The effect of *Psidium guajava* hydroalcoholic extract on reducing the expression of Resistin and TNF-α genes in articular chondrocytes of patients with knee osteoarthritis

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**ARTICLE INFO**

**Original paper**

Article history:
Received: August 24, 2021
Accepted: December 08, 2021
Published: December 30, 2021

Keywords:
Articular chondrocytes, Hydroalcoholic extract, knee osteoarthritis, *Psidium guajava*

**ABSTRACT**

Knee osteoarthritis is the most common joint disease and the most important cause of disability in elderly patients. Due to the side effects of nonsteroidal anti-inflammatory drugs (NSAIDs) in the treatment of osteoarthritis, the use of herbs is recommended as an alternative treatment. The guava fruit can be considered as a natural medicine due to its antioxidant and anti-inflammatory properties. In this study, the effect of *Psidium guajava* hydroalcoholic extract was considered on reducing the expression of the resistin gene and TNF-α gene in articular chondrocytes of patients with knee osteoarthritis. For this purpose, cartilage chondrocytes of 10 Chinese patients with knee osteoarthritis, who were indicated for arthroplasty, were used for evaluating the effect of guava fruit on the expression of these genes. Articular chondrocytes were cultured under appropriate conditions and their viability was assessed by MTT assay. To increase the level of cytokines, cells were treated with lipopolysaccharide (LPS). They were kept in an incubator with 90% humidity at 37°C for the next steps. Then, the expression of the resistin gene and TNF-α gene was investigated by RNA isolation and cDNA preparation. The results showed that the expression of the resistin gene and TNF-α gene were reduced to 56.59% and 51.86%, respectively, by hydroalcoholic extract of guava fruit. Therefore, according to the results of this study and previous researches, the hydroalcoholic extract of guava fruit (*Psidium guajava*) can be considered as an effective complementary and alternative treatment for osteoarthritis of the knee.

**Introduction**

Knee osteoarthritis is the most common human joint disease in the world and its prevalence is reported to be about 16% in the age group of 15-82 years (1). Osteoarthritis of the knee is characterized by intra-articular cartilage erosion and stimulation of ossification in the subcutaneous bone (2). It is known that this disease is not only a degenerative disease but also some degree of synovium inflammation in the joints (3). It has been shown that during the chondrocyte disease process, Synoviocyte are activated and mononuclear cells (monocytes and macrophages) accumulate in the joint. These activated cells produce inflammatory cytokines (including TNF-α, iIL-1β, and resistin) and they increase the production of NO in PGE2 by expressing the COX-2 and iNOS enzymes (4). These proinflammatory substances play a role in increasing cartilage destruction by known mechanisms such as stimulating the synthesis of metalloproteinases and inhibiting the production of tissue inhibitors of metalloproteinases, stimulating the apoptosis of chondrocytes, and inhibiting the synthesis of proteoglycans (5).

As mentioned, resistin is one of these pro-inflammatory factors secreted by adipose tissue that is involved in insulin resistance (6). This protein gets its name from insulin resistance in mice and is derived from the words “resistance” and “insulin”, which means insulin resistance agent (7). Resistin is a dimer protein with 114 amino acids and is rich in cysteine. The compound, with a molecular weight of 12.5 kDa, was first isolated from the abdominal fat of obese mice (8). Resistin is found in adipocytes and various cells, including peripheral blood mononuclear cells, macrophages, and bone marrow cells, and is involved in the development of inflammatory conditions in humans. However, in humans, only small amounts of resistin are secreted from adipose tissue (9). But it has
been found that with obesity and increased visceral fat, more resistin is expressed in cells in macrophages, liver, spleen, and lungs. Due to the widespread expression of the resistin gene in monocytes and macrophages and the role of these cells in promoting inflammation, the involvement of resistin in the development of inflammation of adipose and cartilage tissue and the recall of more macrophages to the area is important (10).

Every year, millions of people with osteoarthritis are forced to take painkillers, especially nonsteroidal anti-inflammatory drugs (ibuprofen, diclofenac sodium and naproxen), and steroids to relieve pain. Nonsteroidal anti-inflammatory drugs (NSAIDs) have gastrointestinal and renal side effects, and steroids have side effects, including diabetes, high blood pressure, and osteoporosis (11). The high prevalence of osteoarthritis, especially knee osteoarthritis, the high volume of synthetic drugs and the high rate of side effects of these drugs lead to the imposition of very high costs on the health budget of countries (12). Therefore, after determining the role of inflammation in the pathogenesis of osteoarthritis, in recent years, many researchers have been trying to determine the anti-inflammatory role of various types of herbs to reduce the chances of using analgesics and anti-inflammatory drugs with fewer side effects for patients (13).

Various pharmaceutical experiments in many in-vitro and in-vivo models have shown that extracts and metabolites of *Psidium guajava* leaves and fruits are useful in biological activity due to the phenolic, flavonoid, carotenoid, terpenoid and triterpene components (14). *Psidium guajava* fruit is an important food and medicinal herb in tropical and subtropical regions (15). Guava has been used to treat a variety of ailments, such as diabetes, diarrhea, high blood pressure, tooth decay, wound healing, and as a pain killer or fever control. Analgesic, anti-inflammatory, and antioxidant activities, possibly due to the essential oils and flavonoids in guava, have been shown to have analgesic, anti-inflammatory, and antioxidant effects (16).

Nutritional studies in the treatment of osteoarthritis have always been a controversial challenge (17). This study aimed to evaluate the effect of guava fruit on the improvement of knee osteoarthritis by reducing the expression of the resistin gene and TNF-α gene in articular chondrocytes.

**Materials and methods**

**Preparation of hydroalcoholic extract of Guava fruit**

Guava fruit was disinfected in sodium hypochlorite solution for two hours and then chopped into small pieces. After washing with distilled water and drying, it was pulverized. 500g of powdered samples were sterilized in 78 liters of 70% ethanol (Merck, analytical grade) for 78 hours. After filtration, the extract was dried by freeze dryer (Christ, Germany) and 21% guava extract was obtained with a light brown color. To extract the appropriate amounts of the extract for biological experiments, the extraction process was repeated three times. Finally, the hydroalcoholic extract of the fruit was exposed to 4°C.

**Preparation of articular chondrocytes**

In this study, ten Chinese patients with a diagnosis of severe osteoarthritis of the knee were selected based on clinical and radiological evidence (according to the criteria of the American Rheumatological Association) who were candidates for knee arthroplasty. At the time of the joint arthroplasty, ten samples of cartilage with osteoarthritis and ten samples of apparently healthy cartilage from the same patients were taken at the same time. Patients’ written consent was obtained before surgery and sampling. After rinsing the cartilage surface with normal saline, a 5 by 5 mm parallel piece was removed from the cartilage surface. After two washes with 1M PBS buffer (pH7.2), they were treated with Collagenase Type II enzyme for 16 hours, at 37°C and then the cells were separated by a sterile filter and placed in a special flask for subculture passage. It was transferred and kept in an incubator with 5% CO₂, 37°C and 95% humidity until 85% of the flask volume was covered by the single-cell layer.

**MTT assay**

In the MTT assay, after treating the cells for 24 hours, the supernatant was removed and the cells were washed with phosphate buffer (PBS) solution and 100 µL solutions (5 mg/mL) of MTT diluted in PBS was added to each well and the plate was incubated for 24 hours at 37°C in the dark. Then, the top solution of
each well was removed and 100µl of DMSO (Dimethyl sulfoxide) was added to each well, and it was incubated and rotated at 37°C for 15 to 20 minutes, then its light absorption was read at 570 nm and the cell survival curve was calculated to draw the cell survival percentage as:

\[
\text{(Amount of light absorption of the treated group / Amount of light absorption of the control group) } \times 100
\]

All experiments were repeated three times independently.

**Studied groups**

The cartilaginous chondrocytes in this study were evaluated in seven groups (Table 1). In group 1, in order to measure the expression level of the genes in normal conditions, it only had cartilage cells from healthy tissue. The second group included cartilage cells and guava extract for considering the effect of the extract on the expression of genes in chondrocytes of healthy tissue. The third group consisted of cartilage cells from arthritic tissue that were also stimulated by LPS, for more assurance. The fourth group, for evaluating the effect of guava fruit extract on the cells and guava extract for considering the effect of guava extract. In group 5, in order to determine whether PBS as a placebo affects the expression of the studied genes, it included osteoarthritis cartilage cells plus LPS and guava extract. In group 6, it only had cartilage cells from healthy tissue. The second group included cartilage cells from arthritic tissue that were also stimulated by LPS, for more assurance. The fourth group, for evaluating the effect of guava fruit extract on the cells and guava extract for considering the effect of guava extract. The fifth group, for evaluating the effect of guava fruit extract on the expression of genes in osteoarthritis conditions, included osteoarthritis cartilage cells plus LPS and guava extract. In group 7, in order to determine whether PBS as a placebo affects the expression of the studied genes, it included osteoarthritis cartilage cells plus LPS and PBS. In the sixth and seventh groups, dexamethasone (group 6) and naproxen as NSAID (group 7) were used to compare the effect of guava extract. In group 5, in order to determine whether PBS as a placebo affects the expression of the studied genes, it included osteoarthritis cartilage cells plus LPS and guava extract. In group 7, in order to determine whether PBS as a placebo affects the expression of the studied genes, it included osteoarthritis cartilage cells plus LPS and PBS. In the sixth and seventh groups, dexamethasone (group 6) and naproxen as NSAID (group 7) were used to compare the effect of guava extract with common drugs in the treatment of osteoarthritis.

**Treatment by LPS**

Although cartilage tissue from patients with severe knee osteoarthritis was used in this study, 20 ng/ml LPS was used to ensure the test result and increase the expression of proinflammatory cytokines. First, 6×10^6 cells are cultured in a culture medium. After 72 hours, 100 ng of LPS was added to the medium. It was then stored in a CO2 incubator for 24 hours to express the pro-inflammatory cytokines resistin and TNF-α.

**RNA extraction and cDNA synthesis**

In this study, the Jena Bioscience column kit was used to extract RNA. According to the protocol, ethanol was added to the cell solution to bind RNA to the column. The resulting compound is then transferred to a silica column, and by rotating the column in a centrifuge, excess compounds are removed from the column, leaving only the RNA attached to the column. Other residues on the surface of the column are removed by the wash buffer, and finally, pure RNA is released from the column by the wash buffer. Finally, UV-2100 Spectrophotometer was used to determine RNA concentration. In the presence of DNA contamination, the enzyme DNase is used to remove excess DNA. To convert RNA to cDNA, cDNA Synthesis Kit (Sigma-Aldrich, USA) was used according to the relevant protocol. Finally, the concentration of the generated sample was increased on cDNA by PCR.

Specific primers were used for each of the studied cytokines and the GAPDH gene was used as the housekeeping gene (Table 2). Standard PCR conditions were performed and the PCR product was used in 1.5% gel agarose with ethidium bromide.

**Table 1.** Details of the studied groups; A) Guava extract, B) LPS, C) PBS, D) Dexamethasone, E) Naproxen

<table>
<thead>
<tr>
<th>Group</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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<tr>
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<td>Not treated</td>
<td>Not treated</td>
<td>Not treated</td>
<td>Not treated</td>
</tr>
<tr>
<td>2</td>
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<td>Not treated</td>
<td>Not treated</td>
<td>Not treated</td>
<td>Not treated</td>
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<tr>
<td>3</td>
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<td>Treated</td>
<td>Not treated</td>
<td>Not treated</td>
<td>Not treated</td>
</tr>
<tr>
<td>4</td>
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<tr>
<td>5</td>
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<tr>
<td>6</td>
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<td>Treated</td>
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<td>Treated</td>
<td>Not treated</td>
</tr>
<tr>
<td>7</td>
<td>Not treated</td>
<td>Treated</td>
<td>Not treated</td>
<td>Not treated</td>
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</tbody>
</table>

**Table 2.** Primers’ sequence of GAPDH, Resistin, and TNF-α

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward 5’-GTCTCCCTCCTGACTTCAACAGCG-3’; Reverse 5’-ACACCTCCTGTAGGCAACAA-3’</td>
</tr>
<tr>
<td>Resistin</td>
<td>Forward 5’-GAGATCTATGAAAGCCTCCTGTCTCCTCCTG-3’; Reverse 5’-GGAATTTCCCTCAGGGCTGACACGA CA-3’</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward 5’-TCCTCTGTGCTGCTAGCTTTG-3’; Reverse 5’-ATGGGCTACAGGCTTTGTCACT-3’</td>
</tr>
</tbody>
</table>

**Statistical Analysis**

Data analyses were performed with SPSS vol.17 software and Student- Newman REST version 2000.
software, and they were analyzed by one-way ANOVA and Tukey test. \( P<0.05 \) was considered for significant differences between the experimental groups. The obtained values were reported as Mean ±standard error. Reaction data were analyzed by the \( 2^{ΔΔct} \) method.

**Results and discussion**

**The effect of guava fruit hydroalcoholic extract on resistin gene expression in articular chondrocytes**

The hydroalcoholic extract of guava fruit did not alter the expression of resistin in group 2 cells, but in group 3, it was associated with a 100% increase in expression (Figure 1). This expression, in group 4 with the opposite change, reduced the expression of resistin to 56.59%, in cells stimulated by LPS. It should be noted that in group 5, where a placebo was used for treatment, there was no change in the expression of the desired gene. The expression levels of resistin were significantly reduced in the sixth group, which was treated with dexamethasone, and in the seventh group, which was treated with the non-steroidal anti-inflammatory drug naproxen. A reduction of 56.59% by the hydroalcoholic extract of guava fruit was at a significant level.

This decrease was analyzed by ANOVA statistical analysis and confirmed at a significant level of 0.016 with \( P<0.05 \). Using the software, REST Vol.2009, the rate of reduction of resistin expression was remarkable in comparison with the third group, which was treated with LPS, and the sixth and seventh groups that treated with dexamethasone and naproxen plus LPS, respectively.

![Figure 1. The effect of guava fruit hydroalcoholic extract on Resistin and TNF-α genes in articular chondrocytes using RT-PCR by Student’s New-manKeuls test method (mean±1 SD, n=3)](image)

**The effect of guava fruit hydroalcoholic extract on TNF-α gene expression in articular chondrocytes**

The hydroalcoholic extract of guava fruit did not change the expression of TNF-α enzyme in group 2 cells, but in group 3, it was associated with a 100% increase in expression, and this increase in expression in group 4 was reversed (Figure 1). In other words, it reduced the expression of the TNF-α enzyme in LPS-stimulated cells to 51.86%. This change was significant. It should be noted that in group 5, where a placebo was used for treatment, there was no change in the expression of the desired gene. The expression of cytokines in the sixth group, which was treated with dexamethasone, and the seventh group, which was treated with naproxen, had a significant decrease. The reduction of 51.86% by the hydroalcoholic extract of guava fruit was significant. This decrease was analyzed by ANOVA statistical analysis and was confirmed at a significant level of 0.032 with \( P<0.05 \). The reduction rate of cytokine expression was remarkable in comparison with the third group which was treated with LPS, and the sixth and seventh groups that treated with dexamethasone and naproxen plus LPS, respectively.

The present study was performed to compare the effectiveness of guava fruit hydroalcoholic extract with dexamethasone and naproxen in the treatment of knee osteoarthritis on cartilage chondrocytes by evaluating the expression of the resistin gene and TNF-α gene. The results showed the therapeutic effect of guava fruit hydroalcoholic extract by reducing the expression of resistin and TNF-alpha genes to 56.59 and 51.86%, respectively. Therefore, the hydroalcoholic extract of this fruit can be considered as an effective complementary alternative to improve knee osteoarthritis.

New approaches are needed to increase the safety and effectiveness of treatment and the desired effect on the disease process. Some substances that are naturally present in the body may be valuable in preventing or treating osteoarthritis. Although most research is in its infancy, it is possible to use natural ingredients to heal injuries or speed up cartilage repair (18).

The high popularity of complementary and alternative drugs can be due to their advantages such
as widespread availability, no side effects or low side effects, good effectiveness and low cost compared to synthetic drugs. Because osteoarthritis is an inflammatory disease associated with joint destruction, modulatory cytokines and nitric oxide produced by chondrocytes play a key role in disease progression (18).

Guava fruit hydroalcoholic extract has analgesic, anti-inflammatory, antimicrobial, hepatic and antioxidant protective activities. Therefore, guava can be a good alternative to other drugs and methods. Some studies have reported high levels of phenolic compounds with antioxidant activity in white guava and red guava leaves compared to other species of this plant (19-22). A study also showed that guava leaf essential oil in high doses of hydroalcoholic extract (1000 mg/kg) treated and cured pathological symptoms of rats with knee osteoarthritis. Destructive articular surface leads to impaired cartilage and bone metabolism, which stimulates the extracellular gland, causing pain, restricted movement, swelling, and dysfunction in patients. These symptoms were reduced in those knee osteoarthritis rats which were treated with 1000 mg/day guava leaf hydroalcoholic extract (22). The main components of guava leaf essential oil are fatty acids containing the glycerol molecule, known as monounsaturated fatty acids (MUFAs), which contain 95 to 98% of the oil, and the other ingredients include phenols and sterols (23). One of the most important therapeutic benefits of guava oil is its high concentration of monounsaturated fatty acids (mainly oleic acid) (24). In addition, scientists have found that the main phenolic substance is responsible for the anti-inflammatory properties of pure guava oil. These newly discovered substances act as a natural anti-inflammatory agent, similar to naproxen, which is used to treat arthritis and osteoarthritis of the knee (25). These compounds, as same as naproxen and ibuprofen, have been shown to inhibit the function of cyclooxygenase-1 and cyclooxygenase-2 enzymes in the biosynthesis pathways of prostaglandins (26). Prostaglandins cause inflammation in patients with osteoarthritis, and therefore inhibition of its biosynthesis helps to reduce inflammation and pain caused by this disease (27).

On the other hand, inhibiting bone resorption is one of the main requirements in minimizing the symptoms of osteoarthritis. *Psidium guajava* has been shown to be very effective in treating this disease by blocking NF-Kβ. Many traditional uses have been confirmed by scientific research (23). Toxicological studies in rat and other animal models, as well as controlled human studies, show that both the leaves and the fruit have no side effects and are safe. For example, Diyanat et al. (28) conducted research on 60 female rat without ovaries to investigate the anti-osteoporotic effects of guava fruit hydroalcoholic extract. The hydroalcoholic extract of guava fruit increased the weight and volume of the femur, the density of femoral ash, the number of osteocytes and osteoblasts, and the trabecular volume of the bones compared to the group that had only a dose-dependent ovarian harvest. Another study by Budin et al. (29) found that guava fruit had a significant antioxidant effect in diabetic rats. This study showed that the levels of superoxide dismutase and glutathione in the experimental group were significantly higher than in the control group. Jahagirdar et al. (30) showed a high arthritic effect of the hydroalcoholic extract of guava in three different doses in the back of the rat's hind paw. Claw size, body weight, tibiotarsal joint diameter and the total number of leukocytes in the blood were measured. The results showed a significant treatment and dose-dependent improvement. Kuo and Chien (31) conducted a study designed to explore the defense mechanisms of combining guava juice with trehalose in rats with type 2 diabetes. The results showed that this compound has more protective effects on the pancreas and kidneys against inflammation caused by hyperglycemia and oxidative damage. Tanide et al. (32) reported a significant effect of guava leaf essential oil in the space between the joint and a decrease in osteophytes. Radiography showed that joint space stenosis with increased osteophyte formation was higher in arthritic rats compared to rats treated with guava fruit hydroalcoholic extract. The results of our study also showed that the hydroalcoholic extract of guava fruit could improve the function of cartilage chondrocytes in osteoarthritis by reducing the expression of the resistin gene and TNF-α gene.

The use of plant extracts to treat diseases is still being researched and the findings of these calves are increasing day by day. In recent years, researchers have focused on the use of traditional medicines based on medicinal plants such as *Curcuma longa* plant.
species that have a long and proven history in treating various diseases (33). In this regard, more studies are needed to discover the potential of guava fruit in the treatment of joint diseases (34-37).

Conclusions
In the current study, the effect of Psidium guajava hydroalcoholic extract was evaluated on reducing the expression of Resistin gene and TNF-α gene in articular chondrocytes of patients with knee osteoarthritis. The results showed the therapeutic effect of guava fruit hydroalcoholic extract by reducing the expression of resistin and TNF-alpha genes to 56.59 and 51.86%, respectively. Therefore, the hydroalcoholic extract of this fruit can be considered as an effective complementary alternative to improve knee osteoarthritis.

Acknowledgements

None.

Interest conflict

None.

References