ^b \pm 5.57	248.4 ^b \pm 12.99	249.2 ^b \pm 8.88	263.9 ^b \pm 12.82	272.6 ^b \pm 12.74
Osteoarthritis control	196.5 ^a \pm 5.09	164.5 ^a \pm 12.26	168.3 ^a \pm 12.20	164.1 ^a \pm 13.89	146.3 ^a \pm 11.00
Ibuprofen 40 mg/kg	194.3 ^a \pm 4.31	217.6 \pm 6.68	221.0 \pm 8.87	239.7 ^b \pm 2.14	243.6 ^b \pm 3.77
CSE 100 mg/kg	194.7 ^a \pm 5.10	193.9 \pm 6.25	192.5 ^a \pm 8.16	218.0 \pm 7.09	219.0 ^b \pm 5.63
CSE 200 mg/kg	196.6 ^a \pm 5.86	208.8 \pm 14.84	214.1 \pm 21.97	225.2 ^b \pm 12.88	235.1 ^b \pm 12.79
CSE 300 mg/kg	196.3 ^a \pm 8.06	212.1 \pm 6.22	232.9 ^b \pm 14.45	244.5 ^b \pm 9.78	251.9 ^b \pm 12.23

^a $P \leq 0.05$ indicates significant differences in comparison with the sham group; ^b $P \leq 0.05$ versus osteoarthritis control group; CSE, *Crataegus sinaica* methanolic extract.

The radiographic findings

Rats showed variable grades of muscular atrophy and slight joint effusion in the injected joint compared to the contralateral limb in the ventrodorsal view (Fig. 1 and Table 3). Radiographically, the right knee joint of the experimental animals in the sham group showed normal joint capsule radiodensity without effusion, radiolucent joint space, and clear normal articular surfaces either femoral condyles or tibia surface (Fig. 1A). The radiographic inspection of the knee of the osteoarthritis control group revealed high radiodensity of articular space which was extremely narrow and nearly disappeared with articular surface osteophytic changes in both femoral condyles and tibial surface (Fig. 1B). Moreover, in the ibuprofen group; the knee joint capsule showed neither distension nor effusion, and the joint space showed moderately increased radiodensity with osteophytic reactivity and moderate femoral condyle and proximal tibia surfaces changes in all animals treated with ibuprofen (Fig. 1C). In the CSE-100 group, the joint capsule showed

distension displayed with minor elevation in the radiodensity and the space, along with moderate radiodensity. The proximal tibia and the femoral condyle were almost even with moderate evidence of osteoreactivity expressed as increased radiodensity and space narrowing, whereas in some treated animals the joint space showed high osteophytic reactivity (Fig. 1D). While in CSE-200, the joint capsule and space were almost normal radiodensity except capsule distension was noticed in some treated animals. The femoral condyles and the proximal tibia were nearly regular without evidence of osteoreactivity, except some treated rats demonstrated very low osteophytic reactivity and the knee showed narrow joint space and increased radiodensity (Fig. 1E). The CSE-300 group revealed normal radiographic density of the knee joint capsule in most scanned rats. The joint space showed almost normal radiodensity and diameter while the femoral condyle and proximal tibia showed some irregularity without evidence of osteophytic reactivity in all treated animals (Fig. 1F).

Table 3. Effect of CSE on the radiographic findings.

Groups	Joint space radiodensity	Osteophytic reactivity	Bone changes
Sham	Normal (-)	Normal (-)	Normal (-)
Osteoarthritis control	Extreme (++++)	Extreme (++++)	Extreme (++++)
Ibuprofen 40 mg/kg	Moderate (++)	Low (+)	Moderate (++)
CSE 100 mg/kg	High (+++)	Moderate (++)	Moderate (++)
CSE 200 mg/kg	Very low (+/-)	Low (+)	Low (+)
CSE 300 mg/kg	Very low (+/-)	Very low (+/-)	Very low (+/-)

CSE, *Crataegus sinaica* methanolic extract.

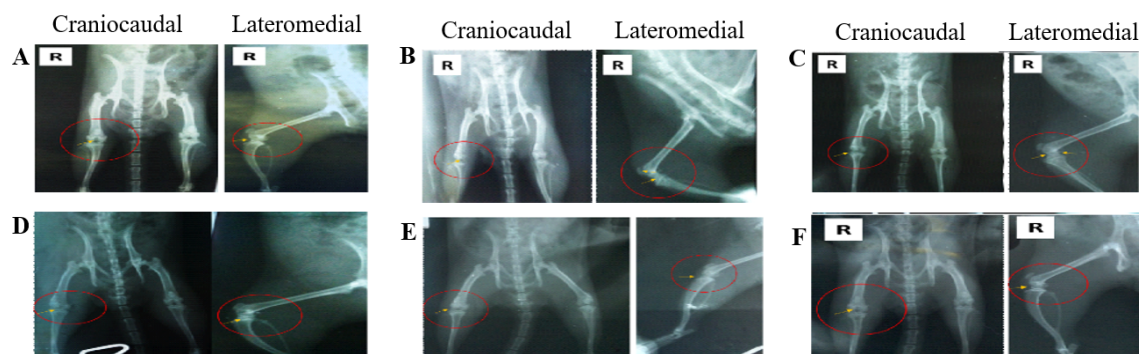


Fig. 1. Photographs of the right knee joint of rats at different radiographic positions. Craniocaudal and lateromedial in different experimental rat groups: (A) sham, (B) osteoarthritis control, (C) ibuprofen 40 mg/kg, (D-F) *Crataegus sinaica* methanolic extract at 100, 200, and 300 mg/kg.

Effect on serum inflammatory cytokines

As depicted in Fig. 2, the OA rat group (control group) displayed a significant elevation in serum TNF- α , IL-6, IL-8, CTX-II, and HA compared to the sham rats, by 110.9%, 71.1%, 45.4%, 157.5%, and 70.8%, respectively. CSE administration resulted in a dose-dependent decrease in TNF- α , IL-6, IL-8, CTX-II, and HA serum levels compared to the osteoarthritis control group.

Histopathological findings

Normal histological pictures of the regular articular surface, articular cartilages containing chondrocytes, and synovial membrane of tibiofemoral and patellofemoral joints were recorded in the sham control group in H&E-stained sections (Fig. 3A). On one hand,

dysregulation of the articular surface together with ulceration of articular cartilages and erosion which is filled with fibrous tissue which replaced areas of articular cartilage was observed in the osteoarthritis control group (Fig. 3B). Chondrocytes were with either weakly stained nuclei or karyolytic nuclei. The number of chondrocytes was markedly reduced (Fig. 3B). Moreover, the synovial membrane was markedly widened and thickened due to the proliferation of their synovial lining cells and the proliferation of fibroblasts with mononuclear inflammatory cell aggregation in the sub-lining layer. Whilst, the groups treated with ibuprofen (Fig. 3C) and CSE at 100, 200, and 300 mg/kg revealed minimal histopathological lesions that were previously mentioned in the sham group (Fig. 3D-F).

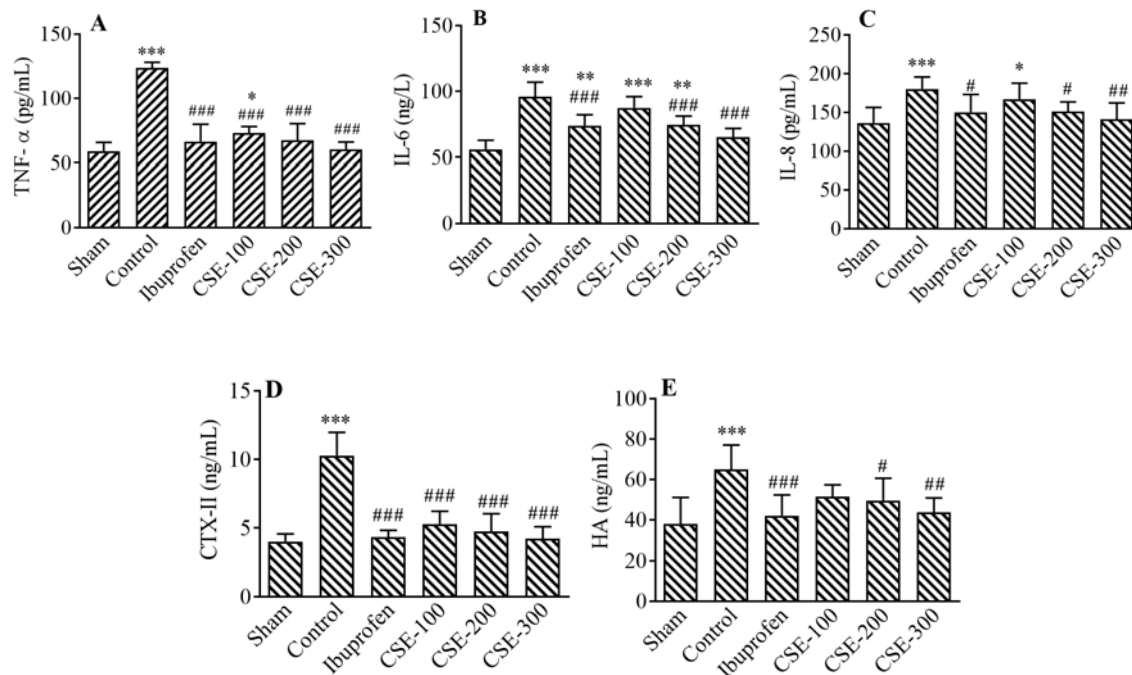


Fig. 2. The effect of CSE at 100, 200, and 300 mg/kg and ibuprofen at 40 mg/kg on (A) TNF- α , (B) IL-6, (C) IL-8, (D) CTX-II, and (E) HA serum levels of eight animals. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$ indicate significant differences compared to the sham group; and # $P \leq 0.05$, ## $P \leq 0.01$, ### $P \leq 0.001$ against osteoarthritis control group. TNF- α , Tumor necrosis factor-alpha; IL, interleukin; CTX-II, C-telopeptide of type II collagen; HA, hyaluronic acid; CSE, *Crataegus sinaica* methanolic extract.

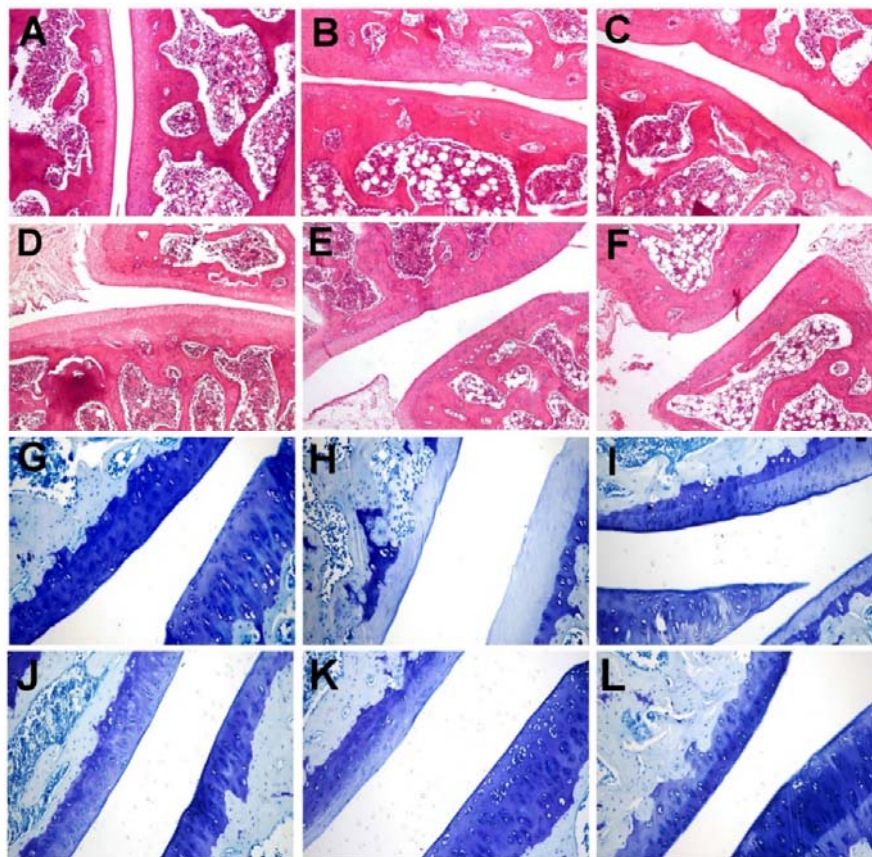


Fig. 3. Photomicrographs of joints collected from different experimental rat groups. (A-F) Hematoxylin and eosin-stained sections, magnification $\times 100$. (A) Sham group displaying normal articular surface, articular cartilages, and chondrocytes; (B) osteoarthritis control group showing irregular articular surface, articular cartilages containing few numbers of chondrocytes, weakly stained, or necrosed chondrocytes and area of articular cartilage replaced by fibrous tissue; (C) ibuprofen at 40 mg/kg showing irregularity of articular surface with decrease in chondrocytes' numbers; (D) CSE-100 showing moderate necrosis of chondrocytes; (E) CSE-200 and (F) CSE-300 groups showing individual chondrocytes necrosis. (G-L) Toulidine blue-stained sections magnification $\times 200$. (G) Sham group showing cartilaginous matrix with a normal distribution of proteoglycan; (H) osteoarthritis control group demonstrating obvious decrease with the complete vanishing of proteoglycan content in some parts in the cartilaginous matrix; (I) ibuprofen at 40 mg/kg showing reduction of proteoglycan content of cartilaginous matrix especially in the articular surface; (J-L) CSE at 100, 200, and 300 mg/kg showing the cartilaginous matrix with the uniform spreading of proteoglycan content. CSE, *Crataegus sinaica* methanolic extract.

The articular surface of tibiofemoral and patellofemoral joints was regular, and articular cartilages with individual cell necrosis and synovial membrane showed slightly proliferated cell lining and slight thickening of the sub-lining layer. Regarding OARSI scoring of arthritis and the grading of synovitis (Fig. 4A-E), the osteoarthritic control group revealed a significant elevation in both OA score and synovitis grade compared to the sham group; and ibuprofen, CSE-100, CSE-200, and CSE-300 groups exhibited a significant reduction when compared to the osteoarthritic control group. Concerning

toluidine blue-stained sections, normal uniform distribution of proteoglycan (blue color) in the cartilaginous matrix was observed in the sham group (Fig. 3G). While marked irregular and decrease in proteoglycan content with complete loss of proteoglycan content (very pale blue color) were observed in many areas of the cartilaginous matrix in the osteoarthritis control group (Fig. 3H). However, in the cartilaginous matrix, an even and diffused distribution of proteoglycan content (blue color) was observed in CSE-100, 200, and 300 groups (Fig. 3J-L).

Immunohistochemistry of TGF- β 1 and caspase-3 expression

The immunohistochemical investigation of protein expression of TGF- β 1 was detected in both the synovial lining and sub lining areas (Fig. 5A-E). There was a significant elevation of TGF- β 1 in the osteoarthritis control group in comparison with the sham group and a significant decrease of TGF- β 1 protein expression in CSE-100, CSE-200, and CSE-300 groups compared to the osteoarthritis control group (Fig. 6A). In addition, the immunohistochemical

investigations of active caspase-3 expression (Fig. 5F-L) in the chondrocytes of the superficial (superficial and upper intermediate cartilaginous layers) and deep zone (lower intermediate and deep cartilaginous layers) revealed that there was a significant increase in the percentage of caspase-3 immune-positive chondrocytes in osteoarthritic control compared to the sham group and a significant decrease in that percentage in CSE-100, 200, 300 treated groups compared to osteoarthritis control as shown in Fig. 6B.

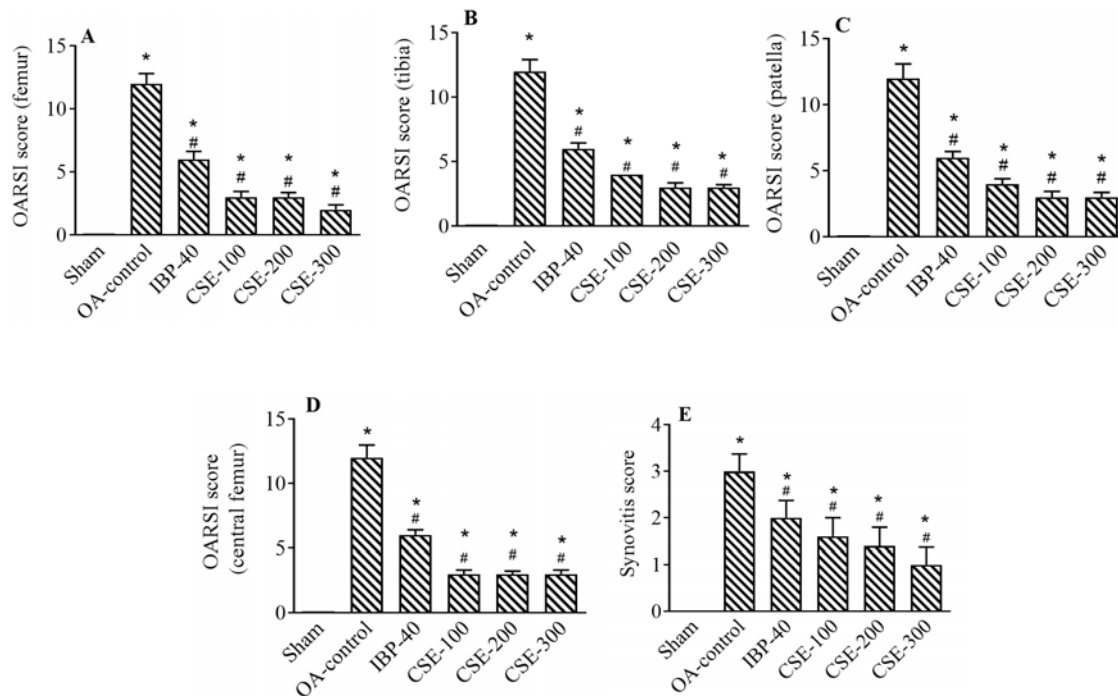


Fig. 4. OARSI scoring of articular cartilage and grading of synovitis. Articular cartilage degeneration in the patellofemoral joint and synovial membrane using the OARSI scoring. (A-B) OARSI score of tibiofemoral joint: (A) femur and (B) tibia; (C-D) OARSI score of patellofemoral joint: (C) patella and (D) central femur; and (E) synovitis score. Data represent the mean \pm SEM of eight animals. * $P \leq 0.05$ indicates significant differences compared to the sham group, and # $P \leq 0.05$ against the osteoarthritis control group. OARSI, Osteoarthritis cartilage histopathology assessment system; CSE, *Crataegus sinaica* methanolic extract; IBP, ibuprofen.

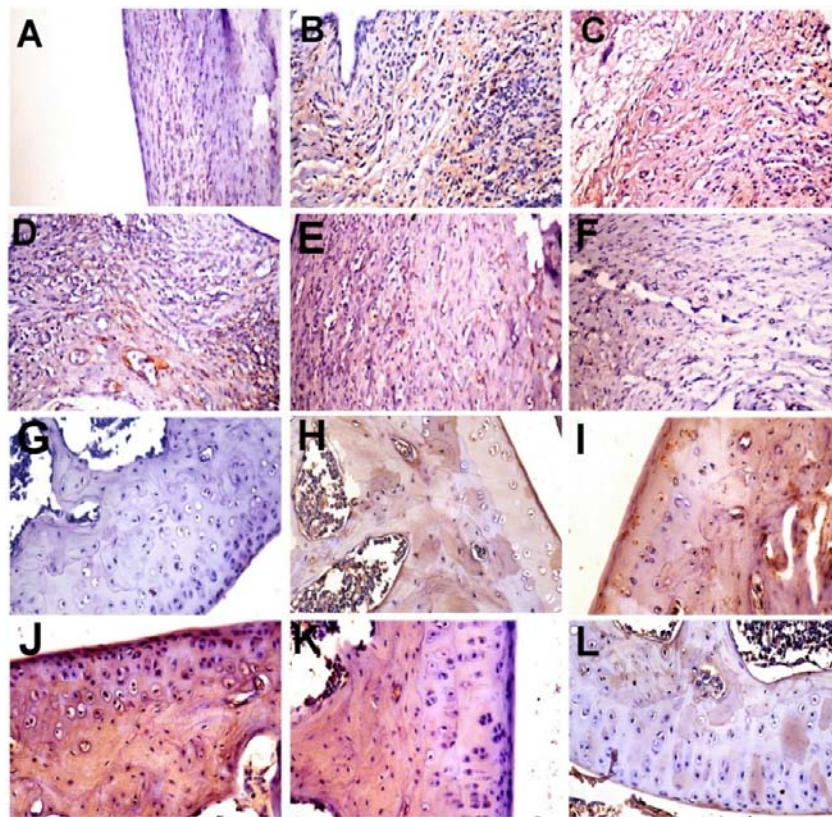


Fig. 5. Photomicrograph of immunohistochemistry of TGF-β1 and caspase-3 protein expressions. (A-F) TGF-β1 protein expression in the synovial membrane, magnification ×400. (A) Sham group exhibiting very weak immune-positive reaction; (B) osteoarthritis control group revealing strong immune-positive reaction in the synovial lining, synovial and sub lining layers; (C) ibuprofen at 40 mg/kg group showing moderate immune-positive reaction; (D-F) CSE at 100, 200, and 300 mg/kg groups showing a weak immune-staining reaction. (G-L) protein expression of caspase-3 in the articular cartilage, magnification ×400. (G) Sham group exhibiting very weak immune-positive reaction in chondrocytes; (H) osteoarthritis control group showing a marked increase in the number of immune-positive chondrocytes (brown color); (I) ibuprofen at 40 mg/kg showing a moderate number of immune-positive chondrocytes; (J-L) CSE at 100, 200, and 300 mg/kg showing few immune-positive chondrocytes. TGF-β1, transforming growth factor-beta; CSE, *Crataegus sinaica* methanolic extract.

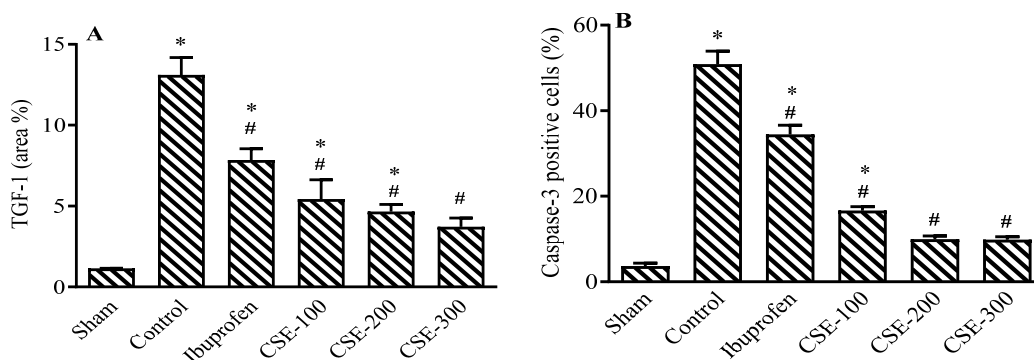


Fig. 6. The effect of CSE on the protein expression of (A) TGF-β1 and (B) caspase-3 by analyzing the immunohistochemistry image. Data represent the mean ± SEM of eight animals. *** $P \leq 0.05$ indicates a significant difference compared to the sham group, and ### $P \leq 0.05$ against the osteoarthritis control group. CSE, *Crataegus sinaica* methanolic extract; TGF-β1, transforming growth factor-beta.

DISCUSSION

In a former study on the tested defatted CSE, many phenolic compounds were identified based on UV absorption maxima, molecular weight, and tandem mass fragmentation patterns using UPLC/PDA/ESI-MS analysis (13). Additionally, the metabolomic profiling verified the existence of several phenolic constituents such as catechin and proanthocyanidin derivatives and flavonoids C-glycosides such as vitexin which was confirmed in the literature (11). Other studies reported several phytoconstituents of *Crataegus* species, such as caffeoylquinic acid derivatives, quinic acid, cratenacin, quercetin-*O*-pentoside (21), caffeoylshikimic acid derivatives (11), anthocyanins, phenolic acid glycosides, dihydrochalcone glycoside phlorizin (22), syringic acid-*O*-hexosides (23), flavonoids such as luteolin dihexoside (24), and quercetin-di-*O*-rhamnosyl-hexoside, (Epi)catechin-*C*-hexoside (25).

MIA is a metabolic inhibitor that breaks down the cellular aerobic glycolysis pathway through the inhibition of glyceraldehyde-3-phosphate dehydrogenase in chondrocytes when injected intra-articular and consequently induces cell death. MIA induces dose-dependent cartilage degradation and histopathological changes in the synovial membrane, which are similar to the pathological changes of human osteoarthritis (26).

MIA injection triggers an inflammatory response associated with joint edema along with monocyte and neutrophil infiltrations. Thereby, the osteoarthritis model using MIA is a proper animal model to examine the pharmacological effects of new drugs on osteoarthritis with the progression of pain-like behavior. MIA injection into the knee joint gives rise to a biphasic response, starting with an initial inflammatory reaction, then subsequent injury of the entire cartilage provoked after 2 weeks (9).

In the current study, the osteoarthritis control group exhibited a significant elevation in the knee joint diameter in comparison to the sham group after one-week post-MIA injection. However, rat groups orally administered CSE (100, 200, and 300 mg/kg), significantly

reduced the joint diameter one week post-treatment and till the end of the experimentation when compared to the osteoarthritis control group. The anti-edematous effect of CSE could be owed to its content of polyphenolic compounds, such as flavonoids and phenolic acids; and the capability of the extract for scavenging free radicals which could be partly responsible for its anti-inflammatory effect. Moreover, various *Crataegus* species were reported to have anti-inflammatory and antioxidant effects. For instance, *C. pinatifida* ethanolic extracts were found to be rich in vitexin-2-*O*-rhamnoside, rutin, and hyposide, accordingly possessing free-radical scavenging activity and diminishes the paw edema in a carrageenan-induced paw edema model. Moreover, *C. pinnatifida* extract decreased the number of inflammatory cells, mostly eosinophils in the murine model of asthma (27). Tadić *et al.* reported significant gastroprotective, anti-inflammatory, and antimicrobial effects of the hawthorn berries extract (*C. monogyna* and *C. oxyantha*; 1:1) (28).

Knee osteoarthritis is a well-known cause of pain and disability. Cartilage damage and inflammation contribute to pain flares in osteoarthritis and might result in sustained sensitization and thus aggravate chronic pain (29). The MIA model is a well-established pain model of osteoarthritis due to joint degeneration, and subchondral bone lesions, sharing many histological features with human osteoarthritis. The mechanisms that are mediating bone pain are complex and may involve nerve damage, the release of inflammatory cytokines, and persistent osteoclastic activity (30).

In accordance with previous studies, our data showed that intra-articular injection of MIA in rats developed significant pain-like behavior. Our findings revealed impairment of joint mobility and augmented pain behavior that was demonstrated by a decreased duration spent on the rotarod. The osteoarthritic group demonstrated a significant reduction in the rotarod latency recorded during the 4 weeks of experimentation compared to the normal group. CSE demonstrated a dose-dependent increase in the rotarod latency.

The analgesic effect of hawthorn was reported by several previous studies. For instance, Bor *et al.* reported remarkable antinociceptive, anti-inflammatory, and antioxidant activities of *C. orientalis* leaves extract (31). They also proposed that the analgesic effect of *Crataegus* extract is mediated by the opioid mechanisms and the extract is more powerful analgesia at the spinal level than at the supraspinal level. Another study by Alsayari *et al.* revealed that *C. oxyacantha* significant analgesic activity is due to its richness in terpenoids and steroids (32).

It is noteworthy to mention that the biochemical disruptions that arise within the articular cartilage in osteoarthritis are associated with alterations in the joint cartilage and bone as a result of the imbalance between the synthetic and degradative pathways. This leads to a change in the bone contour and the formation of subchondral cysts. Additionally, the subchondral bone is hypo-mineralized, due to abnormal bone remodeling (33).

Findings of the radiographic investigation of the knee of the osteoarthritis control group showed high radiodensity of articular space which was extremely narrow and nearly disappeared with articular surface osteophytic changes in both femoral condyles and tibial surface. Moreover, ibuprofen-treated rats revealed moderately increased radiodensity with osteophytic reactivity and moderate femoral condyle and proximal tibia surface changes. Remarkably, CSE administration exhibited dose-dependent improvement of the joint radiodensity and osteophytic changes. For instance, the CSE-300 group revealed the normal radiographic density of the knee joint capsule in most scanned rats with almost normal radiodensity of the joint space and normal diameter while the femoral condyle and proximal tibia showed some irregularity without evidence of osteophytic reactivity in all treated animals.

Several studies highlighted the involvement of immune cells in the progression of osteoarthritis. Under osteoarthritis conditions, cytokines and chemokines are produced by chondrocytes and synoviocytes in the osteoarthritic joints. As well, synovial fibroblasts are another source of pro-inflammatory cytokines and matrix-degrading enzymes in osteoarthritis. In osteoarthritis,

amplification of the inflammatory process occurs *via* the secretion of cytokines, such as IL-6 and IL-1 β , from the immune cells like activated neutrophils and macrophages. Further enhancement of infiltration of leukocytes such as macrophages, neutrophils T and B lymphocytes in the synovium (9).

It is well-established that TNF- α plays a critical role in the development and progression of osteoarthritis based on considerable evidence derived from studies on animal models and in culture. Besides, TNF- α is a catabolic cytokine that stimulates the degradation of chondrocytes to the surrounding cartilage matrix and arouses the secretion of destructive enzymes and prostaglandin E2 (34).

The obtained data demonstrated that rats in the osteoarthritis control group revealed a significant elevation in serum levels of TNF- α , IL-6, and IL-8 compared to the sham group. In contrast, CSE treatment exhibited a dose-dependent decline in TNF- α , IL-6, and IL-8 serum levels compared to the osteoarthritis control group. Supporting our findings, Nguyen *et al.* (5) stated that the antioxidative and anti-inflammatory effects of *C. laevigata* berry extract is owing to the existence of two active compounds, chlorogenic acid and (-)-epicatechin. Moreover, the protective effect against the inflammatory response induced by lipopolysaccharide involved the inhibition of the NF- κ B pathway and TNF- α . Further, an *in vitro* study on mouse macrophage cell lines exhibited that hawthorn fruit aqueous extract suppressed the expression of different inflammatory cytokines, such as IL-6, IL-1 β , and TNF- α (35).

In osteoarthritis, the crosslinked CTX-II is considered a marker of cartilage turnover predominantly in the cartilage extracellular matrix, and thus it is a predictor of the destruction of articular cartilage. Additionally, elevated serum levels of HA reflect synovitis and cartilage degradation in osteoarthritis, since it is a vital molecule in lubricating and maintaining the integrity of cartilage surfaces in the diarthrodial joints. Moreover, the disruption of HA by cellular metabolism might influence its lubricating effect on the articular cartilage and provoke joint deterioration. Therefore, high serum levels of HA are an indicator of synovitis and cartilage degradation in osteoarthritis (9).

The obtained findings revealed a significant increase in serum levels of CTX-II and HA in osteoarthritis control group, compared to the sham group. In contrast, CSE treatment revealed a dose-dependent decrease in serum CTX-II and HA compared to the osteoarthritis control group.

Intra-articular injection of MIA induces a dose-dependent cartilage degradation and histopathological changes in the synovial membrane, which are similar to the pathological changes of human osteoarthritis (26).

In this study, the histopathological investigation of the knee joint of the osteoarthritis control rat group showed the erosion and ulceration of articular cartilages and consequently irregularity of articular surface, with a significant increase in OARSI score of synovitis. The number of chondrocytes was markedly reduced with either weakly stained nuclei or karyolytic nuclei. Moreover, the synovial membrane was markedly widened and thickened due to the proliferation of their synovial lining cells and proliferation of fibroblasts with mononuclear inflammatory cell aggregation in the sub-lining layer. Groups treated with ibuprofen, CSE at 100, 200, and 300 mg/kg revealed minimal histopathological lesions that previously mentioned in the osteoarthritis group. The articular surface of the tibiofemoral joint was regular, and articular cartilages with individual cell necrosis and synovial membrane showed slightly proliferated cell lining and slight thickening of the sub-lining layer. Moreover, marked irregular and decreases in the proteoglycan content, in the form of a very faint blue color were observed in many areas of the cartilaginous matrix in osteoarthritis control rats; in the toluidine blue-stained sections. However, in the cartilaginous matrix, an even and diffused distribution of proteoglycan content (blue color) was observed in the groups treated with CSE at 100, 200, and 300 mg/kg.

During osteoarthritis development and progression, the TGF- β 1 signaling pathway implements unique and a critical role in chondrocytes, mesenchymal stem cells, and synovial lining cells, by driving chondrocytes toward hypertrophy, promoting osteoprogenitor cell differentiation into osteoblasts and angiogenesis in the subchondral bone. Further stimulation of the synovial lining cells towards

expansion and fibrosis also results (36). Moreover, caspase-3 is an effector caspase that is triggered by apoptosis signals; therefore, caspase-3 positive chondrocytes are highly relevant to progressive cartilage degradation. Thus, targeting the inhibition of caspase is a good therapeutic candidate.

Protein expression of TGF- β 1 in the immunohistochemical analysis showed significant elevation in the osteoarthritis control group when compared with the sham group. However, CSE-100, CSE-200, and CSE-300 groups revealed a significant decrease in TGF- β 1 protein expression relative to the osteoarthritis group. Furthermore, the immunohistochemical investigation of the active caspase-3 expression in the chondrocytes of the superficial and deep zone revealed a significant increase in the percentage of caspase-3 immune-positive chondrocytes in osteoarthritis control and a significant decrease in that percentage in the groups treated with CSE at 100, 200, 300 mg/kg compared to osteoarthritis control.

Herbal preparation extracted from *C. oxyacantha* down-regulated the gene expressions of different inflammatory markers including IL-1 β , tumor TNF- α , and TGF- β 1, and subsequently protect against liver fibrosis (37). Another study by Liu *et al.* revealed that corosolic acid, a pentacyclic triterpenoid that is an active component of *C. pinnatifida*, significantly decreased the protein level of TGF- β 1 in the liver tissues and decreased fibrosis (38). Many studies highlighted the effect of *C.* species and their metabolites on the prevention of caspase-3 activation. For instance, epicatechin gallate from *C. oxyacantha* extract inhibited caspase-3 activation (39). Liu *et al.* reported that hawthorn polyphenol extract suppressed the activation of caspase-3/9 and inhibited apoptosis (40).

CONCLUSION

To sum up, the current study revealed that the defatted CSE was efficient in curtailing the inflammatory immune response and alleviating arthritic joint pain in a rat model of osteoarthritis. CSE significantly diminished joint inflammation and pathological joint damage and decreased the serum levels of TNF- α , IL6, HA, and CTX-II. Added to that,

CSE likewise significantly abolished MIA-induced pain behavior. In conclusion, our data supported that CSE has chondroprotective effects and can inhibit pain associated with osteoarthritis.

Conflict of interest statements

All authors declared no conflict of interest in this study.

Authors' contributions

IA. Alsharif, RM. Abd-Elsalam, MS. Amer, and AH. El-Desoky participated in the practical experimentation and revised the manuscript. RF. Abdel-Rahman participated in the practical experimentation, carried out the statistical analyses, and wrote the manuscript. The final version of the manuscript was approved by all authors.

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