Original Article

The Possible Therapeutic Effect of Annona Muricata Leaf and Fruit Extract on Experimentally Induced Temporomandibular Joint Osteoarthritis in Albino Rats (Histological, Histochemical, and Immunohistochemical Study)

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ABSTRACT

Introduction: Osteoarthritis is lately considered as a low-grade inflammatory disease, causing discomfort, stiffness, and function loss. Therefore, we conducted this study to evaluate the therapeutic effect of Anonna muricata against Complete Freund's adjuvant (CFA)-induced osteoarthritis in Temporomandibular Joint (TMJ) of albino rats.

Material and Methods: Thirty adult male, pathogen free, white albino rats, weighing 150-250 gms were selected and divided into 3 groups (10 rats each): Control group receiving only phosphate buffered saline (PBS), Arthritic group which was subjected to induction of osteoarthritis by intra articular injection of two doses of CFA (50 µl) 1st dose on day one of experiment while 2nd dose was on day 14, followed by oral administration of 20 mg/ kg of PBS Annona group: this group was subjected to induction of osteoarthritis as arthritic group, then was treated by Annona extract 100 mg/kg/day for 14 days post CFA 2nd injection. After arthritis confirmation animals were sacrificed after three weeks. Demineralized tissue sections were stained with Hematoxylin and Eosin, Anti TNFα, Anti MMP3, Picrosirius red stain then were subjected to digital image analysis followed by one way ANOVA statistical analysis.

Results: Annona Muricate treated group revealed improvement in TMJ regeneration in comparison with the arthritic group in H&E sections with decreased disc thickness, increased condylar cartilage and more organized bone trabeculation. Immunohistochemically, Annona treated group showed decreased expression of TNFα and MMP3 ($P < 0.001$) when compared to arthritic group. While picrosirius red stain sections showed more organized and less inflamed collagen fibers in articular disc of Annona treated group.

Conclusion: Annona Muricata leaf and fruit extract can be possible candidates for alleviation of osteoarthritic conditions in rats.

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Key Words: Annona muricata, complete freund's adjuvant, MMP3, TMJ osteoarthritis, TNFα.

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INTRODUCTION

Osteoarthritis is one of the most prevalent types of arthritis[1]. Temporomandibular joint osteoarthritis (TMJOA) is defined by degenerative changes in bone, cartilage, and supporting tissues including osteophytes and flattening of the condyle surface[2]. Masticatory muscle and TMJ pain, stiffness, joint instability, joint noises, and reduced range of movements are all inflammatory clinical symptoms of the illness[3].

Complete Freund's adjuvant (CFA) has been commonly used to induce acute or chronic TMJ inflammation by intra-articular injection[4] The exact mechanism of action of CFA is not known, but it mainly triggers inflammatory reaction via the activation of the cellular immune response[5].

Tumor necrosis factor α (TNFα) is usually the first cytokine released in OA. It activates osteoclasts and stimulates nociceptors directly, resulting in both joint injury and pain[6]. Cytokines have an impact on the cartilage microenvironment by upregulating hypoxia-inducible factors (HIFs). Matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF) are advanced in OA through HIF1α-mediated pathways causing aberrant vascular formation and extracellular matrix breakdown in the cartilage[7].

Annona muricata belong to the Annonaceae family, it is a fruit tree with a long history of medicinal use[8]. Annona muricata leaf extract exhibits anti-inflammatory properties by inhibition of inflammatory mediators as TNF-α, IL-1β, IL-6 and nitric oxide[9]. The fruit and leaves were found to be rich in flavonoids, phenolic substances, tannins, saponins, glycosides and acetogenins that have been proved to have a potent antioxidant effect through decreasing reactive oxygen species (ROS) production[10]. Herein we tried to test whether Annona muricata leaf and fruit extract could ameliorate CFA induced osteoarthritis in TMJ of albino rats. The null hypothesis was that there is no effect.

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MATERIALS AND METHODS

All experimental procedures were approved by the ethical committee of Faculty of Dentistry, Mansoura University and complied to ARRIVE reporting guidelines for experimental research.

Thirty adult male, pathogen free, white albino rats, weighing 150-250 gm were selected. They were housed in individual cages, kept in 12/12 dark and light cycle with relative 50% humidity. They were acclimatized for one week before the experiment with free access to standard laboratory animal diet and water. Then the 30 rats were divided into 3 equal groups (10 rat each) as follows:

I. **Group I** (control group): animals received only PBS.

II. **Group II** (arthritic group): animals were subjected to induction of osteoarthritis by intra articular injection of two doses of CFA (50 µl) 1st dose on 1st day, while 2nd dose was on 14th day.

III. **Group III** (Annona group): animals of this group were subjected to arthritis induction as group II, then after 2nd dose of CFA injection, animals of this group were treated with 100mg/kg/day of (Graviola Herb Tincture 100 ml Soursop-indigo Herbs Glastonbury, United Kingdom) orally for 14 days.

Induction of osteoarthritis

Animals were subjected to induction of arthritis following adjuvant-induced arthritis (AIA) model. The joint was palpated 5-10 mm next to the lateral canthus of the eye at the same time the mandible was manipulated to sense the movement of the condyle to identify the joint. The needle was inserted until the needle tip touched the mandibular condyle. One volume 50 µl of CFA (Sigma Aldrich, St. Louis, Missouri, United States) was injected slowly into the joints over a time span of 2 minutes. Arthritis was induced by double intra-articular injections bilaterally into the TMJ, 1st on the start of experiment and the 2nd on 14th day to provide sustained inflammation[11].

Extract supplementation

Annona muricata leaf and fruit extract was provided by (Graviola Herb Tincture 100 ml Soursop-indigo Herbs Glastonbury, United Kingdom) and given orally, with a dose of 100 mg/kg once a day between days 1 and 14 post 2nd CFA injection via oral gavage[12].

Biopsy collection

After 3 weeks, animals of three groups were euthanized by overdose of halothane. TMJs were harvested in buffered formalin, decalcified, and processed for histological examination by routine Hematoxylin and Eosin (H&E), immunohistochemically for Tumor necrosis factor alpha (anti-TNFα) to detect early inflammatory responses to arthritis and Matrixmetaloproteinase-3 (anti-MMP3) Polyclonal antibodies for detection of cartilage deterioration, and histochemical staining by Picrosirius red stain (SR) to investigate the organization of collagen fibers in the articular disc. The sections stained with picrosirius were examined through polarized microscope then photographed using Olympus digital camera attached to Olympus polarized microscopy in Geology department, Faculty of science, Mansoura University.

Image analysis and statistical analysis

Ten fields were examined using thresholding technique to measure the intensity of positive immunostaining reaction in all groups. Normally distributed data were coded, tabulated using Statistical Package for Social Science software computer program version 25 (SPSS, Inc., Chicago, IL, USA). Data were presented in mean and standard deviation (SD). One-way analysis of variance (ANOVA) followed by post-hoc LSD tests were used. *P value* less than 0.05 was considered statistically significant.

RESULTS

H&E stain results

Negative control group revealed normal TMJ structure with normal thickness of articular disc, mandibular condyle with its four distinct zones: fibrous layer, proliferative cell layer, hypertrophic chondrocytes layer and endochondral ossification layer. Arthritic group showed increased thickness of articular disc, irregularity in fibrous layer, thinning of condylar cartilage, widening of marrow spaces, irregular and disordered trabecular bone. While the Annona treated group revealed decreased thickness of articular disc, increase of fibrous layer thickness, and increased bone trabeculation (Figure 1).

IHC staining results

Negative control group showed mild positive reaction to both Anti-TNFα and Anti-MMP3 as brown deposit in articular cartilage and trabecular bone with mean and standard deviation (.6177±.07203) (.5170±.18803) respectively. Arthritic group showed highest expression of both Anti-TNFα and Anti-MMP3 with mean and standard deviation (5.4762±.42922) (4.6164±.37457) respectively. While Annona treated group revealed decreased reaction when compared to arthritic group for Anti-TNFα and Anti-MMP3 with mean and standard deviation (1.7632±.23260) (1.5310±.35899) respectively (Figure 2, Table 1).

Picrosirius red stain results

This stain was used to show the organization of collagen fibers in the articular disc that revealed three colors: yellow, green that indicate healthy disc with organized collagen fibers and red related to inflamed one. Control group showed organized collagen fibers in the articular disc with green and yellow refraction with mean and standard deviation (23.4334±.42795). Arthritic group showed marked decrease in the organization of disc fibers and increased red color with mean and standard deviation (.8440±.06613). Where Annona treated group showed increased organization of collagen fibers when compared to arthritic one (11.4720±.44981) (Figure 3, Table 1).

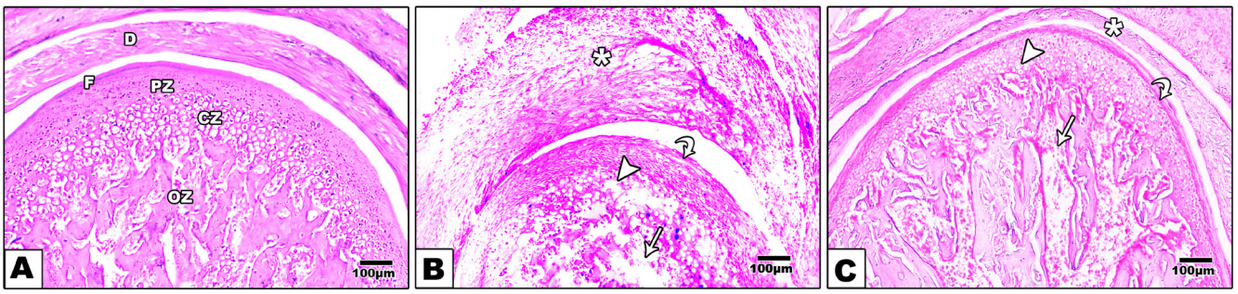

Fig. 1: Photomicrograph showing control group with normal thickness of articular disc D, four zones of condyle: fibrous layer (F), proliferative cell layer (PZ), cartilage zone (CZ) and ossification zone (OZ) (A), arthritic group revealed increased disc thickness (*) and irregular bone trabeculae (arrow) (B), Annona group decreased disc thickness and increased bone trabeculation (C) (H&E x 100).

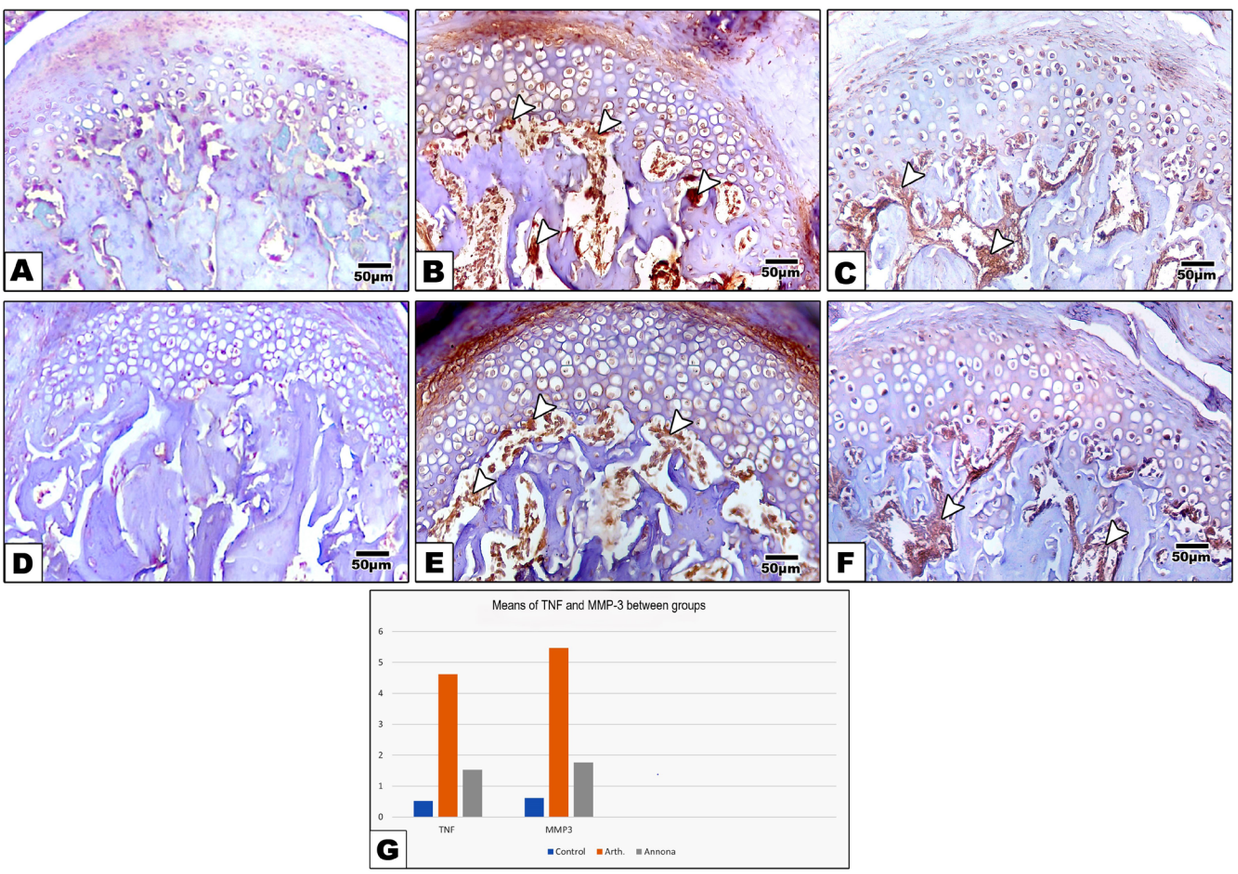

Fig. 2: Photomicrograph of TNF-α mild expression in control group (A), arthritic group showing high intensity (B), Annona treated group showed moderate expression (C). MMP-3 showed mild reaction in control group (D), arthritic group with highest expression (E), while Annona showed moderate reaction (F) The chart bar shows levels of expression of TNF-α and MMP3 expression in all groups analyzed by One-way ANOVA. Where there is significant difference between all groups (G) (IHC x 200).

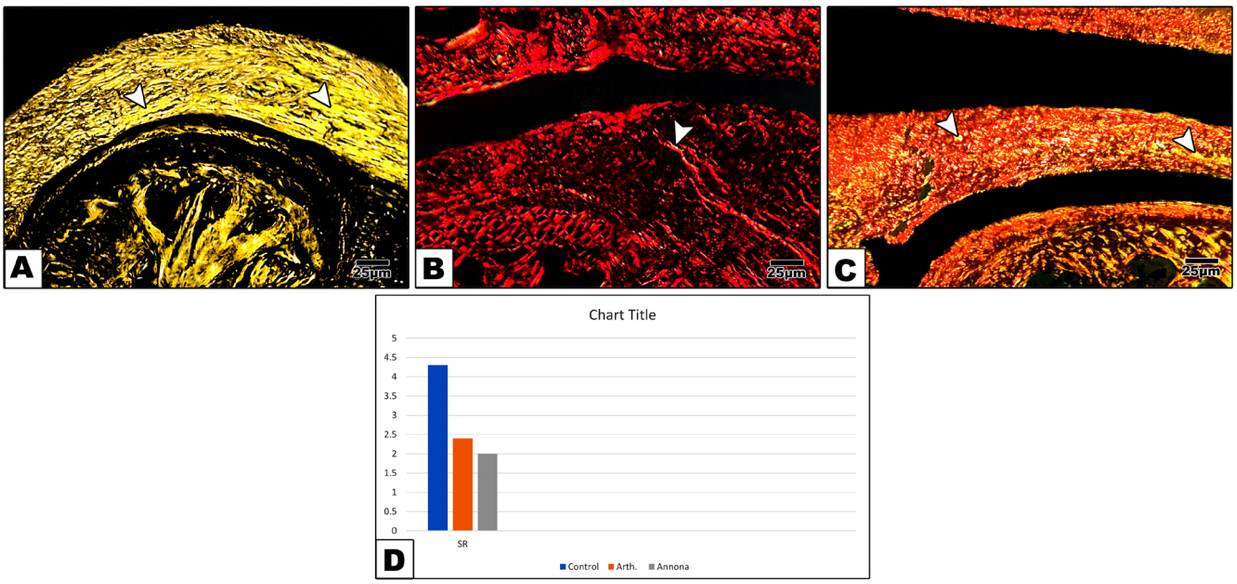

Fig. 3: Photomicrograph showing control group with normal collagen with yellow and green reflection (A), arthritic group showing high red intensity (B), Annona treated group showed moderate yellow reflection (C). The chart bar shows collagen distribution in all groups analyzed by One-way ANOVA. Where there is significant difference between all groups (D) (SR x 400).

Table 1: Mean ±Standard deviation (SD) of TNFα, and MMP3 expression levels and SR stain distribution in all groups

Groups	N	TNFα		MMP3		SR	
		Mean	SD	Mean	SD	Mean	SD
Control	10	.6177	.07203	.5170	.18803	23.4334	.42795
Arthritis	10	5.4762	.42922	4.6164	.37457	.8440	.06613
Annona	10	1.7632	.23260	1.5310	.35899	11.4720	.44981
Total	30	2.6190	2.12703	2.2215	1.79959	11.9165	9.39163

DISCUSSION

TMJ diseases are usually studied due to its high prevalence among population and its deleterious effect on cartilage, subchondral bone, synovial membrane, and other hard and soft tissues[13]. Male rats have been used in this study as anatomical and histological features in rats TMJ mimic that of human joint, and males were particularly used to exclude hormonal factors that may affect cartilage and bone metabolism[14].

In the present study, H&E sections for negative control group showed normal arrangement of bone trabeculae. While the arthritic non-treated group revealed increased articular disc thickness, decreased thickness of condylar cartilage with disorganization of its layers and abnormal bone trabeculation. Similarly, Wang *et al*. reported the occurrence of adaptive changes in subchondral bone in CFA induced arthritis group with increase in both MMP3, IL-1β expression in disc and condyle[15].

In agreement with these results Xu *et al*. stated that CFA induction reduced cartilage thickness, number of chondrocytes and amount of proteoglycans after 2 weeks. Also, the subchondral bone showed trabecular separation and larger marrow cavities[16].

In Annona treated group there were improvement in TMJ when compared to arthritic group, these findings conformed to Quilez who stated that oral treatment with Annona muricata leaves extract significantly inhibited the paw edema of rats, as the presence of phenolic compounds as tannins, flavonoids and phenolic acids have anti-inflammatory properties, and the possible synergistic effect between such compounds potentiate this effect[17]. Additionally, Ishola *et al*. found that Annona fruit extract had analgesic effects through interaction with opioidergic pathway and anti-inflammatory activity via inhibiting inflammatory mediators[18].

With regard to the IHC staining with Anti-TNFα results, control negative group revealed mild reaction while the arthritic group revealed significant increase in Anti-TNFα, these results were in approval with Moussa *et al*. who found that in osteoarthritis, the increased levels of IL-6, TNFα and COX-2 and the decreased TGF-β and intracellular interleukin, IL-4, IL-10 and IL-13, concentrations expose the cartilage to chronic inflammatory conditions[19].

Annona treated group showed decreased expression of TNFα when compared to arthritic group these findings where in accordance with Foong *et al*. who reported that A. muricata reduced inflammation by suppression of TNFα

and IL- β in xylene-induced ear edema[20]. Furthermore, Attiq *et al* stated that fruit, leaf, and seed extract from A. squamosa and A. montana declined level of TNFα and IL-6 in lipopolysaccharide stimulated macrophage that are produced because of inflammation[21].

In our study MMP3 expression was mild in control negative group, while the arthritic group revealed significant increase in MMP3 expression. These results agreed with Chen *et al*. who reported that MMP-3 protein expression in synovial tissue of osteoarthritic patients was significantly higher than in control, and the expression level was positively associated with osteoarthritis severity[22].

Annona treated group showed significant reduction of MMP-3 in comparison with arthritic group, these results were compatible with Haleagrahara *et al*. who conducted that flavonoids and antioxidant significantly alleviated joint inflammation by declining circulating cytokines levels and MMPs resulting in significant decrease in the expression of TNFα and MMP genes in the ankle joints of arthritic mice[23].

Picrosirius red staining revealed well organized collagen fibers in normal groups compared with the arthritic one in which inflamed collagen fibers were disordered. The inflamed discs showed apparent reddish refraction when seen under polarized light. Similarly, Cui *et al*. found that inflammation caused by arthritis decreased green intensity and increased red intensity of disorganized collagen fibers of the disc[24].

On contrary Lemos *et al*. reported that sections stained with picrosirius, red-orange color is seen in the normal group articular disc, whereas arthritic group exhibited reddish-orange coloration of lower intensity, proposing disruption of these fiber. Also, they conducted that in picrosirius red stain sections, articular disc in animals of control group showed intense red orange color indicating a high degree of compaction and arrangement of collagenous fibers, while arthritic group showed greenish color which suggests distribution and destruction of articular disc fiber network[25].

In Annona treated group there was significant arrangement of collagen fibers in articular disc with increased yellow refraction compared to the arthritic one. According to Yuce *et al* stated that the inflammation-induced thickening of the articular disc was found to be significantly less in groups treated by antioxidants either orally or by injected way by increasing the expression levels of collagen type I and type X in matrix[26].

CONCLUSION

With the limitations of this study, Annona muricata leaf and fruit extract may be a promising candidate for treatment of TMJ arthritis via antioxidant and anti-inflammatory potential by reducing the expression of inflammatory cytokines as TNF-α and MMP-3 so limiting extracellular matrix degradation and decreasing disc, cartilage, and bone destruction.

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CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

التأثير العلاجي المحتمل لمستخلص أوراق وفاكهة القشطة على الالتهاب المستحث في المفصل الصدغي الفكي في الفئران البيضاء (دراسة هستولوجية وهستوكيميائية وهستوكيميائية مناعية)

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مقدمة: هشاشة العظام تعتبر في الآونة الأخيرة مرض التهابي منخفض الدرجة، يسبب عدم الراحة،التيبس،الوهن و فقدان الوظيفة. ولذلك قمنا بهذه الدراسة لتقييم التأثير العلاجي ل مستخلص فاكهة القشطة ضد التهاب المفاصل العظمي الناجم عن مادة فرويند (CFA) في المفصل الفكي الصدغي (TMJ) في الفئران البيضاء.

المواد والطرق: تم اختيار ثلاثين ذكراً بالغاً، خالياً من مسببات الأمراض، من فئران ألبينو البيضاء، وزنها ١٥٠-٢٥٠ جرام وتقسيمها. تم تقسيمها إلى ٣ مجموعات (١٠ فئران لكل منها): المجموعة الضابطة التي تتلقى فقط محلول ملحي بالفوسفات (PBS). المجموعة المصابة بالتهاب المفاصل والتي تعرضت لتحفيز التهاب المفاصل العظمي عن طريق الحقن داخل المفصل لجرعتين CFA (٥٠ ميكرولتر) الجرعة الأولى في اليوم الأول من التجربة بينما الجرعة الثانية في اليوم ١٤، تليها تناول ٢٠ ملغم/كغم PBS عن طريق الفم . مجموعة مستخلص فاكهة القشطة: تم إخضاع هذه المجموعة للتحريض بالتهاب المفاصل العظمي كمجموعة التهاب المفاصل، ثم عولجت بمستخلص الفاكهة ١٠٠ ملغم / كغم / يوم لمدة ١٤ يومًا بعد الحقن الثاني لـ .CFA تم التضحية بالحيوانات بعد تأكيد اصابتها بالتهاب المفاصل بعد ثلاثة أسابيع. وتم صباغتها بكل من H&E و Anti TNFα و Anti MMP٣ و Picrosirius red ثم تم إخضاعها لتحليل الصور الرقمية متبوعة بالتحليل الإحصائي One-way ANOVA.

النتائج: أظهرت المجموعة المعالجة بمستخلص فاكهة القشطة تحسنا في تجديد المفصل الصدغي الفكي مقارنة بمجموعة التهاب المفاصل حيث اظهرت H&E انخفاض سماكة القرص وزيادة الغضروف اللقمي وجعلت العظام أكثر تنظيماً. من الناحية الكيميائية المناعية، أظهرت المجموعة المعالجة بمستخلص الفاكهة انخفاضًا في ظهور TNFα وMMP٣ ($P < 0.001$) عند المقارنة بمجموعة التهاب المفاصل. بينما أظهرت مقاطع الصبغة الحمراء للبيكروسيريوس ألياف كولاجين أكثر تنظيماً وأقل التهاباً بشكل خاصفي المجموعة المعالجة.

الخلاصة: يمكن أن يكون مستخلص أوراق وفواكه القشطة من المرشحين المحتملين للتخفيف من حالات هشاشة العظام في الفئران.