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Evaluation of the Healing Effect of Psidium GuajavaL. Hydro-alcoholic Fruit Extracton Induced Osteoarthritis in Male Rats

Abstract

Background: Osteoarthritis (OA)is a major public health consideration which leads to disability because of chronic pain, stiffness, sleeping disorder and depression.Among the herbal products *Psidium Guajava L.* fruit could be regarded as apotential natural remedy due to its anti-oxidant characteristics. The aim of this study was to evaluate the healing effect of*Psidium GuajavaL*. fruit onosteoarthritisof the knee in rats.

Methods: In this experimental study, Forty male Sprague-Dawley rats withinduced osteoarthritis by intra-articular injection of 500U of type 2 collagenase he left knee were recruited in four treatment groups (n=10): (A) control, (B) Piascledine (10mg/kg)as positive contı , (C)oral administration of hydro-alcoholic extract of *Psidium GuajavaL.* fruit(500mg/kg), and (D) oral administration hyc -alcoholic extract of *Psidium Guajava*L. fruit(1000mg/kg). After 8 weeks of daily administration, the outcome was histopathological and radiographic assessments.The data were analyzed using Kruskal-Wallis nonparametric in SPSS version 23.00, and P<0.05 was considered statistically significant.

Results: *Psidium Guajava*extractexhibited good radical scavenging activities with IC50 of 0.46 ± 0.13 mg/r (quercetin as a positive control). Histopathological assessments of the cartilage showed smooth and continuous articular surface with columnar cell distribution in the experimental group that received 1000mg/kg *Psidium GuajavaL*. fruithydro-alcoholic extract which healed better than others,especially in the control group (p-value =0.02). The radiological assessments of the knee joints showed similar findings in histopathological assessment.

Conclusion: Psidium GuajavaL. fruithydro-alcoholic extractmight be regarded as an effective complementary and alternative treatment forosteoarthritisof theknee in rats.

Key words: Osteoarthritis, Knee, Radiography, Pathology, Psidium, Rats

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[1]Sara Sadat Nabavizadeh, MD; [1]Mohsen Ahmadi Zarei, MD; [2]Cambyz Irajie, PhD; [3]Aida Iraji, PhD; [4]Parvin Ghaemmaghami, PhD; [3]Omid Koohi Hosseinabadi, MSc; [5]Ida Hasssanpour, PhD; [6]Maryam Mojahed Taghi, MSc; [7]Mahshid Jamshidi, PhD; [8]Sajad Daneshi, PhD; [9]Nader Tanideh,PhD

1.Student Research Committe Shiraz University of Medical Sciences, Shiraz, Iran
2. Biochemistry department, Shiraz University of Medical Sciences, Shiraz, Iran
3.Central Research Laboratory, Shiraz University of Medical Sciences, Shiraz, Iran
4.Department of biostatistics, school of medicine, Shiraz University of Medical Sciences, Shiraz, Iran
5.Surgery Department, Veterinary School, Shiraz University, Shiraz, Iran
6.Pharmacology Department, Shiraz University of Medical Sciences, Shiraz, Iran
7.Solace, medclinic, Toronto, Canada
8.Stem Cells Technology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
9.Stem Cells Technology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
Pharmacology Department, Shiraz University of Medical Sciences, Shiraz, Iran
*** Correspondence**
Nader Tanideh
Email: tanidehn@gmail.com

Osteoarthritis (OA) is amultidimensional problem, involving complex and interacting mechanical, biological, biochemical, molecular, and enzymatic feedback loops with cartilage degeneration as the common final effect[1, 2]. It is a major public health consideration because of its high prevalence and costs. Moreover, slightly progressive process of OAin synovial joints, would lead to disability because of chronic pain, stiffness, sleeping disorder, and depression[3, 4]. OA incidence is correlated withobesity, repetitive joint trauma, fracture and diabetes. Moreover, some studies revealed that genetics can affect the susceptibility to OA by expressing human leukocyte antigen, vitamin D receptor, insulin-like growth factor I and cartilage oligomeric proteins [5].

Yet, there is no exact cure for OA, but some treatments can help to relive thesymptoms and decrease the chance of exacerbation of the patients' arthritis. Non-steroidal anti-inflammatory drugs (NSAIDS), Analgesics, Capsaicin cream, herbal medicine, cold and warm therapy, Transcutaneous Electrical Nerve Stimulation (TENS), knee braces, Intra-articular (IA) steroid injections, IA Hyaluronic acid injections, and surgery are some of the current treatments [6, 7].

Different pharmacological experiments in a number of in vitro and in vivo models have been carried out; then, the extracts and various metabolites of the leaves and fruits of *PsidiumGuajava Linn*showed to be useful in biological activities mainly due to phenolic, flavonoid, carotenoid, terpenoid and triterpene components[8].

Psidium GuajavaLinn fruit isan important food crop and medicinal plant in tropical and subtropical countries.*Psidium Guajava*is usedto cure various diseases, for instance in diabetes, diarrhea, hypertension, caries, wound healing, and as pain killer or to control fever[9].

*Psidium Guajava*illustrated analgesic, anti-inflammatory and anti-oxidant activities that are probably due to the essential oils and flavonoids present in the plant [10].

Nutritional studies in the treatment of osteoarthritis have always been a controversial challenge.The aim of this study was to evaluate the healing effect of *Psidium Guajava*L. fruit on osteoarthritis of the knee in rats.

Method

Animal design

This experimental study was performed in the center of laboratory animals, Shiraz University of Medical Sciences in 2019, using 40 male rats weighing 220±20 gr and 10-12 weeks old. The animals were housed in standard cages under a 12-hr light/dark cycle (lights on at 7:00 pm) with an ambient temperature of 22 ± 2ºC, and rel humidity of 55± 5%. Rats were given at 3-day acclimation period with free access to normal chow and water, ad libitum before the experiment. Animals were randomly divided into four groups of 10 animals each. The first group of rats served as controls and did not receive any treatment; group 2 was treated with 10mg/kg Piascledine; group 3 was treated with 500 mg/kg hydro-alcoholic extracts of *Psidium Guajava*; and group 4 was treated with 1000mg/kg hydro-alcoholic extracts of *Psidium Guajava*[11] The hydro-alcoholic extracts of *Psidium Guajava*fruit was given orally after induction of osteoarthritis during a daily study. After 8 weeks, all animals were sacrific $CO_2$70% and then the samples were harvested. All the experiments were in accordance with the recommendations of the European Council Directive (86/609/EEC) of ember 24, 1986, regarding the protection of animals used for experimental purposes (http://data.europa.eu/eli/dir/1986/609/oj). The study protocol was approved by the Ethics Committee of Shiraz University of Medical Sciences (No. 94-01-01-9757).

Osteoarthritis induction

In the process of osteoarthritis induction, the animals were anesthetized with 100 mg/kg ketamine 10 (Alfasan, Netherlands) and 10 mg/kg xylazine 2% (Alfasan, Netherlands). Then, the animals received two injections, on days 0 and 3, with 500 U of type II collagenase from Clostridium histolyticum (Sigma-aldrich,St.louis,MO, USA) whichwas dissolved in saline and filtered through a 0.22 µm membrane prior to injection. Animals were randomly assigned to each group before the first injections.

Hydro-alcoholic extract of *Psidium Guajava*L. fruit preparation

*Guava*fruits (Genus: Psidium, Species: guajava; herbarium number 771)were prepared from Chabhar city in the south-east of Iran (Figure 1).*Guava* fruits were disinfected for 2 hours in sodium hypochlorite solution and then were cut into small pieces, rinsed in distilled water, dried and finally powdered. 500 g of the pulverized samples were percolated in about 3 litres of ethanol (70%, Merck, analytical grade) for 78 hours to obtain the ethanol (crude) extract, followed by filtration. Then, the extract concentrated under reduced temperate and lyophilized (Modulyo D Freeze Dryer, Thermo Scientific). The extract of *Psidium guajava L* yielded 21% with light brown color. Extraction procedure repeated tree times to collect appropriate amounts of extract for biological experiments. At the end, the fruit hydro alcoholic extract stored at 4 ºC in an amber flask [12].

Figure 1: Fresh fruit of *Psidium Guajava L*.

Determination of total phenolic content (TPC)

Total phenolic components of hydro-alcoholic extract were determined according to method described by Folin-Ciocalteu with modifications. Briefly, 50 µL of *Psidium guajava* extract dissolved in dH_2O (extract with 1 mL of distilled water) were mixed with 200 µL of Folin-ciocalteu´s reagent and 3 mL dH_2O. Afterwards 600 µL of 25% sodium carbonate (Na_2CO_3) solution were added to the mixture, followed by incubating at room temperature in the dark for 2 hours. The absorbance against a blank was measured at 725 nm. All samples were assayed in triplicate. Gallic acid was used to prepare a standard curve and he same procedure was repeated for the standard solution of gallic acid. The results were expressed as mg gallic acid equivalents (GAE)/g dry extract [13].

Total antioxidant activity assay

The antioxidant activity of the extracted oil was evaluated by free radical scavenging effect on 1, 1 – dipheny 1-2-picrylhydrazyl (DPPH). The determination was based on the method described by with some modification. The 1 mg/mL of sto solution ofextract were prepared in a series of concentrations ranging from 6.2 -200 µg/mL. 200 µL of each concentration was then mixed with 5 µL of 1 mM DPPH. The mixtures were incubated in the dark at room temperature for 30 min. As the extract reduce the DPPH radical, the solution change color from purple to yellow (arising of diphenylpicrylhdrazine). The reduction in capability of the DPPH radical is determined by decrease in its absorbance (abs) at 517 nm. The lower IC_{50} demonstrate for higher antioxidant power. Quercetin was used as standard and DPPH mixture without any sample served as blank. All samples were assayed in triplicate. Percentage of inhibition (I%) of DPPH radical was determined using formula as below:

$$I\% = \frac{\text{Abs blank} - \text{Abs sample}}{\text{Abs blank}} \times 100\%$$

Median inhibitory concentration (IC_{50}) was determined from the graph [14].

Histopathological assessments

To evaluate histopathological, the distal femoral and the proximal tibial plateau were removed and fixed in 10% buffered formalin. The samples were decalcified and cut into four pieces. All pieces were embedded in paraffin. Serial sagittal sections(5µm) were prepared and stained with hematoxylin and eosin.Forty slices for the distal femoral and the proximal tibia plateau were evaluated. This observation was performed by a blinded pathologist. The severity of the articular cartilage lesions was graded, using a modified histological grading method provided by Yanai et al. (ICRS)[15]. This scoring system is based on the following repair indices: surface, matrix,cell distribution, cell population viability, subchondral bone, and cartilage mineralization (Table 1). A lower score indicates more severe damage. All morphometric parameters were recorded with Olympus DP12 Digital lens system (Olympus Optical, Tokyo, Japan).

Table1. Histopathological scoring using internatio cartilage repair society (ICRS).

Variable	Score
Surface	
Smooth/continuous	3
Discontinuous/irregular	0
Matrix	
Hyaline	3
Mixture: hyaline +fibrocartilage	2
Fibrocartilage	1
Fibrous tissue	0
Cell distribution	
Columnar	3
Mixture/ columnar cluster	2
Cluster	1
Individual cell/ disorganized	0
Cell population variability	
Predominantly viable	3
Partially viable	1
<10% viable	0
Subchondral bone	
Normal	3
Increase remodeling	2
Bone necrosis/granulation tissue	1
Detached/fracture/cell at base	0
Cartilage mineralization	
Normal	3
Abnormal/inappropriate location	0

Table 2. Radiological evaluations for knee osteoarthritisin rat					
Radiographic OAfeature of the medial compartment		Grade0	Grade1	Grade2	Grade3
Joint space width		normal	reduced	absent	NA
osteophytes	Medial tibial condyle	absent	small	moderate	sever
	Medial femoral condyle	absent	small	moderate	sever
	Medial fabella	absent	present		NA
Total osteophyte				0-7	
Global OA score				0-9	

Radiographic assessments

To investigate radiological indices, digital images of the knee joint were taken by using an Axiom Multix M radiographic unit (Siemens, Germany). Radiographical images were taken in both AP and lateral position. (Table 2)

Statistical analysis

All qualitative data were presented as mean and standard deviation (SD) and analyzed using Kruskal-Wallis nonparametric (distribution free) by SPSS version 23.00 and P<0.05 was considered as the significance level.

Results

The TPC assay showed 495.36 ± 7.88 mg GAE/g dry extract of phenolic content. Given the promising phenolic content observed for*Psidium Guajava*extract,it was essential to check the potential antioxidant activity.

*Psidium Guajava*extractexhibited good radical scavenging activities with IC50 of 0.46 ± 0.13 mg/mL (Quercetin as a positive control demonstrate IC50=3.01±1.72 µM).

The results indicated a strong correlation between anti-oxidative activities and phenolic compound, suggesting that phenolic compounds probably account for the anti-oxidative activities of *Psidium Guajava.*

The histopathological findings of the femoral and tibia articular cartilage 8 weeks after the beginning of treatment are demonstrated in Figures 2,3, 4.

Articular surface healing index and matrix distribution index in *Guava* fruit hydro-alcoholic extracted groups 1000mg/kg were statist significantly different as compared to the control group (p-value =0.01).

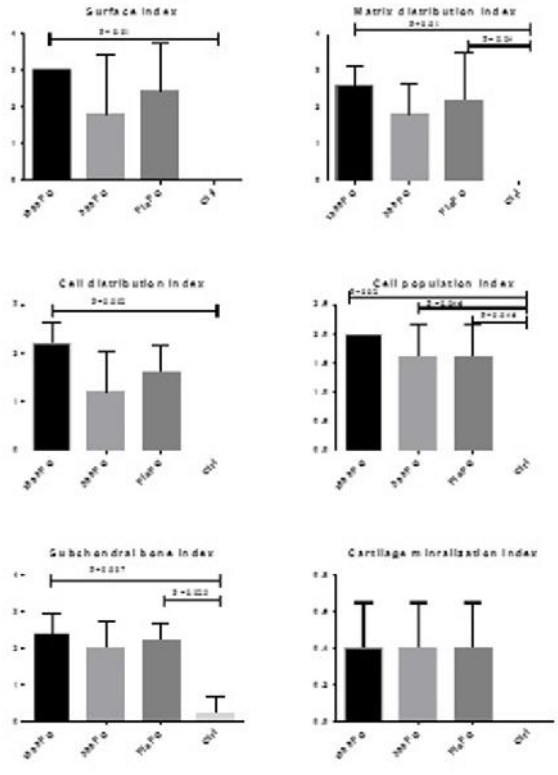

Figure 2: Graph pad prism representation of the histological scores based on international cartilage repair society.Values shown as median and interquartile range. Significant differences marked with for p < 0.05.

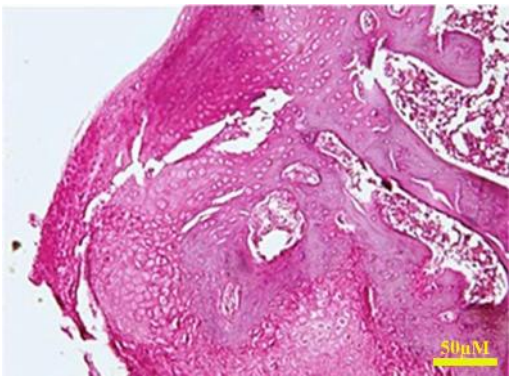

Figure 3:Histopathological examination of the rat knee articular cartilage in control group, the surface in the control group had irregularity, and the cells were arranged in clusters (H&E ×100).

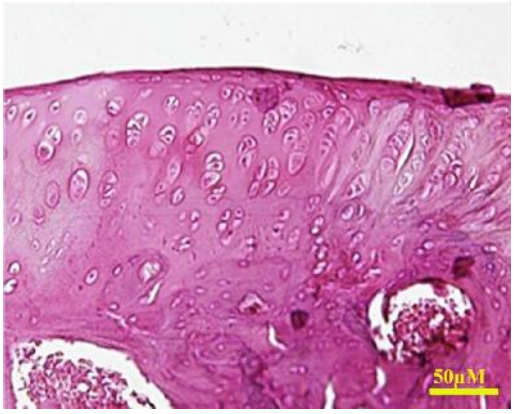

Figure 4;Histopathological examination of th knee articular cartilage after administration of 10(mg/kg hydro-alcoholic extract of*Psidium Guajava*L.fruit intra-articularly which showed repair with smooth and continuous articular surface along with columnar cell distribution (H&E ×10

The mineralization of the femoral and tibia articular cartilage index among the groups was not significantly different. The cell distribution score of the femoral and tibia articular cartilage in the high dose experimental group (1000mg/kg) was significantly higher than the controls. (Pvalue =0.02).

Also, the viable cell population index of the femoral and tibia articular cartilage was higher in all groups compared to the controls. However, the high dose (1000mg/kg) *Psidium GuajavaL.* fruit hydro-alcoholic extract had more considerable p-value. (P-value =0.02).

Overall, the pathological findings revealed better healing of both femoral and tibial articular surfaces in the treatment group(1000mg/kg).Radiological findings were similar to histopathological assessment in which the improvement of the articular cartilage was much better in the experimental group (1000mg/kg) in comparison to the controls. (Figure5)

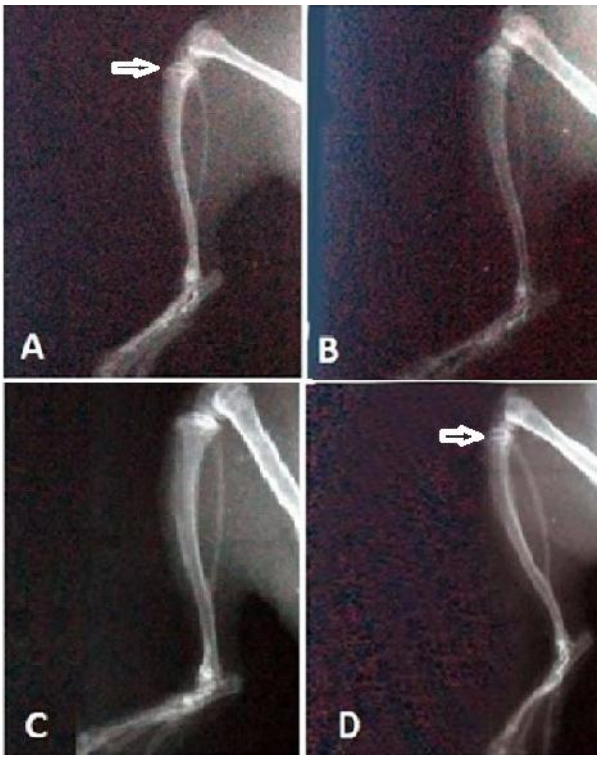

Figure 5: Evaluation different treatment - induced arthritis using collagenase type -ray films on 42th day. Control animals without treatment showed severe osteoarthritis in form of a white line at joint space above the tibia (arrow) (A); Animal treated with standard drug "Piascledine" (B); Animal treated with 500mg\kg of Psidium Guajava (C); Animal treated with 1000 mg\kg of Psidium Guajava showed mild osteoarthritis in form of a white line at joint space above the tibia (arrow) (D).

Medial tibial and femoral osteophyte index was statistically significant in high dose (1000mg/kg) *Psidium GuajavaL*. fruithydro-alcoholic extract compared to the control group. (p value =0.003, 0.009, respectively) However, Medial febellar osteophyte index only showed a significant p value among oral Piascledine and control groups. (P-value =0.012).

The joint space width index among all groups was significantly different compared to the controls, while high dose (1000mg/kg) *Psidium GuajavaL.* fruithydro-alcoholic extract showed a better p value.(P value =0.0(*Psidium Guajava*L. fruit hydro-alcoholic extractmight be regarded as a great supplementary treatment forosteoarthritisof the knee in rats.

Discussion

The present studywas conducted to compare the efficacy of *Psidium GuajavaL*. fruithydro-alcoholic extract in healing osteoarthritis during an eight-week period. The results showed more healing effect of *Psidium GuajavaL*. fruit hydro-alcoholic extract.*Psidium Guajava*L. fruit hydro-alcoholic extractmight be regarded as an effective complementary and alternative treatment forosteoarthritisof the knee in rats.

New approaches are, therefore, needed both to increase the safety and efficacy of symptomatic treatment and to exert a favorable influence on the course of the disease. A number of substances that occur naturally exist in the body may have value for the prevention and/or treatment of OA. Although much of the research is in its early stages, the possibility that natural substances can be used to heal the degradation, or enhance the repair, of the joint cartilage is intriguing. This high popularity in applying complementary and alternative medicine can be due to its advantages such as widespread availability, no or fewer side effects, moderate efficacy, and low cost as compared with synthetic drugs [16].

As osteoarthritis is an inflammatory disease which is accompanied by joint destruction, mediator-like cytokines and nitric oxide produced by chondrocyte play a key role in progression of the disease[17].

Guajava hydro-alcoholic extract has analgesic, anti-inflammatory, antimicrobial, hepatoprotective, and antioxidant activities. Therefore, Guajava can be a good alternative for other drugs and procedures [18].

Jimenez-Escrig et al. and Patthamakanokporn et al. reported the presence of higher amounts of phenolic compounds with antioxidant activity in the leaves of white (*Psidium Guajava*var. pyrifera L.) and red guajava (*Psidium Guajava*var. pomifera L.)when compared with other vegetable species[19, 20].

Similar to pervious experimental study that evaluate the effect of *Psidium GuajavaL.* leaf essential oil on induced osteoarthritis of knee in male rats[11], pathological indices related to knee osteoarthritis like cell population, cell distribution and articular surface showed better healing and improvement in whichin those rat with osteoarthritis that received the high dose (1000mg/kg*Psidium GuajavaL.* fruit hydro-alcoholic extractthe population of chondral cells were in articular cartilage was predominant,distributed cell were columnarand articular surface was smooth and continuous.Destructive articular surface leads to metabolism disturbanceof cartilage and bone which make subchondral bonedamage, pain, limitation of movement, swelling and dysfunction in patients.

Also, Chen et al. found Gallic acid, Catechins, epicatechins, rutin, naringenin and kaempferol in the leaves. The major components *in Guava* leaves oil are fatty acids containing glycerol molecule, known as mono-unsaturated fatty acids (MUFA) which contribute to 95-98%of oil and the remaining constitutes include phenolics and sterols [21].

The major health benefits of *Guava* oil are attributed to the high concentration of mono-

unsaturated fatty acids (which is mainly oleic acid).Moreover, scientists discovered that the major phenolic component responsible for anti-inflammatory properties in extra virgin *Guava* leaves oil is oleocanthal. This newly discovered substances act as a natural anti-inflammatory substance, similar to that of ibuprofen used in treatment of arthritis. It is found that this compound, like ibuprofen, inhibits the action of cyclooxygenase enzymes-1 (COX-1) and cyclooxygenase enzyme-2 (COX-2)in prostaglandins biosynthesis pathways. Prostaglandins cause inflammation in osteoarthritis patients and hence inhibit its biosynthesis helps in reducing the inflammation and pain associated with this disease[22, 23].

In-vitro and in-vivo research indicated the notable inhibitory effects of Nitric oxide (NO) on chondrocytescollagen and proteoglycan synthesis[24].

An experiment revealed positive effects of oleocanthal, phenolic compound in extra virgin *Guava* leaves oil, on lipopolysaccharide-induced NO synthase (NOS2) reduction chondrocyte with no harmful effects on cell viability [25].

Furthermore, it seems that tyrosol and hydroxyl-tyrosol, phenolic component, are responsible for higher amounts of blood calcium in laboratory animals with *Guava*leaves oil intake[26].

Bone reabsorption inhibition is one of the key requirements in minimizing the symptoms of OA; *Guava*has been proved to be very beneficial in treatment of such disease by blocking NF-kβ[27].

Many of the traditional uses have been validated by scientific researches. Toxicity studies in mice and other animal models as well as controlled human studies show that both leaves and fruit are safe without any side effects[28].

For example, Diyanat et al. (2018) conducted a research on sixty ovariectomize female rats to investigate the anti-osteoporotic effects of the hydro-alcoholic extract of the *Psidium Guajava*(PG) fruit. The hydro-alcoholic extract of the PG fruit increased the femoral weight and volume, femoral ash density, number of osteocytes and osteoblasts, and trabecular volume of the bones in comparison with the group which only got ovariectomized in a dose-dependent manner[29].

Another study conducted by Siti Balkis Budin in 2013 showed significant anti-oxidative effect of *Psidium Guajava*fruit in diabetic rats. This study showed that superoxide dismutase (SOD) and glutathione (GSH) levels were significantly higher ($p<0.05$) in the experimental group in comparison to the control group[30].

Kirti Jahagirdar in 2010 showed a great ar - arthritic effect of hydro-alcoholic extract in*Psidium Guajava*lin in three different dosages in the subplantar region of the hind paw of rats. The paw volume, body weight, diameter of the tibiotarsal joint and total leukocyte count in the blood were measured. Results showed a noticeable and dose-dependent improvement[31].

Kuo and Chien disclosed a study that was designed to explore the defensive mechanisms of *Guava*juice combination with trehalose in the rats with diabetes type 2. The outcomes showed that the combination of *Guava*juice and trehalose had more protective effects in the pancreas and kidney against hyperglycemia-induced inflammation and oxidative injury [32].

Unfortunately, our study faced some limitations as to theevaluation of antioxidant indexes, anti-inflammatory markers and some gene expressions for inflammation.

Tanideh et al (2018)reported significant effect of *Psidium GuajavaL*. leaf oil in joint space width and decreasing osteophytes which confirms our radiographic findings.Radiographs showed narrowing joint space along with increasing in osteophytes formation in osteoarthritis rats compared to rats treated with *Psidium GuajavaL.* fruit hydro-alcoholic extract especially in the high dose (1000mg/kg). Reduction in joint space is

related to destruction of hyaline articular cartilage[11].

Similar to the findings of our previous studyabout **e**valuation of *Psidium Guajava*L. leaf oil extract effect on induced osteoarthritis in male rats[11],histopathologicaland radiographic resultsof the present study showed a significant difference between hydro-alcoholic extract of *Psidium GuajavaL*. fruit (1000mg/kg) as the treatment group and the control group in terms of the surface, matrix distribution, cell population, subchondral bone and cell distribution indices and alsojoint space width, medial tibial condyle osteophytes and medial femoral condyle osteophytes, respectively.

In recent years, emphasis of research has been on utilizing traditional medicines that have a long and proven history of treating various ailments. In this regard, further studies are required to be carried out to explore P. guajava L fruit for its potential in treating joint diseases. Stereological evaluation and using gel form instead of hydro-alcoholic extract can be the limitation of the current study.

Conclusion

Hydro-alcohol extract *Psidium GuajavaL* showed considerable effectiveness in all histopathological indexes except cartilage mineralization index. Furthermore, radiographic results demonstrate better outcome of the joint width index, medial tibial osteophyte index and medial femoral osteophyte index in the high dose group compared to the control group rats.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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