Pomegranate Juice Inhibits Periodontal Pathogens Biofilm In Vitro

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Received date: March 3, 2018. Accepted date: September 1, 2018. Published date: September 28, 2018.

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DOI: http://dx.doi.org/10.26912/sdj.v2i3.2572

ABSTRACT

Background: Pomegranate (Punica granatum) fruits are commonly regarded as medicinal plant in Indonesia, and the polyphenols found in pomegranate juice (punicalagin and ellagic acid) have been shown to have antibacterial properties.

Objectives: Using monospecies and multispecies biofilms, we sought to examine the effects of pomegranate juice on the viability of three periodontal pathogens: Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, and Treponema denticola. Methods: Biofilm assays were performed using crystal violet. Pomegranate juice was obtained from pomegranates using a juicer, and the juice was then diluted into different concentrations with phosphate saline buffer. The three pathogens were cultured in both monospecies and multispecies plates. Pomegranate juice was then added to each biofilm well. These were then incubated for 1h, 6h, or 24h, after which the optical density (OD) of the biofilm mass was measured using a microplate-reader (490 nm). Biofilm without treatment was used as a negative control and 0.2% chlorhexidine gluconate as a positive control. Data were analyzed with a one-way ANOVA; the level of significance was set at p<0.05. Results: Compared to the negative control, biofilm mass was significantly decreased after treatment with pomegranate juice across all concentrations and incubation times, for both monospecies and multispecies biofilm (p<0.05). The best results were achieved with P. gingivalis biofilm, with 100% concentration (OD 0.34 ± 0.03); A. actinomycetemcomitans, 50% concentration (OD 0.22 ± 0.01); and T. denticola, with 25% concentration (OD 0.87 ± 0.08), as well as with a multispecies biofilm with a 50% concentration in 1h incubation time (OD 0.09 ± 0.02). Conclusion: Pomegranate juice effectively inhibited the biofilm formation of P. gingivalis, A. actinomycetemcomitans, and T. denticola. Pomegranate juice may therefore be used as an alternative therapy in preventing periodontal disease. Additional research should explore this effect in an environment that mimics oral cavities.

Keywords: Aggregatibacter actinomycetemcomitans, biofilm, Punica granatum, pomegranate, Porphyromonas gingivalis, Treponema denticola
Background

Dental caries and periodontal disease have become global health problems. The accumulation of dental biofilm, which is accompanied by a change in bacterial composition, can lead to the onset of dental diseases, such as dental caries, gingivitis, and periodontal disease. Biofilm is defined as an aggregation of oriented microorganisms attached to each other or to a surface and covered in a self-generated extracellular polymeric substance. Bacterial colonization of the mouth begins from the bottom layer of dental biofilm; early colonists bind to the teeth through adhesion to dental pellicles, and later colonists bind to the previous colonies. Over 700 bacterial species have been detected in oral cavities, including in dental biofilms. Their distribution is not random; most species prefer specific environments, such as the anaerobic conditions provided by periodontal pockets. The specific bacteria isolated in human dental biofilm consist primarily of anaerobic Gram-negative rods, such as Treponema denticola, Porphyromonas gingivalis, and Aggregatibacter actinomycetemcomitans; these are known to be involved in periodontal disease.

Within the field of dentistry, chlorhexidine gluconate (CHX) is the most widely used antimicrobial agent and is widely considered to promote plaque reduction due to its effectiveness against oral pathogens. Nevertheless, the use of chlorhexidine remains controversial as it also stains the teeth and detrimental effects on vital tissues and lead to the development of hypersensitivity. The need therefore exists for the development of new agents that inhibit the growth of biofilm-forming bacteria, as one strategy to prevent oral diseases.

The use of “plant products”—secondary metabolites produced by plants—as an alternative method for controlling pathogenic microorganisms has become popular in recent years. These substances commonly serve as plants’ own defense mechanisms against microorganisms, insects, and herbivores. Pomegranates (Punica granatum L.), which are widely grown in southern China, India, and Southeast Asia, are often used in traditional medicine, as well as for consumption and ornamentation. Pomegranates are rich in polyphenols, which possess anti-inflammatory, antioxidant, antibacterial, and anti-cancerous properties. Several fatty acids, sterols, triterpenes, anthocyanins, flavonoids, and tannins have been identified in pomegranates’ pericarp juice, leaves, and seeds.

Previous research has suggested that pomegranate’s anti-inflammatory effects may be beneficial in treating periodontal disease. One placebo-controlled study found that pomegranate juice decreased plaque formation and reduced periodontal pocket depth over a three-month period. However, research has not yet established whether pomegranate juice can eradicate dental biofilm formation. The present study was sought to evaluate the effects of pomegranate juice on the growth of both monospecies and multispecies oral bacteria that are known as periodontal pathogens. To this end, we examined the effects of pomegranate (Punica granatum L.) juice on the viability of P. gingivalis, A. actinomycetemcomitans, and T. denticola in both monospecies and multispecies bacterial cultures. We then compared the biofilm mass in each in vitro culture.

Methods

Pomegranate juice preparation

Pomegranate juice was obtained using a juicer. The resultant juice was then filtered using a Whatman® filter no. 1 to remove the seeds, and was subsequently re-filtered using a 0.22 μm Acrodisc® filter for sterilization. The sterile juice was then diluted into different concentrations (12.5%, 25%, 50%, 100%) using phosphate buffer saline (PBS, pH 7.3–7.5).

Microbial strains and growth conditions

P. gingivalis ATCC 33277T, A. actinomycetemcomitans ATCC 33384T, and T. denticola ATCC 35405T were cultured in brain heart infusion (BHI) agar (Thermo Scientific, Waltham, MA, USA) at 37°C for 48 hours in anaerobic conditions (10% CO2, 10% H2, 80% N2). They were then re-cultured in BHI broth for 48h at 37°C and were incubated in a GasPak jar system (Becton Dickinson, Franklin Lakes, NJ, USA).

Biofilm Assay

Biofilm assays were performed using crystal violet assays. Samples of 200 μL, each containing 1×10^7 CFU/mL of periodontal pathogen (either P. gingivalis,
A. actinomycetemcomitans, T. denticola, or a mixture of the three), were distributed into individual 96-well plates. The samples were incubated for 48h to form biofilms and were then washed using PBS. Subsequently, different concentrations (12.5%, 25%, 50%, 100%) of pomegranate juice were distributed into well plates that contained biofilms. These were incubated for 1h, 6h, or 24h. Biofilm samples without the addition of any juice served as negative controls. For the positive control, we applied 0.2% CHX to biofilm samples. Results were compared by measuring the optical density (OD) of each sample’s biofilm mass. OD was measured using a microplate reader (490 nm). All experiments were performed in triplicate.

Statistics analysis

We used the Shapiro-Wilk test for normality and Levene’s test to assess homogeneity of variance. Biofilm differences between the experimental groups were analyzed using a one-way ANOVA with Post-Hoc test. P<0.05 was considered statistically significant. Statistical calculations were performed using SPSS Statistics v.20 software for Windows (IBM, USA).

Result

We found that, compared to the untreated negative control, biofilm mass was significantly decreased after treatment with pomegranate juice in all concentrations and all incubation times, across all monospecies and multispecies samples. The mean OD (±SD) of the negative controls were 1.31 (±0.11), 1.01 (±0.17), 1.97 (±0.16), and 1.32 (±0.12) for P. gingivalis, A. actinomycetemcomitans, T. denticola, and the multispecies samples, respectively. As further shown in Fig. 1–4, P. gingivalis was maximally inhibited using concentration of 100% juice in 1h (OD 0.34±0.03); A. actinomycetemcomitans was maximally inhibited using 50% juice in 1h (OD 0.22±0.01); T. denticola was maximally inhibited using 25% juice in 1h (OD 0.87±0.08); and the multispecies culture was maximally inhibited using 50% juice in 1h (OD 0.09±0.02). Moreover, a one-way ANOVA test showed that the biofilm decrease was significant (p<0.05) across all concentrations and incubation times.

**Treponema denticola** biofilms

![Graph](image)

**Figure 1.** The reduction of T. denticola biofilms (as measured by optical density) after application of pomegranate juice (12.5%, 25%, 50%, 100%), compared to negative and positive controls. Biofilm without treatment was used as a negative control and chlorhexidine gluconate (0.2%) as a positive control.
**Porphyromonas gingivalis** biofilms

![Graph showing biofilm mass (optical density) over time for different concentrations of Punica granatum juice.](image)

**Figure 2.** The reduction of *P. gingivalis* biofilms (as measured by optical density) after application of pomegranate juice (12.5%, 25%, 50%, 100%), compared to negative and positive controls. Biofilm without treatment was used as a negative control and chlorhexidine gluconate (0.2%) as a positive control.

**Aggregatibacter actinomycetemcomitans** biofilms

![Graph showing biofilm mass (optical density) over time for different concentrations of Punica granatum juice.](image)

**Figure 3.** The reduction of *A. actinomycetemcomitans* biofilms (as measured by optical density) after application of pomegranate juice (12.5%, 25%, 50%, 100%), compared to negative and positive controls. Biofilm without treatment was used as a negative control and chlorhexidine gluconate (0.2%) as a positive control.
Multispecies biofilms

![Graph showing Biofilm Mass (Optical Density) over Concentrations of Punica granatum juice for different time points: 1 hour, 6 hours, and 24 hours, with control groups at 12.5%, 25%, 50%, and 100%](image)

**Figure 4.** The reduction of multispecies biofilms (as measured by optical density) after application of pomegranate juice (12.5%, 25%, 50%, 100%), compared to negative and positive controls. Biofilm without treatment was used as a negative control and chlorhexidine gluconate (0.2%) as a positive control.

**Discussion**

We found that pomegranate juice effectively inhibited periodontal pathogens biofilm formation. More specifically, our results indicated an inhibitory effect of pomegranate juice on biofilm formation in monospecies and multispecies *P. gingivalis, A. actinomycescomitans,* and *T. denticola* cultures. In all samples, biofilm OD decreased after 1h of incubation with pomegranate juice.

In the present study, pomegranate juice’s antimicrobial effects were observed to gradually decline with longer incubation times. At 1-hour incubation, the formation of biofilm enters an adhesion stage, at which point its attachment is reversible and the attached bacteria can be easily detached from the pellicle layer. The 24h incubation period yielded the highest amount of bacterial biofilm because it allowed the biofilms to enter the maturation stage, at which point biofilm pathogens can increase their antibacterial resistance. Thicker biofilms make it more difficult for antibacterial agents to penetrate into the biofilm, possibly due to the biofilm’s glycocalyx matrix, which protects the microorganisms inside and thus increases their immunity to any antimicrobial and antibiotic agents. This could also be related to the presence of dormant persister cells; it is plausible that the bacterial populations in the biofilms might develop higher antimicrobial resistance during stationary phases as the persister cells synthesize glycocalyx matrixes to enhance their immune systems. It has been previously shown that at the end stage of antimicrobial activities from many antimicrobial agents, persisters can start to re-induce biofilm formation, thus further increasing their immunity.

A previous study tested the antimicrobial effects of pomegranate juice against several Gram-positive and Gram-negative bacteria and found that pomegranate juice effectively inhibited the growth of *B. cereus, B. coagulans, B. subtilis, E. coli, K. pneumoniae, P. aeruginosa,* and *S. aureus.* This likely occurred due to...
Pomegranate juice’s previously demonstrated antibacterial activity. It possesses polyphenolic compounds which can affect bacterial cell walls, inhibit enzymes with oxidized agents, interact with proteins, and interfere with the co-aggregation mechanism of microorganisms. However, other research has found that the antibacterial compounds of pomegranates are relative to the amount and concentration of the pomegranate juice used, and the minimum concentration of pomegranate juice needed to inhibit bacterial growth could vary depending on the method of extraction, type of solvents, and variety of bacterial strains used. Although fresh pomegranate juice typically consists of approximately 85.4% water and 15.6% dry substances (including pectins, organic acids, hydrolyzing tannins, anthocyanins, and polyphenols), if the juice is extracted from whole fruits then the extracts may also contain significant amounts of polyphenols derived from the fruit’s rind and seeds, including punicalagins. However, the percentage of these components may vary according to the fruit’s variety, storage methods and length, and degree of ripeness.

Among plants, flavonoids are the most widely distributed polyphenolic compound and are considered good antimicrobial agents due to their ability to inactivate efflux pumps in microbial cytoplasmic membranes, leading to cell lysis and death. Phenolic compounds also inhibit and inactivate the bacterial enzymes required for protein biosynthesis. One study found that it could inhibit nucleic acid biosynthesis and bacteria’s energy metabolisms, thus suggesting an explanation for their broad range of antimicrobial effects against Gram-positive and Gram-negative bacteria. Pomegranate juice’s antibacterial effects may also be related to the presence of hydrolyzed tannins and polyphenols, especially punicalagin and gallagic acid. Tannins, in both hydrolysable or condensed forms, are water-soluble, bioactive polyphenol compounds that exhibit both bacteriostatic and bactericidal effects against bacteria due to their ability to chelate the metal ions required in bacteria’s biosynthesis of nucleic acids. It has also been reported that punicalagin (a major component of the ellagitannin compound found in pomegranate juice) has an antibacterial effect; these ellagitannin compounds result in turn from the hydrolysis of tannin compounds. Ellagic acids are considered toxic to bacteria due to their ability to disrupt bacterial membranes, potentially leading to bacterial cell lysis. This would seem to indicate that the presence of ellagic acid in pomegranate juice is related to the present study’s finding that pomegranate juice inhibits the viability of monospecies and multispecies P. gingivalis, A. actinomycetemcomitans, and T. denticola biofilms.

Our results also indicated that the multispecies cultures were more significantly inhibited by the pomegranate juice than the monospecies cultures. A potential explanation for this result is that the different metabolic states provided by each strain of bacteria inside the biofilm, combined with the limited nutrients available and the presence of antimicrobial agents, suppressed bacterial growth and thus the formation of multispecies biofilms. We also noted that the most effective concentration of pomegranate juice was different for each bacterial strain. Since we used crude extracts of pomegranate juice in this study, the extracts may have contained many soluble and non-soluble impurities. These impurities, especially the non-soluble and half-soluble particles, could lead to false positive or negative results. Further studies should therefore take care to use dilution to reduce impurities from samples.

Conclusion

Pomegranate (Punica granatum) juice has antibacterial effects against the biofilm development of P. gingivalis, T. denticola, and A. actinomycetemcomitans, in both monospecies and multispecies cultures. Pomegranate juice might therefore be used as an alternative antibacterial agent for periodontal pathogens, although additional research is needed to analyze pomegranate juice’s active component mechanisms.

Acknowledgment

The authors would like to thank Microbiology Centre of Research and Education Laboratory (MiCORE Laboratory) for their invaluable support for this study. Furthermore, we want to express appreciation to Stella, S.Si and Aradhea Monica Drestia, S.Si for their laboratory assistance.
Conflicts of Interest

The authors declare that they have no conflicts of interest.

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