Evaluation of the Effect of Hydroalcoholic Extract of *Psidium Guajava* Linn. (Myrtaceae) Leaf with Synovium-derived Stem Cells and Platelet Rich Plasma (PRP) on Induced Osteoarthritis of the Knee in Male Rats

Abstract

**Background:** The available synthetic drugs for treatment of Osteoarthritis (OA), a degenerative arthritis, have serious side effects and low efficacy. Therefore, using herbal medicines, with mesenchymal stem cells, as an important component of alternative and complementary medicine, have an effective role in the treatment of diseases. *Psidium guajava* leaves are used as antioxidant, anti-inflammatory and antinociceptive/analgesic drugs. The present study aimed to evaluate the effect of injection of the hydroalcoholic extract of *Psidium Guajava* leaf with stem cells into the articular cartilage of rats’ knees.

**Methods:** Ninety male rats were chosen and collagenase type II was prepared for an intra-articular knee injection, for induction of OA. Then, the rats were divided into nine treatment groups (10 rats in each group): (a) Sham, (b) Control, (c) Hyaluronic Acid intra-articular injection, (d) Stem Cells intra-articular injection, (e) PRP injection, (f) Hydroalcoholic extract injection, (g) Hydroalcoholic extract injection (PGL), (h) PGL with PRP injection, (i) PGL with stem cells injection, and (j) PGL with stem cells and PRP injection. After 5 months, the results were assessed by a radiologist and histopathologist.

**Results:** Radiological and histopathological findings showed higher improvement of the articular cartilage in groups with PGL, stem cells and PRP groups, compared with the control group (P<0.05).

**Conclusions:** *Psidium guajava* leaves, with polyphenol compounds, can be suggested as an effective complementary and alternative treatment for knee OA.

**Keywords:** Knee Osteoarthritis, *Psidium guajava*, Platelet rich plasma, Stem Cell, Rat

**Introduction**

Osteoarthritis (OA) is the most common adult joint disease in the whole world. About 25 to 40 million people in the United States suffer from this disease\(^1\). OA is a common degenerative disorder of the joint cartilage, associated with hypertrophic bone changes\(^2\,3\). In addition to articular cartilage, changes occur in the synovial fluid, and the beneath bone (subchondral), articular capsule, and other joint structures\(^5\,6\). OA develops first in weight-bearing joints, such as knees and pelvis, which include cartilage degeneration and changes in the subchondral bone. There is currently no clinically available drug to definitely prevent the disease progression. The current treatment includes controlling pain and inflammation with various analgesic and anti-inflammatory drugs or herbal medicines\(^7\,8\). Mesenchymal Stem Cells (MSCs) have high reproductive power, capable of differentiating into mesenchymal and even non-mesenchymal derived categories\(^9\,10\).

The results of some studies indicate that the reproductive power of synovial tissue-derived mesenchymal stem cells is 10 times higher than that of mesenchymal stem cells derived from bone marrow, periosteum, adipose tissue and muscle. In addition, in these studies, it has been proven that in terms of differentiation power into cartilage and adipose tissue, this cell category is stronger than all of its similar types\(^11\).
With regard to the above, it seems that these cells (synovial-derived mesenchymal stem cells) have a special position, used in cell therapy protocols and tissue engineering. PRP is a relatively new and fundamental cell therapy in medical science. PRP treatment, a rich environment containing high concentrations of various growth factors, can be used as a solution to stimulate these cells to replicate and repair damaged joint and bone tissues\(^{12, 13}\). Regarding the widespread acceptance of PRP in improving sports injuries, researchers are seeking to use PRP to repair ligament and tendon injuries, knee osteoarthritis, knee cartilage erosion, chronic elbow tendonitis, and muscle rupture\(^{14}\).

The use of herbal remedies was common since a long time ago in ancient civilizations, and today herbal medicine is common in many parts of the world and it has attracted particular attention. Until now, plants such as avocado, red pepper, ginger, devil’s claws, white leaves, nettle and bush trees have been used to treat OA\(^ {15}\). Guava plant extracts and metabolites, especially the leaf and fruit, have useful pharmacologic activity\(^ {16}\). Guava leaf extract contains tannins, phenolic compounds, flavonoids, glycolic acid, triterpenes, guavojavirine, quercetin, alkaloids and saponin\(^ {16, 17}\).

Polyphenolic and triterpenoid compounds in the ethanolic extract of the leaf can have anti-inflammatory effects. Considering that tumor necrosis factor and interleukin-1 are important cytokines for inflammation and osteoarthritis that inhibit the synthesis of proteoglycan and type II collagen, and cytokines are the most important causes of granuloma production, therefore, flavonoids in Guava leaf can reduce granulomas and show anti-inflammatory properties by inhibiting cytokines\(^ {18}\). Flavonoids and quercetin in the leaf also have spasmolytic properties, antimicrobial and anti-inflammatory effects\(^ {19, 20}\). Due to the fact that intra-articular injection of Guava extract is used for the first time, the present study aimed to determine the restorative effect of hydroalcoholic extract of Guava leaf, stem cells and PRP via intra-articular injection on cartilage formation in rat model osteoarthritis, evaluated by histopathology and radiology.

**Methods**

**Induction of osteoarthritis**

General anesthesia was induced in rats by 90 mg/kg ketamine (GmbH, Germany) and 8 mg/kg zylazine (Alfasan, Netherlands). The left knee of the rats was shaved and prepared for injection. In their left knee, 4 mg collagenase type II (clostridium histolithicum) dissolved in sterile PBS (Phosphate Buffered Saline), was intra-articularly injected after passage from a 0.22 μm filter. The second injection of collagenase was repeated with the same dose after 3 days\(^ {21, 22}\). After injections, the rats were kept motionless and kept in standard condition. After three months, the damage was examined by radiography.

**Preparation of hydroalcoholic extract of Guava**

Guava leaf was collected from Chabahar province in Sistan and Baluchestan province and identified by a botanical expert of Pharmacy Faculty (SFPH-771). First, the leaves were cleaned, dried in the shade, powdered by electric mill after drying, and used directly for extraction. Extraction was carried out using ethanol %50. The extracts were concentrated by rotary desiccator at 40°C, and then the concentrated extracts were transferred to Freeze-drier and finally the leaf extract was used as a lyophilized powder\(^ {23}\).

**Assessing the antioxidant capacity**

The antioxidant capacity was assessed by radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) \(^ {24}\). Different concentrations were made to determine 50% of inhibition. Absorption was read at 517 nm using by a spectrophotometer device. Finally, the inhibition percentage and IC50 were calculated\(^ {25, 26}\).
Folin-Ciocalteu method
This method determines the total phenolic compounds. Folin-Ciocalteo is an oxidizing compound. Phenols and polyphenols restore this composition by transferring their single electron in an alkaline medium, and create a blue color in the environment (27).

Preparing synovial-derived stem cells
The synovial membrane was prepared from rats by aseptic surgery and transferred to stem cells laboratory; tissue homogenization was performed under range hood, and then treated for digestion with 1 mg/ml collagenase type D solution in α-MEM medium at 37°C for 2 hours. The digested tissue was then passed through a nylon filter with a 0.22 μm membrane and the remaining tissue was discarded. After this step, the resulting cells were washed with PBS, the suspension was centrifuged (for 4 minutes at 1300 rpm), and the precipitated cells were washed with PBS. At this step, the nucleus cells were incubated in cell culture flasks containing DMEM, Pen/Strep 1%, and FBS 10% for 48 hours at 37°C with 95% humidity and 5% CO2 (28). Mesenchymal stem cells, extracted from synovial tissue, were used for injection after 4 passages. After culture and passage of isolated cells, to determine the cells’ identity, fluocytometry was used by specific antibodies of CD73 and CD105 (Fig. 1).

Investigating the cytotoxicity of Guava leaf extract
To evaluate cell growth inhibition, MTT assay (Methy Thiazol Tetrazolium) was used. In this method, 100 μl culture medium containing 3000 cells was placed in 96-well plate. After incubation of plates, appropriate concentrations were added from the extract to the plate wells and the cells were placed in the incubator. Then, 20 μl MTT was added to each well and incubated for 4 hours in the dark. Ultimately, a light absorption of 570 nm was read by the ELISA Technology Inc., Stat Fax 2100, USA.

Platelet-rich plasma (PRP) plasma
Venous blood was taken from the heart by aseptic technique and centrifuged after transferring to test tubes, and then the plasma was separated (5810 R; Eppendorf AG, Hamburg, Germany) and re-centrifuged. The top two-third, which consisted of platelet-poor plasma (PPP) was removed and the remaining layer (the final one-third) was considered as PRP (29). PRP passed through a filter with a 0.22μ membrane.

Platelet count
The number of platelets in the whole blood and the isolated PRP fraction was assessed. Therefore, to evaluate platelet count, the Sysmex XT-1600i system (30).

Animals
In this study, 90 adult male Sprague Dawley rats with weight range of 200±20 and age range of 10-12 weeks were used. This randomized animal trial was performed in the Center of Comparative and Experimental Medicine, Shiraz University of Medical Sciences and kept at 22±2 °C, at 12 hours light/darkness and a relative humidity of 55 ± 5% with standard water and food supply, freely provided.

Grouping
Rats were grouped randomly into 9 groups of 10.
1. The sham test group: four saline injections (50 Landa) during treatment course, and no treatments with the only stress of knee injection
2. Negative control group: received no treatment after induction of osteoarthritis
3. Positive control group (Hyalgan): After induction of osteoarthritis during the study, 50 Landa Hyalgan with a concentration of 1% (Fidia Italy) was injected intra-articular four times.
4. Test group number 1 (Leaf): After induction of osteoarthritis during the study, the hydroalcoholic extract of Guava leaf with a dose of 500 mg/kg was injected intra-articular four times
5. Test group number 2 (leave + PRP + Stem Cells): After induction of osteoarthritis during the study, the hydroalcoholic extract of Guava leaf with a dose of 500 mg/kg was injected intra-articular and PRP with a dose of 50 Landa four times and stem cells with a concentration of 10^6 cells was injected intra-articularly once.
6. Test group number 3 (Leave + Stem Cells): After induction of osteoarthritis during the study, the hydroalcoholic extract of Guava leaf with a dose of 500 mg/kg was injected intra-articular four times and stem cells with a concentration of 10^6 cells was injected intra-articularly once.
7. Test group number 4 (leave + PRP): After induction of osteoarthritis during the study, the hydroalcoholic extract of Guava leaf with a dose of 500 mg/kg was injected intra-articular and PRP with a dose of 50 Landa four times.
8. Test group number 5 (Stem Cells): After induction of osteoarthritis during the study, stem cells with a concentration of 10^6 cells was injected intra-articularly once.
9. Test group number 6 (PRP): After induction of osteoarthritis during the study, PRP was injected intra-articular with a dose of 50 Landa four times.

Preparation of radiological images
Three months after injection of collagenase type II and induction of osteoarthritis and also at the end of the study period, the animals were subjected to radiography from the left knee and detailed examination by the radiologist at Radiology Department of Faghihi hospital, in standard conditions. At the time of imaging, the radiographic device was adjusted to 45 kV and 20 mA/s and evaluated according to table 1.

Histopathological evaluation
For histopathologic evaluation of the recovery process, the treatment and control groups of rats were sacrificed ethically after taking radiographs. After sacrificing, the left knee was cut and the muscles and ligaments were pulled off to expose the knee joint; then, femoral condyles and the upper segment were separated and placed in a 10% buffered formalin solution. Samples were stored in this solution for one week. After the stabilization step, the samples were decalcified in 4% hydrochloridric and 5% formic acid, and then paraffin blocks were prepared from them. Then the tissue sections were stained with hematoxylin and eosin (H&E) and examined under optical microscope. The International Cartilage Repair Society (ICRS) grading system was used to evaluate and grade the tissue sections (Table 2) (32). The histopathologic evaluation was done by the pathologist.
Statistical analysis
The data were analyzed using One Way ANOVA method. Also Kruskal-Wallis and Mann-Whitney tests were used to compare the difference between groups. These tests were performed using SPSS 18 software and the mean differences were considered significant at the level of p < 0.05.

Results
Histopathologic findings
Histopathologic study of the knee articular cartilage (Figure 2) showed a significant difference between the treatment groups, compared to the control and PRP group had a regular cartilage surface; the articular cartilage surface of PRP group than stem cells group, Guava extract with stem cells group, Guava extract with PRP group, Guava extract with stem cells and PRP group, and Guava extract alone were irregular and there was significant difference (p <0.05). Also, stem cells group did not differ significantly in terms of articular cartilage surface than Guava extract with stem cells group, Guava extract with stem cells and PRP group, and the group receiving Guava alone (P > 0.05).

The knee cartilage matrix in Hyalgan group, PRP group, and the control group was significantly lower than other treatment groups (p <0.05). Also, the knee cartilage matrix in stem cells group did not differ significantly from the groups receiving Guava with stem cells, Guava extract with stem cells and PRP, and the group receiving Guava alone (P > 0.05). The mean cell distribution score in the group receiving Hyalgan, PRP and control group significantly decreased (p <0.05). This mean significantly decreased in PRP group compared to stem cells groups, Guava extract with stem cells and PRP group, Guava extract

<table>
<thead>
<tr>
<th>Feature Score</th>
<th>Smooth/continuous</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface</td>
<td>Irregularities discontinuous</td>
<td>0</td>
</tr>
<tr>
<td>Matrix</td>
<td>Hyaline</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Mixture: hyaline/fibrocartilage</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Fibrocartilage</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Fibrous tissue</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Columnar</td>
<td>2</td>
</tr>
<tr>
<td>Subchondral bone</td>
<td>Partially viable</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>&lt; 10 % viable</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>3</td>
</tr>
<tr>
<td>Cartilage mineralization (calcified cartilage)</td>
<td>Normal</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Abnormal/inappropriate location</td>
<td>0</td>
</tr>
<tr>
<td>Type I collagen staining of the matrix</td>
<td>Normal or nearly normal</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Slight</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Abundant</td>
<td>0</td>
</tr>
<tr>
<td>Type II collagen staining of the matrix</td>
<td>Normal or nearly normal</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Slight</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>0</td>
</tr>
</tbody>
</table>

*ICRS, International Cartilage Repair Society
with stem cells group, and the group receiving Guava extract alone (p <0.05).

Figure 2. Histopathologic observations in groups: (A) control group, (B) sham group, (C) the treatment group receiving hydroalcoholic extract of Guava leaf, PRP, and stem cells (×10 magnification)

Also, this mean significantly increased in the group receiving stem cells alone, compared with the group receiving Guava extract with PRP (p < 0.05). The cell population viability was significantly higher in stem cells groups, Guava extracts with stem cells and PRP group, Guava extract with stem cells, and the group receiving Guava extract alone, than the control group (p <0.05). There was no significant difference between other groups.

Subchondral bone in Hyalgan group, PRP group and control group showed a clear reduction in regeneration compared to other treatment groups (p <0.05). Also, PRP group showed less Subchondral bone regeneration compared to Guava extract group, Guava extract with PRP group, Guava extract with PRP and stem cells group, and Guava extract with stem cells group (p<0.05). There was no significant difference between the group receiving stem cells and other groups receiving Guava extract, Guava extract with PRP, Guava extract with PRP and stem cells, and Guava extract with stem cells. The mean cartilage mineralization was significantly different among the groups, compared to the control group (p<0.05). This difference was not significant between PRP group and stem cells group, compared to other groups. Finally, the pathologic findings in the group receiving Guava extract with PRP and stem cells, Guava extract with stem cells, and Guava extract group were significantly different from that of the control group (p<0.05) (Chart 1).

Radiological findings

Radiologic findings in the present study indicated significant articular injury in rats with induced osteoarthritis, presenting with injury in the medial part of tibial and femoral condyle, articular space, and medial fabella (sosamoid bones behind the knee joint) (Chart 2). In this study, 3 months after injection of collagenase enzyme, osteoarthritis formation was assessed, which showed articular surface damage, decreased articular space, and osteophyte formation. At the end of the treatment period, according to chart 1, osteophytes were formed in medial tibial condyle, medial fabella condyle, and medial femoral condyle in different groups; the amount of osteophytes formed in the control group was higher than the other groups, indicating development of acute osteoarthritis during the study. Also, the mean osteophytes formation had a significant difference and was lower in the groups receiving Guava extract with PRP and stem cells, Guava extract with stem cells group, and Guava extract group than the control group (p<0.05).

Also, the joint space width had a significant difference with the control group and increased in the groups receiving Guava extract with PRP and stem cells, Guava extract with stem cells, and Guava extract group, compared with the control group (P<0.05). And the articular space decreased in PRP group compared to stem cells group, Guava extract with stem cells group, Guava extract group, and Guava extract with PRP and stem cells group.
Chart 1. The results of histopathologic assessments. This chart shows significant difference in the groups receiving Guava leaf with PRP and stem cells, Guava leaf, and Guava leaf and stem cells with the control group.
Chart 2. The results of radiologic assessments. This chart shows significant difference in the groups receiving Guava leaf with PRP and stem cells, Guava leaf, stem cells, and Guava leaf and stem cells with the control group.
cell group with a significant difference (p<0.05). And the joint space in stem cells group increased, compared to Guava extract with PRP group and control group (p<0.05). Stem cells group had a significant difference considering global OA score with control group, Hyalgan group, and PRP group (p<0.05).

**Discussion**

Rheumatologic diseases such as osteoarthritis and rheumatoid arthritis are very common. The synthetic drugs used to treat these diseases have low efficacy and significant adverse effects. Several methods are used to supplement and replace synthetic drugs for treatment of these diseases. One of these methods is the use of herbal medicines. Guava is a medicinal plant and an important food product in tropical and subtropical countries. Studies have shown that polyphenolic compounds, flavonoids, tannins, alagic acid and triterpenoid in the ethanolic extract of the leaf can have anti-inflammatory effects (34). The leaf extract of this plant is also capable of inhibiting tumor necrosis factor and interleukin 1, which are important cytokines for inflammation and osteoarthritis and inhibit the synthesis of proteoglycan and type II collagen (35). Leaves are rich in flavonoids, especially quercetin, and most of Guava’s therapeutic effect is attributed to flavonoids (36).

The antioxidant activity of Guava leaf extract shows that the hydroalcoholic extract shows outstanding activity in capturing free radicals. The results showed that the 50% inhibitory concentration (IC50) of Guava leaf’s extract was 172±3.592 ng/ml. The total amount of phenolic compounds in this extract was 252.67±2.71 mg GAE/g of dry extract. It seems that Psidium guajava plays an important role in preventing cellular damage against ROS.

The results of radiographic evaluation in this study, by Kruskal-Wallis statistical tests, showed that the mean number of osteophytes, the articular space, and degree of osteoarthritis had a significant difference at p<0.05 in the groups receiving stem cells with hydroalcoholic extract of Guava leaf, Guava leaf with stem cells and PRP, and Guava leaf extract with stem cells, compared to other therapeutic and control groups.

Histopathologic findings were significantly higher in the group receiving Guava extract with PRP and stem cells, Guava extract with stem cells, stem cells group, and Guava extract group than the control group and was different in PRP and Hyalgan group with significant increase in cartilage repair and regeneration (p<0.05).

In 2001, Altman performed a study on 61 patients with moderate to severe acute osteoarthritis and the radiologic evaluations showed that the use of Zinger extract twice daily decreased the signs of knee osteoarthritis in these patients over a period of 6 weeks (37). This study is consistent with the results of radiographic evaluation in this study in the groups receiving Guava extract, Guava extract with stem cells, and Guava extract with stem cells and PRP.

Kwon and colleagues studied the effect of PRP injection for 4 weeks and macroscopic and histopathological assessments showed cartilage repair and regeneration in the knee of rabbits with osteoarthrosis after 9 weeks of collagenase injection. However, this study did not match the results of histopathologic evaluations performed in our research on PRP group (38). This difference could be due to the different PRP volume, different stage of osteoarthritis, and the type of animal that differed from our study.

Horie and colleagues reported the result of intra-articular injection of Luc/LacZ + synovium-MSC into the knee of rats and observed the effects of meniscal regeneration and type II collagen production in the knee of rats with osteoarthrosis after 12 weeks (39). Therefore, it is compatible with the results of our study on treatment groups receiving stem cells, Guava leaf extract with stem cells, and
Guava leaf extract with stem cells and PRP with statistically significant changes (p<0.05). The high reproductive capacity of MSCs synovium appears to play an important role in improving the meniscus. The hydroalcoholic extract of Guava leaf, due to the presence of triterpenes, can provide favorable conditions for growth and differentiation of stem cells to chondrocytes and ultimately repair cartilage.

Iwata et al. reported that injection of hyaluronate in patients with knee osteoarthritis and shoulder periarthritis is a safe and effective treatment. But in our study, hyalinization of knee cartilage in the Hyalgan group was significantly lower than the sham group (p<0.05). This study does not match the results of our study on the Hyalgan group. Although Hyalgan is a useful drug in reducing symptoms of osteoarthritis, but is not the final treatment, and we did not find a positive and beneficial outcome because we used Hyalgan in acute phase of the disease. Perhaps, we would observe beneficiary results, if we used it in the early phase of osteoarthritis or as prevention of osteoarthritis.

In this study, in osteoarthritis induced in rat’s knee, in histopathologic and radiological evaluation, Guava leaf extract, due to phenolic compounds via inhibiting COX enzyme and lipooxygenase, could reduce inflammation in the joint and was able to create favorable conditions for growth and differentiation of stem cells injected into the articular space for chondrocytes. Therefore, in the presence of Guava leaf extract, these cells could repair and treat damaged cartilage tissue, including resolution of knee cartilage osteophyte, restoration of articular space, and reduction osteoarthritis severity in knee joint. Tissue restorations are likely due to the presence of triterpenes in the hydroalcoholic extract of Guava leaf, which reduces inflammation in the cartilage and can provide favorable conditions for growth and differentiation of stem cells to chondrocytes and ultimately to repair cartilage. Therefore, based on previous studies and anti-inflammatory results derived from this study, it can be suggested that the prophylactic effects of Guava leaf extract on osteoarthritis may be due to flavonoids and triterpenes present in this plant and because of these strong antioxidants, it can prevent damage due to free radicals, involved in the process of OA.

**Conclusion**

This study showed that Guava leaf extract can be considered as a complementary and alternative treatment for osteoarthritis. Of course, more studies are required to confirm these results, investigating other animal models and sterological tests. Further studies are needed to understand the metabolism, mechanism of action, effectiveness, safety, and the effective components of hydroalcoholic extract of Guava. Therefore, accurate phytochemical analysis of the hydroalcoholic extract of Psidium Guajava is essential for investigating the mechanism of articular cartilage repair and regeneration. Therefore, it is suggested that further considerations be given to the procurement of pharmaceutical products, with using Guava leaf extract as a complementary, alternative, and useful treatment for patients with osteoarthritis.

**Acknowledgments**

The staff of stem cells Laboratory and the Laboratory Animal Center of Shiraz University of Medical Sciences, who helped us in this study, are sincerely appreciated.
References


