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Evaluation of Psidium Guajava L. Leaf Oil Extract Effect on Induced Osteoarthritis in Male Rats

Abstract

Background: Osteoarthritis (OA) is widespread degenerative joint disease. Although many therapeutic policies exist, no clear preventive remedy exists. Due to various side effects caused by conventional medication; we aim to evaluate the effect of leaf of Psidium guajava essential oil on animal model of OA.

Methods: This 8-week animal model study was conducted on 25 male rats with induced osteoarthritis with collagenase. The rats were divided into 5 groups randomly, the first group that serves as negative control group and received intra-articular injections of saline, the second group received olive oil, the third group intra articular(IA)Hyalgan, the fourth group received low dosage (25 λ) essential oil of Psidium guajava L. leaf, and the last group which received high dosage (50 λ) essential oil of Psidium guajava L. leaf. All groups received their medication for 4 weeks. After 8 weeks from beginning of study, all of the animals were euthanized with CO2 and samples harvested. All the samples underwent histopathological and radiological evaluation and data were collected and analyzed by Kruskal-Wallis nonparametric test (distribution free) by SPSS version 23.00.

Results: In histopathological findings illustrate a considerable difference between high dose (50λ) treatment group and the control group. Moreover; in radiographic findings, significant difference was observed between groups in evaluation of distribution in joint space width. (P value =0.0001)

Conclusion: Essential oil of Psidium guajava L. leaf has a considerable positive effect on osteoarthritis.

Keywords: Osteoarthritis, Knee, Radiography, Pathology, Psidium, Rats

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Introduction

Osteoarthritis (OA) is a slowly progressive degenerative joint disease in synovial joints which counts as a major public health problem due to its high prevalence, costs, and levels of pain and disability caused by chronic pain and at times stiffness [(1)]. Representative prevalence rates for radio-graphically apparent OA of the knee in the general population older than 35 years of age are approximately 15% in males and 20% in females with females generally at a higher risk specially after menopausal age [(2,3)]. Conventional pharmacological treatment of OA consists primarily of nonsteroidal anti-inflammatory drugs (NSAIDs) and analgesics. While these medications often relieve in symptoms, they are far from ideal therapeutic agents. NSAIDs, in particular, can cause serious side effects, including peptic ulcer and (less commonly) hepatic or renal failure. Neither of these classes of medications prevents or delays the progression of OA. Many evidences show acceleration in articular destruction due to NSAIDs administration, both in experimental animal and human with OA. [(4)]. New approaches are therefore needed, both to increase the safety and efficacy of symptomatic treatment and to exert a favourable influence on the course of the disease. A number of substances that occur naturally in the body may have value for the prevention and/or treatment of OA. Some preliminary data reveal that some of these compounds show some considerable effect in the exacerbation of the disease.

Although much of the research is in its early stages, the possibility that natural substances can be used to prevent the degradation, or enhance the repair of 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 [5,6]. Guava leaf extract illustrated analgesic, anti-inflammatory and anti-oxidant activities, mainly stems from phenolic, flavonoid, carotenoid, terpenoid and triterpene components; in several previous studies. hence, we hypothesized that the joint structures destruction in OA could be prevented by use of Psidium guajava L. leaf essential oil [7,8].

The present study was conducted to observe the effect of Psidium guajava L. leaf essential oil on induced Osteoarthritis of knee in male rats.

Methods

Osteoarthritis induction

Twenty-five adult male rats were anesthetized with 90-100 mg/kg Ketamine 10% (Alfasan, Netherlands) plus 5-10 mg/kg Xylazine 2%(Alfasan, Netherlands) through IM route. Animals received a bacterial collagenase intra-articular injections in the right knee. Each shot included 1 mg of collagenase type II from Clostridium histolyticum (Sigma-Aldrich, St. Louis, MO, USA) dissolved in saline sterile phosphate buffered saline (pH 7.4) and filtered through a 0.22 µm membrane(142N), then slowly injected into the left knee joint cavity; and repeated after 3 days.

Animal design and housing

Twenty five male adult Sprague dawley rats weight of 220±250 gr and age of 10-12 weeks old were prepared and approved by the Ethics Committee in Shiraz University of medical science (ethical code: 94-01-01-9732). All animals housed in standard cages under a 12-h light cycle (lights on at 7:00 pm) with an ambient temperature of 22±2 ºC, and 55±5% relative humidity. Rats were given standard chow and water ad libitum. All Animals were randomly divided into five groups, (each, n=5). The first group of rats served as negative control which received once weekly intra-articular (IA) injections of saline (50µL) for 4 weeks after OA induction. The second group received olive oil (50 µL, IA) (extra-virgin olive oil, ETKA, Iran) and the third group were treated with Hyalgan(10mg/kg, 50µL, IA) . Group 4 received 25 λ essential oil of Psidium guajava L. Leaf while animals in group 5 were treated with 50λ of Psidium guajava L. leaf essential oil. First injections were done right after the induction of OA and then next 3 injections in each group were done in two weeks interval. Finally, after 8 weeks, all of the animals got euthanized with CO_2 and samples harvested.

Preparation essential oil

Psidium guajava Linn. Leaves were collected from a local area of Chabahar port, Sistan and Baluchestan province, southeast of Iran (Herbarium no.771). Essential oil was extracted from leaves by the process of steam distillation. As the yield in using an all glass still is just around 0.11% and hence a large amount of Psidium Guajava leaf would be required, modified Paint could be used as a distilling pot. Also, a small hot plate was used as a heat source. Moreover, a 250 ml round bottom flask was used as a receiver. Then, 450 gr of Psidium guajava leaf and 400 ml distilled water were added to the distilling pot and began heating the system slowly. The heat was adjusted so that the distilling rate would be equal to 20 drops per minute. After that, about 150-200 ml of distillate was collected. It should take to consideration that the presence of the olive oil in the condensate would cause the drops forming in the condenser to be cloudy. Then, the distillate was transferred to a 250 ml separatory funnel, 50 ml of Methylene Chloride and extract of Psidium guajava leaf oil were added, the Methylene Chloride layer was drained off into a 250 ml Erlenmeyer flask and then, The Methylene Chloride with a little Anhydrouse Magnesium Sulphate got dried. This is required stage because trace amounts of water will dissolve in the Methylene Chloride since Anhydrouse Magnesium Sulfate will absorb the water and produce solid Magnesium Sulphate Heptahydrate. The system was allowed to dry for 10-15 minutes. After that, the liquid was decanted into a 250 ml round bottom flask. Used Rotovap to strip off the Methylene Chloride from the Psidium guajava leaf oil. At the end, Psidium guajava leaf oil was transferred to label 4-dram vial and stored at 4°C.

GC-MS Analysis

The steam-distilled oil was analysed by GC/MS allowing the constituents to be identified. moreover, qualitative and quantities analysis to the volatile compounds were carried out using a model

6890 gas chromatograph (GC) equipped with 30m_0.25 mm i.d. HP-(5%cross linked phenyl-methyle siloxane) column with 0.25 im i.d. film thickness and an agilent model 5973N MSD mass spectrometer (MS) with a 7683 auto sampler (Agilent, Palo Alto, CA). The initial oven temperature was held at 40˚ C for 6 minutes. It was then increased at 2.5 C/min to 150˚ C and finally at 90˚ C/min to 250 ˚C; the injection port and ionizing source were kept at 250˚ and 280˚ C, respectively. The split ratio was 10:1 with 2 ml of sample injected. there was a solvent delay of 2 min after which mass spectra were scanned from m/z 35 to 300, generating 5.27 scans/s. Compounds identification were made by visual comparison of the mass spectra and retention times with those of corresponding reference standards (Aldrich Chemical Co., St. Louis, MO; Bedoukian Research, Inc., Danbury, CT; and Keller and Milne, 1974) for all compounds except (Z)-3-hexenoic acid and R-copaene that were identified by the NIST98 library (NIST, Gaithersburg, MD) and reported retention indices. The different components of essential oil were: a-pinene (65.4%), trans-caryophyllene (17.4%), 1,8-cineole (7.6%), limonene (4.0%), a-humulene (3.5%) and a-copaene (2.0%).

Histopathological evaluation

Histopathology is the gold standard for evaluation of OA. Distal femoral and the proximal Tibial plateau were removed and fixed for 24 h in 10% buffered formalin, decalcified for 7 days in 20% EDTA, then embedded in paraffin. Serial sagittal sections (5 µm) were prepared and stained with Haematoxylin and Eosin. This observation was performed by a blind pathologist. The severity of articular cartilage lesions was graded, using a modified histological grading method provided by Yanai et al. (2005). This scoring system is based on the following repair indices: surface, matrix, cell distribution, cell population viability, subchondral bone, and cartilage mineralization. All morphometric parameters were recorded with Olympus DP12 Digital Camera system (Olympus Optical, Tokyo, Japan). (Table 1)

Radiological assessments

Radiologic evaluations were performed in all rats after anaesthesia with 100 mg/kg ketamine 10% and 10 mg/kg xylazine 2% at the end of 8 weeks post operatively. Digital radiographs of the knee joint were taken in both Lateral and Antro-Posterior position, by using an Axiom Multix M radiographic unit (Siemens, Germany) and graded according to Joint space width and osteophyte formation related to Medial Tibial Condyle, Medial Femoral Condyle and Medial fibula. (Table 2)

Finally, the left knee joint cavity was opened and soft tissues around the knee were removed and after that, the rats were euthanized by Euthtanasia co2 70%.

Statistical analysis

All qualitative data were presented as mean and standard deviation (SD) and analysed using Kruskal-Wallis nonparametric (distribution free) by SPSS version 23.00 and $P<0.05$ was considered as significant difference to interpret the results.

Table1. International Cartilage Repair Society Histological Score

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

Result

Histopathology results

In this experiment, 8 weeks after beginning of the treatment, the histopathological findings of the anterior Tibial articular cartilage of femur demonstrate significant differences between groups in all the indexes but cartilage mineralization. To deepen the results about

Table 2. Radiographic feature					
Radiographic OA feature 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	

Table 3.Histological analysis, p-values and comparison						
Index / Group	Surface	Matrix	Cell Distribution	Cell Population Viability	Subchondral Bone	Cartilage Mineralization
Group5	3(0)	2.4(0.55)	2(0.71)	2(0)	2.6(0.55)	0.4(0.55)
Group4	1.8(1.64)	1.4(0.55)	1.6(0.55)	1.6(0.55)	2.0(0.71)	.20(0.45)
Group3	1.2(1.64)	1.0(0.71)	1.20(0.45)	1.0(0.71)	1.40(0.55)	0.0(0.0)
Group2	.60(1.34)	.60(0.55)	.60(0.55)	.60(0.55)	.80(.45)	.40(0.55)
Control	0.0(0.0)	0.2(0.45)	0.2(0.45)	0.2(0.45)	0.4(0.55)	0.0(0.0)
Sig	0.008	<0.0001	<0.0001	<0.0001	<0.0001	0.32

* Surface, C-group5:0.018
*matrix, C-group5: 0.002, J-K:0.033
*Cell Distribution, C-group5: 0.007
*Cell Population Viability, C-group5: 0.004
*Subchondral Bone, C-group5: 0.003, J-K:0.029

Figure 1. Compression between ICRS index among groups

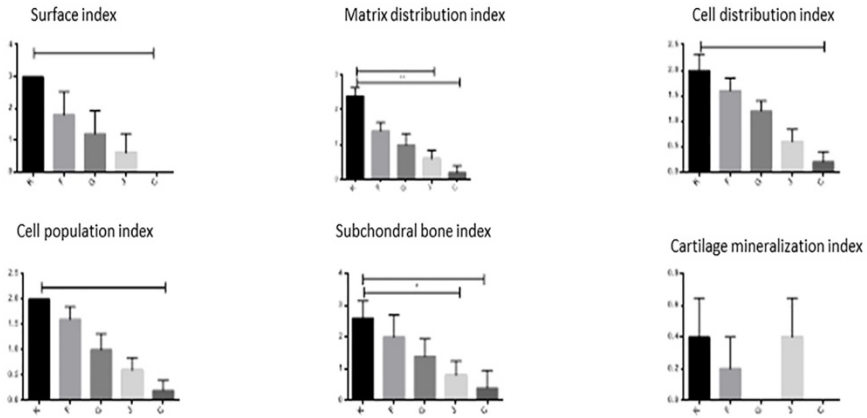


Figure 2. The surface in the control group had irregular, and the cells were arranged in clusters (H&E× 100)

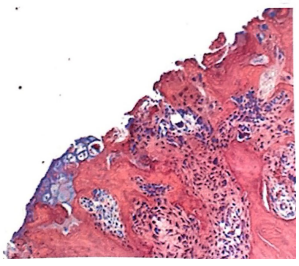


Figure 3. In compression with the control group, the defects in the experimental group was repaired with smooth and continuous articular surface with columnar cell distribution (H&E× 100)

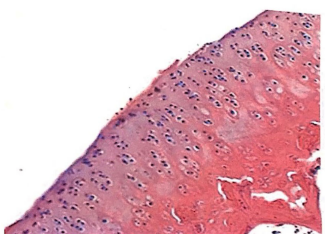
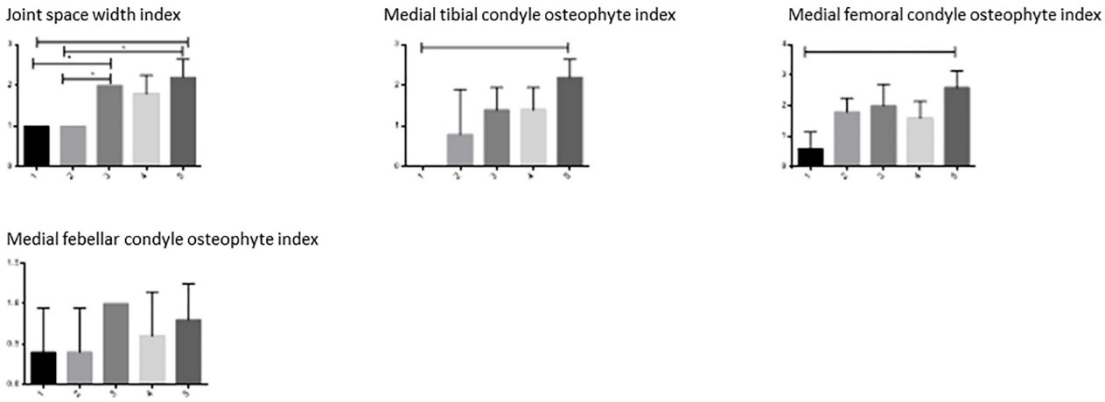


Table4. Radiographic analysis, p-values and comparison				
Index / Group	Joint Space Width	Medial tibial osteophyte index	Medial femoral osteophyte index	Medial febellar osteophyte index
Group5	1(0)	0(0)	6(0.55)	0.4(0.55)
Group4	1(0)	0.8(1.1)	1.8(0.45)	0.4(0.55)
Group3	1(0))	1.4(0.55)	2(0.71)	1.0(0)
Group2	1.8(0.45)	1.4(0.55)	1.6(0.55)	.60(0.55)
Control	2.2(0.45)	2.2(0.45)	2.6(0.55)	0.8(0.45)
Sig	<0.0001	0.005	0.005	0.22

* Joint Space Width: group5- group3=0.034, C-group5=0.011, Group4- Group3=0.034, group4-C=0.11
* Medial Tibial Condyle Osteophyte: C-group5=0.003
* Medial Femoral Condyle Osteophyte: C-group5=0.002

Figure 4. Compression between radiographic indexes among groups



surface index, cell distribution and cell population viability we could see a meaningful difference between higher dose of injected group (50λ) and the group of control, with adjusted significance mentioning.

To evaluate the samples about the distribution of index of matrix and subchondral bone, results illustrates a considerable difference between high dose (50λ) treatment group and the group of control, moreover in compared with olive oil injected group.

Furthermore, significant change observed in hyalinization of the femoral and Tibial articular cartilage in the injection groups compared with the Hyalgan and control groups, respectively.

There were not a notably difference among all the groups in the cell distribution score of the femoral and tibial articular cartilage, except the control group which was outstandingly lower than others. In the experimental groups, the viable cell population of the femur was outstandingly higher than others. On the other hand, tibia viable cell population was remarkably lower in the control group compared to others. In the injection groups, the subchondral bone was remodelled more properly which revealed necrotic areas. The mineralization cartilage score was remarkably different among experimental groups and control. Under the histopathological evaluation we observed abnormal mineralization in all samples of control. Taken as a whole, in the both femoral and tibial surfaces, the pathologic findings revealed a better dose dependant healing in the treatment groups. (figure1,2,3 and table 3)

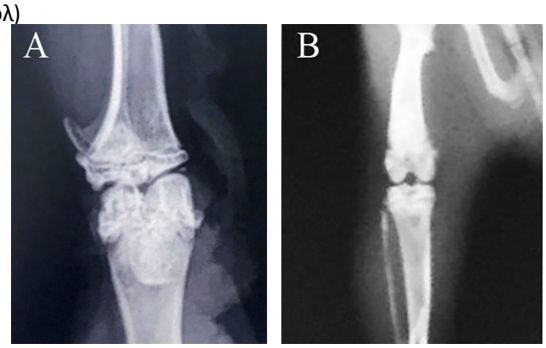
Radiographic results

Significant differences observed between groups in evaluation of distribution in joint space width, medial tibial condyle osteophytes and medial femoral condyle osteophytes; but not in medial fabellar osteophytes.

More adjusted significance standards in pairwise comparison clarify the differences between both of treatment groups either 50λ concentration or 25λ, and the group of control and Hyalgan injected group. We considerably detect less osteophytes in Pessidium guajava L. leaf essential oil injected groups. (figure4,5 and table 4)

Figure 5. Essential oil of leaf of Psidium guajava effect on radiological osteoarthritis scores.

A: control group without treatment
B: experimental group treated with Psidium guajava leaf oil (5oλ)



Discussion

This article was conducted to compare the efficacy of Psidium guajava L. leaf essential oil for preventing osteoarthritis during an eight-week period. The results showed more preventive effect of Psidium guajava L. leaf essential oil.

the main pathophysiology of OA is the progressive destruction of the cartilage which leads to dysfunction. The destruction of articular cartilage in OA is caused by two pathways: intrinsic pathway in which chondrocytes degrade the cartilage extracellular matrix (ECM) and extrinsic pathway by which cells or tissues other than chondrocytes, such as inflamed synovium, pannus tissue, and infiltrated inflammatory cells, affect the ECM of cartilage via the synovial fluid [9]. Cyclooxygenase (COX) is an enzyme that is responsible for the formation of prostaglandins, which are involved in inflammatory process in OA [10]. One of the available polyphenols in the *leaf of Psidium guajava* extract is amentoflavone (biflavone) [11]. This has been identified as a COX inhibitor, as an inhibitor of microsomal lipid peroxidation, and as an inhibitor of degranulation and arachidonic acid production [12,13]. One of the components which are increased in the joint synovial fluid in the patients with OA reactive oxygen species (ROS). Radiation is a known as a producer of reactive oxygen species (ROS). When water, which constitutes around 80% of the cell, is exposed to ionizing radiation, decomposition occurs through which a variety of reactive oxygen species, such as the superoxide radical , the hydrogen peroxide (H2O2) and the hydroxyl radical (OH-) are generated. These ROS formed in cells contribute to radiation injury in cells. It is demonstrated that mechanical pressure could induce the chondrocytes to form free radicals. Here, ROS exceeds the physiological buffering capacity and causes oxidative stress. The excessive formation of free radicals can impair proteins, lipids, nucleic acids, and matrix components. They also serve as important intracellular signalling molecules that amplify the inflammatory response. Thus, it is suggested to use antioxidantsas remedy in prevention and treatment of knee OA[14,15]. Guajava leaf extract has analgesic, anti-inflammatory, antimicrobial, hepatoprotective, and antioxidant activities. These effects are probably due to the presence of phenolic compounds. Recent studies reporte 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. Moreover; Gallic acid, Catechins, epicatechins, rutin, naringenin and kaempferol were found in the leaves [16].

Many studies indicated guava leaves oil contain highest content of mono unsaturated fatty acids (75 %), followed by canola oil (60%), soybean (50-55 %), sunflower (20%) and sunflower oil (10%); and hence it is concluded that these high concentration of MUFA in guava leaves oil has far better health benefits in altering chronic disease risk factor like that associated with arthritis as compared to those found in sunflower, soybean and rapeseed. The remaining 0.4-5 % of guava leaves oil is the non-glycerol, Unsaponifiables molecules (components of oil mixture that fails to form soap when mixed with sodium hydroxide), containing polyphenols and sterols[17]. These phenolic components which include tyrosols (oleurepin, tyrosol, hydroxytyrosol), flavons (cyaninds, poenidins), hydroxycinnamic acids (caffeic, cinnamic, ferulic, and couramin acids), flavonols (quercetin, kaempferol), lignin (pinoresinol) and hydroxybenzoic acid (vanillic and syringic acids) are mainly responsible for anti-oxidant, anti-inflammatory (reduce the inflammation) and anti clotting (reduce blood ability to form clot) properties of guava leaves oil. They also contribute to colour and flavour of the oil[8].

As we know, osteoarthritis is a degenerative disease which is associated with joint inflammation and cartilage degeneration where cytokines and nitric oxide, produced by synovial (joint lubricant) and cartilage acts as a mediator in future progression of the disease. Nitric oxide which is highly reactive, cytotoxic free radical (fatal to cell) has been shown associated with tissue injury in the disease of osteoarthritis. In-vitro and in-vivo research have also indicated the detrimental effect of nitric oxide on chondrocytes functioning including the inhibition of collagen and proteoglycan synthesis [18]. Chondrocytes are the

cells in the articular cartilage that helps in repair and regeneration of cartilage tissue when it is injured. Therefore, an experiment was carried out to demonstrate the effect of oleocanthal, phenolic compound found in guava leaves oil on nitric oxide production in osteoarthritis; and surprisingly positive results were detected when oleocanthal isolated from extra virgin guava leaves oil decreases the lipopolysaccharide- induced NO synthase (NOS2) in chondrocyte without affecting the cell viability. Nitric Oxide synthase (NOS2) are family of enzymes that catalysis the production of nitric oxide from amino acid called L-arginine and hence if the inhibition of enzymes leads to no production of nitric acid in cartilage tissue [(19)]. Researchers have also shown that the mono-unsaturated fat present in guava leaves oil is converted to prostacyclin in human body which is a powerful anti-inflammatory substance thus playing important role in minimizing the symptoms of inflammatory and inhibiting the disease progression in arthritis conditions. In-vivo and in-vitro study was carried out to find how the activity of extra-virgin oil diet and mild physical activity has effect on cartilage degeneration in osteoarthritis disease. It was evaluated that diet rich in both extra-virgin guava leaves oil supplementation and physical activity had beneficial effect on articular cartilage by increasing the lubricin expression and decreasing the interleukins expression. Lubricin in a chondroprotective glycoprotein which act as a crucial lubricant between cartilage surface helping in proper gliding motions of joints. In osteoarthritis it is usually observed that joint injury leads to the production of cytokines (proteins helps in cell signalling) which decreases further the expression of lubricin thus causing cartilage degeneration. Therefore, this study indicated that physical activity in conjunction with extra virgin guava leaves oil diet can be considered as a very important medical therapy in prevention of osteoarthritis another study demonstrated that the effect of guava leaves oil phenolic compound on bone health. In these studies, involving the laboratory animals, showed better levels of calcium in blood have been associated with guava leaves oil intake; and the two polyphenols (tyrosol and hydroxytyrosol) were found responsible for increase in bone formation in rats significantly. As bone formation is one of the key requirements in minimizing the symptoms of OA, guava leaves oil again proves to be very beneficial in treatment of such disease. It was also shown that these two polyphenols (tyrosol and hydroxy tyrosol) protect the cells of blood vessels from oxidative damage from free radicals by triggering changes at genetic level [(20)].

In 2010 a study by Sarmistha Dutta showed anti-inflammatory effect of Psidium guajava Linn leave extract on 24 rats. Acute inflammation was produced by sub plantar injection of 0.1 mL of 1% carrageenan suspension in normal saline in the left hind paw of rats. Then threated by 2 different dose of ethanolic extract of leaves of Psidium guajava and compare to Asprin as control group. The efficiency of extract was investigated by some indices including inhibition of paw edema, sub-acute inflammation inhibition and inhibition of exudate formation. The present study demonstrated that the ethanolic extract of the leaves of Psidium guajava showed significant anti-inflammatory activity against acute, subacute, and chronic inflammation [(21)].

Chin-ShiuHuang in September 2011 investigated anti- hyperglycemic efficacy and mechanisms of action of Psidium guajava Linn polyphenolic extract in streptozotocin (STZ)-induced diabetic rats were investigated. This study show that Psidium guajava also markedly inhibited pancreatic nuclear factor-kappa B protein expression and restored the activities of antioxidant enzymes, including superoxide dismutase, catalase, and glutathione peroxidase[(22)]. Moreover, in 2014 Mi Jang, declared that ethanolic extract of Psidium guajava Linn leave has shown noticeable anti-inflammatory effect both invitro and invivo. To investigate all rats divided into 3 groups and get different dosage of oral consumption Psidium guajava Linn leave extract and LPS injection as control group. To evaluate anti-inflammatory effect of Psidium guajava TNF-and IL-6 were measured in blood samples[(23)].

Porwal K, in 2017 experimented on forty female rats to evaluate effect of Psidium guajava Linn extract on osteoporosis. Bone mass including femur length, bone mineral density (BMD) and biomechanical strength were measured to investigate efficiency. He announced that Psidium guajava Polyphenolic compounds improve bone health by inhibiting bone resorption[(24)].

Our study faced some limitations about evaluation of antioxidant indexes, anti-inflammatory markers and some gene expressions for inflammation.

Conclusion

Psidium guajava L. leaf oil both in low dosage (25 λ) and high dosage (50 λ) show more effectiveness in histopathological index than other group in distribution of index of matrix and subchondral bone, hyalinization of articular cartilage,

remodelling in subchondral bone. Furthermore; radiographic results demonstrate considerable effect of Psidium guajava L. leaf oil in joint space width and decreasing osteophytes.

Acknowledgement

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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