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Original Article

Effect of *Punica granatum* L. Flower Water Extract on Five Common Oral Bacteria and Bacterial Biofilm Formation on Orthodontic Wire

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Abstract

Background: Use of herbal extracts and essences as natural antibacterial compounds has become increasingly popular for the control of oral infectious diseases. Therefore, finding natural antimicrobial products with the lowest side effects seems necessary. The present study sought to assess the effect of *Punica granatum* L. water extract on five oral bacteria and bacterial biofilm formation on orthodontic wire.

Methods: Antibacterial property of *P. granatum* L. water extract was primarily evaluated in brain heart infusion agar medium using well-plate method. The minimum inhibitory concentration and minimum bactericidal concentration were determined by macro-dilution method. The inhibitory effect on orthodontic wire bacterial biofilm formation was evaluated using viable cell count in biofilm medium. At the final phase, samples were fixed and analyzed by Scanning Electron Microscopy.

Results: The growth inhibition zone diameter was proportional to the extract concentration. The water extract demonstrated the maximum antibacterial effect on *Streptococcus sanguinis* ATCC 10556 with a minimum inhibitory concentration of 6.25 mg/ml and maximum bactericidal effect on *S. sanguinis* ATCC 10556 and *S. sobrinus* ATCC 27607 with minimum bactericidal concentration of 25 mg/ml. The water extract decreased bacterial biofilm formation by *S. sanguinis*, *S. sobrinus*, *S. salivarius*, *S. mutans* ATCC 35608 and *E. faecalis* CIP 55142 by 93.7-100%, 40.6-99.9%, 85.2-86.5%, 66-4-84.4% and 35.5-56.3% respectively.

Conclusion: *Punica granatum* L. water extract had significant antibacterial properties against 5 oral bacteria and prevented orthodontic wire bacterial biofilm formation. However, further investigations are required to generalize these results to the clinical setting.

Keywords: *Punica granatum* L., Water extract, Antibacterial activity, Bacterial biofilm, Orthodontic wire

Introduction

Use of medicinal plants for treatment of diseases has centuries of history. At present, although a significant portion of drugs is chemical, it is estimated that at least one third of drugs either are plant-derived medicinal products or have a modified botanical origin. Nutritional and medicinal value of the edible plants has always been the focus of attention of the mankind (1). *Punica granatum* L., commonly known as pomegranate is a shrub or small tree of Asia belonging to the family

Punicaceae. The plant grows 5-8 meters tall. It is originated to Iran and has long been cultivated in the Mediterranean region of Asia, Europe and Africa. *P. granatum* L. is rich in bioactive compounds used for the treatment of a wide range of illnesses like cancer, cardiovascular diseases, diabetes, dental diseases, bacterial infections, skin damage due to UV radiation, diarrhea, hemorrhoids, and as a mouthwash for some cases of sore throat. The medicinal parts of the plant include its flowers, bark, fruits, roots and seeds. Bark and roots are used for treatment of parasitic infections, dried flowers of the plant are used for treatment of bronchitis, diarrhea and bloody diarrhea, and its tisane is used for treatment of inflammation of the throat and mouth. Flowers of *P. granatum* L. have long been suggested in ancient Greek literatures an astringent and homeostatic agent and been recommended for treatment of diabetes (2, 3).

Presence of orthodontic bands and wires in mouth results in accumulation of bacterial plaque because of more difficulties to maintain dental care. Furthermore, orthodontic appliances cause a physico-chemical change in the microbial flora of the oral cavity resulting in increased number of cariogenic microorganisms (4, 5). Although oral health has significantly improved in developed countries, oral diseases are still a constant concern. Dental caries affect more than 90% of school-aged children and most of the adults (6). Dental plaque (yellow, sticky and soft biofilm on tooth surface) is a factor responsible for an increase in the incidence of most common oral diseases i.e. dental caries and periodontal disease (7, 8). Oral streptococcus species are recognized as the first plaque-forming bacteria. *Streptococcus sanguinis* comprises 20-50% of dental plaque and appears to be the first plaque-forming microorganism responsible for maturation of dental plaque (7, 9). *Streptococcus salivarius* is a common colonizer of oral mucosal surfaces especially in the dorsal aspect of the tongue, buccal mucosa, and saliva (7, 10). *Streptococcus mutans*and *S. sobrinus* play an important role in development of caries. The cariogenic properties of these bacteria are attributed to the production of insoluble glucans from sucrose, the ability to adhere to tooth surfaces and their acidogenicity(11). Various antibacterial products are commercially available on the market. Chlorhexidine is the most common product of this category (12). Apart from the wide range of antibacterial effects of this mouthwash, it carries some disadvantages as well including the discoloration of teeth, tongue and restorations, unfavorable taste, burning sensation and dryness due to the desquamation of oral mucosa, calculus formation, parotid swelling and rash (12-14).

To date, no study has evaluated the antibacterial properties of *P. granatum* L. water extract and its effect on bacterial biofilm formation on orthodontic wires. Therefore, the present study was designed aiming at in-vitro assessment of the effect of *P. granatum* L. water extract on 5 common oral bacteria and bacterial biofilm formation on orthodontic wire. After procuring the water extract of *P. granatum* L., its antibacterial effects were evaluated on 5 common oral bacteria via determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) and then the magnitude of the inhibitory effect of *P. granatum* L. on bacterial biofilm formation on orthodontic wire was studied.

Materials and Methods

Sample preparation

Shade dried *P. granatum* flowers were obtained from Darab, Fars Province, Iran in June 2011. It was identified under the voucher specimen No.1121 in the Herbarium of the Pharmacognosy Department, School of Pharmacy, Shahid Beheshti Medical University, Tehran, Iran by Mohammad Kamalinejad. Flowers were then ground down to fine powder by a mechanical miller.

Extract preparation

For extract preparation, 1000 ml of boiling water was added to 100 g of *P. granatum* flowers and the mixture was stored at room temperature for 4 hours to allow the infusion process. The obtained mixture was filtered through Whatman No.1 filter paper. The extract was dried by rotary evaporator (Heidolphlaborota 4000). The dried extracts were kept in sterile sample tubes and stored in a refrig-

erator at 4 °C. The yield of extract was 15% (15 g of dried extract from 100g of *P. granatum* flowers).

Understudy bacteria

The understudy bacteria in the present study were *S. mutans* ATCC 35608, *S. sanguinis* ATCC 10556, *S. sobrinus* ATCC 27607, *S. salivarius* ATCC 9222 and *Enterococcus faecalis* CIP 55142 that were purchased in lyophilized form Iranian Research Organization for Science and Technology, Persian Type Culture Collection (PTCC), Tehran, Iran.

Primary evaluation of the antibacterial effect of Punica granatum flowers using the well-plate method:

Saline microbial suspension with a bacterial count of 1.5 x 10^8 Colony Forming Unit (CFU)/ml was prepared. The prepared microbial suspension for each bacterial strain was cultured on Brain Heart Infusion Agar (Merck, Germany) plates and then wells with 6 mm diameter were punched. One hundred µl of the extract prepared with sterile distilled water at 3.1, 6.25, 12.5, 25, 50 and 100 mg/ml concentrations were poured into the wells. All plates were stored in an incubator (Memmert, Germany) at 35° C for 16-24 hours (5, 14). The mentioned phases were repeated for each bacterial strain three times. Afterwards, the diameter of growth inhibition zone was measured in mm and recorded. The test repeated for three times.

Determination of theminimum inhibitory concentration (MIC) of Punica granatum flower water extract

MIC is defined as the minimum concentration of the extract that inhibits visible growth of bacteria. In order to determine the MIC of the *P. granatum* flowers water extract macrodilution method according to the CLSI standard (National Committee for Clinical Laboratory Standard, 2006) with minor modification was used (5, 15). The water extract of *P. granatum* flowers was prepared at 0.195, 0.390, 0.781, 3.1, 6.25, 12.5, 25, 50, 100 and 200 mg/ml concentrations in Brain Heart Infusion Broth medium (Merck, Germany) and inoculated with the microorganism with the bacterial count of 5 × 10^5 CFU/ml. A series of tubes containing the extract were considered as the negative control group while the tube containing culture medium and microorganism was considered as the positive control group. All tubes were stored at 35°C for 24 hours. This test was repeated three times for each microorganism.

Determination of the Minimum bactericidal concentration (MBC) of the Punica granatum flower water extract:

In order to determine the MBC of extract against each microorganism, 50 µl of each tube with no visible growth of microorganism was inoculated on plates containing Brain Heart Infusion Agar. After storing for 16-24 hours in an incubator (Memmert, Germany) at 35 °C, growth of microorganisms was evaluated. Each test was repeated three times (5, 7, 15). MBC was determined as the minimum concentration of the extract at which no bacterial growth was observed.

Bacterial adhesion to orthodontic wire

In order to determine the amount of biofilm formation on orthodontic wire viable cell count method was employed (16, 17). For this purpose, biofilm medium (BM) containing 3% sucrose was used. This medium contained 35 mM NaCl (Merck, Germany), 10 mM (NH4)2SO4 (Merck, Germany), 15 mM KH2PO4 (Merck, Germany), 58 mM K2HPO4 (Merck, Germany), glucose 0.8% wt/vol (Merck, Germany), 0.2% wt/vol Casamino Acids (CAA)(Fluka, Switzerland) and 100 mM MnCl2., 4H2O (pH 7.4)(Merck, Germany). Vitamins including 0.04 mM nicotinic acid (Merck, Germany), 0.1 mM pyridoxine HCl (Sigma, Switzerland), 0.01 mM pantothenic acid (Sigma, Germany), 1 µM riboflavin (Merck, Germany), 0.3 µM thiamin HCl (Sigma, European) and 0.05 µM d-biotin (Sigma, Germany) and aminoacids including 4mM L-glutamic acid (Merck, Germany), 1 mM L-arginine HCl (Merck, Germany), 1.3 Mm L-cysteine HCl (Merck, Germany), 0.1 mM L-tryptophan (Merck, Germany) and 2 mM MgSO4.7 H2O (Merck, Germany) were also added as supplement. At the end, the medium was sterilized with a sterile filter. Sterile orthodontic wire (Stainless Steel, rectangular, 0.016 3 0.022 inch, M Unitek, St. Paul, Minn.) was cut into 2 cm pieces and stored in an incubator (Memmert, Germany) at 35°C for 40 hours along with 1 ml of the MIC of the extract and three lower concentrations

(MIC, 1/2, 1/4 and 1/8 of the MIC) determined for each bacterial strain and prepared using biofilm medium (BM) containing 3% sucrose and also 0.1 ml of microbial suspension with a bacterial count of 10^4 CFU/ml. The biofilm medium containing extract and a piece of sterile orthodontic wire was considered as the negative control group while biofilm medium containing a piece of sterile orthodontic wire and microorganisms was considered as the positive control group. After completion of this time period, each wire segment along with 1 ml of Phosphate Buffered Saline (PBS) [8 g NaCl (Merck, Germany), 0.2 g KCl (Merck, Germany), 1.44 g Na2HPO4 (Merck, Germany), and 0.25 g KH2PO4 (Merck, Germany)] with a pH of 7.2 was placed in Sonicator (Techna 3, Italy, HZ=50-60, V=230±10%, KW=0.13) for 10 minutes. Afterwards, 4 dilutions were prepared of each PBS sample (1/10-1/10,000) and microbial count was done by plate-count method using Brain Heart Infusion Agar medium (Merck, Germany). Plates were kept in an incubator (Memmert, Germany) at 35 °C for three days and after completion of this time the grown colonies were counted. The microbial count of each sample was determined separately (5, 8). All the mentioned steps were repeated three times.

Preparation of samples for SEM analysis:

Orthodontic wires prepared by the a forementioned process and already stored in an incubator for 48 hours were washed three times with 0.1M PBS; 30, 50, 70, and 80% alcohol dilutions were prepared and samples were stored in the mentioned concentrations of alcohol for 10 minutes. In the next phase, all samples were stored in 90% alcohol for 15 minutes and in the final phase, samples were stored in 100% alcohol twice consecutively for 20 minutes. Samples were then placed under the safety cabinet for 24 hours to be prepared for evaluation with electron microscopy (5).

This research project was approved by the Ethics Committee of School of Dentistry, Shahid Beheshti Medical University (Ethics Committee #106, October 2011).

Statistical analysis

Data were presented as mean ± SD in all tables. Graphpad Prism 5.0 (GraphPad Software, Inc., CA, USA) was used for statistical analysis.

Results

In this study primary assessment of the antibacterial effect of *P. granatum* L. flower water extract was done using the well-plate method. The mean diameter of growth inhibition zone (mm) for each bacterial strain exposed to different concentrations of the extract is demonstrated in Table 1. The diameter of growth inhibition zone ranged from 9.5 to 18 mm and was proportional to the extract concentration. The largest growth inhibition zone as the result of exposure to extract was observed for *S. sanguinis* ATCC 10556. Except for *S. sanguinis* ATCC 10556, no growth inhibition zone was observed for other understudy bacteria in extract concentrations in the range of 3.12 to 12.50 mg/ml.

Table1: The mean diameter of the growth inhibition zone for understudy bacteria at various concentrations of the *Punica granatum* L. flower water extract

Microorganisms	Concentration (mg/ ml)					
	100	50	25	12.50	6.25	3.12
S. mutans	14±0.00	10.5±0.70	10±0.00	-	-	-
S. sanguinis	18±0.00	14.5±0.70	13.5±0.70	13±1.4	10±1.4	-
S. salivarius	15±0.00	13.5±0.70	9.5±0.70	-	-	-
S. sobrinus	18±0.00	13±1.4	11.5±0.70	-	-	-
E. faecalis	10.5±0.70	9.5±0.70	-	-	-	-

*Mean diameter of growth inhibition zone± standard deviation in mm/-No formation of growth inhibition zone

The MIC was evaluated using the macro-dilution method. MIC and MBC of the *P. granatum* L. flower water extract against each microorganism are demonstrated in Table 2. MIC and MBC were in the range of 6.25 to 50 mg/ml and 25 to 100 mg/ml, respectively. MIC and MBC of *P. granatum* L. flower water extract for *S. sanguinis* ATCC 10556 were 6.25 and 25 mg/ml, respectively, which were the lowest MIC and MBC values.

Table 2: MIC and MBC values of *Punicagranatum*L. flower water extract against five understudy bacteria

Microorganisms	MIC (mg/ml)	MBC (mg/ml)
S. mutans	50	50
S. sanguinis	6.25	25
S. salivarius	25	100
S. sobrinus	25	25
E. faecalis	50	50

The highest MIC of this extract was 50 mg/ml against *E. faecalis* CIP 55142 and *S. mutans* ATCC 35608. The MBC of this extract for *S. mutans* ATCC 35608, *Enterococcus faecalis*CIP 55142 and *S. salivarius* ATCC 9222 was 50, 50 and 100 mg/ml, respectively.

Viable cell count method was used for evaluation of bacterial biofilm formation on the orthodontic wire. The results of the inhibitory effects of 4 different concentrations of the extract on bacterial biofilm formation on orthodontic wire are demonstrated in Table 3. Various concentrations of the extract had a significant effect on reducing bacterial biofilm formation on orthodontic wire. The extract reduced bacterial biofilm formation on orthodontic wire by *S. sanguinis* ATCC 10556, *S. sobrinus* ATCC 27607, *S. salivarius* ATCC 9222, *S. mutans* ATCC 35608 (fig. 1) and *E. faecalis* CIP 55142 by 93.7%-100%, 40.6%-99.9%, 85.2%-86.5%, 66-4%-84.4% and 35.5%-56.3%, respectively. A lower concentration of the extract was able to reduce significantly bacterial biofilm formation on orthodontic wire by *S. sanguinis* by 93.7%-100%.

Table3: Percentage of reduction in bacterial biofilm formation by the understudy microorganisms on orthodontic wire compared to the positive control group using various concentrations of the *Punicagranatum*L. flower water extract

Microorganisms	Concentration (mg/ ml)						
	50	25	12.50	6.25	3.12	1.56	0.78
S. mutans	84.4	84.4	77.2	66.4	-	-	
S. sanguinis	-	-	-	100	100	100	93.7
S. salivarius	-	86.5	86.5	86.4	85.2	-	-
S. sobrinus	-	99.9	99.0	95.0	40.6	-	-
E. faecalis	56.3	55.2	54.9	35.5	-	-	-

-Not using the mentioned concentrations for the understudy microorganisms

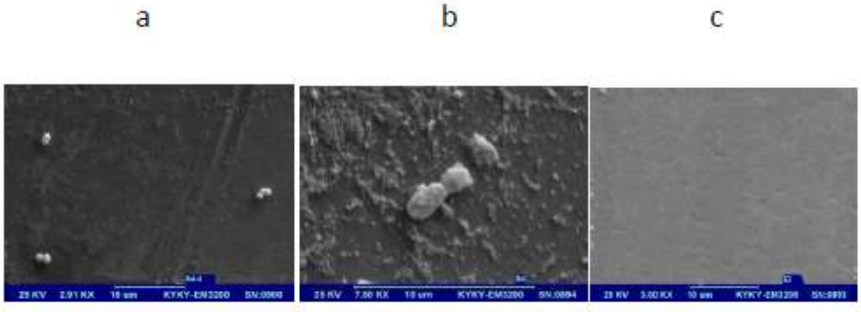

Fig. 1: SEM image of *S. mutans* with the orthodontic wire placed in a: positive control tube b: 50 mg/ml extract concentration and c: negative control tube

Discussion

Demand for orthodontic treatment has greatly increased in the recent years and orthodontists try to obviate patients' esthetic, functional and dynamic needs. Fixed orthodontic appliances enhance adhesion and accumulation of bacterial plaque and are usually associated with inadequate plaque control that consequently results in periodontal disease and dental caries. Considering the constant concern of orthodontics about the development of oral diseases as the result of application of orthodontic appliances and the increasing public interest in the use of herbal medicines for oral health care, the present study was designed aiming at determining the antibacterial effect of water extract of *P. granatum* L. flowers on five oral bacteria and bacterial biofilm formation on the orthodontic wire. The ethanol, water, methanol and acetone extracts of *P. granatum* have shown strong antimicrobial properties against Gram positive and Gram-negative non-oral microorganisms (3). However, a few studies have evaluated the antibacterial properties of this plant on oral bacteria. Hydro-alcoholic extract of the fruit of *P. granatum* was very effective against biofilm forming microorganisms in the clinical setting and resulted in 84% reduction in biofilm formation in the mouth of patients using fixed orthodontic appliances (18). Vasconcelos compared the MIC of pomegranate fruit gel with miconazole against the adherence of *S. mutans*, *S. sanguinis*, *C. albicans* and *S. mitis* to glass and revealed that in comparison with miconazole, pomegranate fruit gel had a greater efficacy for prevention of oral bacteria adherence to glass (19). Lee evaluated the effect of garlic extract on adherence of bacterial plaque to orthodontic wire and demonstrated increased adherence of bacterial biofilm to orthodontic wire in contrast to the significant antibacterial properties of this extract (5). Tannins and polyphenols are the most abundant chemical compounds present in pomegranate plant, its fruits and flowers (20). Tannins have recently been on the spotlight as a substance for prevention of tooth decay.They are capable of passing through the bacterial cell wall and can adhere to the cell surface resulting in eventual reduction of the MIC for microorganisms (17, 19). *S. mutans*, *S. salivarius*, *S. sanguinis* and *S. sobrinus* constitute a great portion of dental plaque. *S. sanguinis* is among the first colonizers on tooth surface (7, 19). Based on the findings of the present studyand SEM images(fig.1) *P. granatum* L. flower water extract had the greatest antibacterial effect with the lowest MIC against *S. sanguinis*. This extract could also reduce the adherence of *S. sanguinis* to the orthodontic wire. Therefore, *P. granatum* L. flower water extract can prevent primary colonization of *S. sanguinis* and subsequent maturation of dental plaque and adhesion of other cariogenic microorganisms. These effects of the extract on reduction of bacterial biofilm adherence to orthodontic wire found in the present study are in contrast to the findings of Lee. This contrast may be due to the different compositions of the two different extracts and sticky consistency of the garlic extract. Positive effects of *P. granatum* L. flower water extract on reducing the adherence of bacterial biofilm to orthodontic wire are attributed to its inhibitory effects due to the impact of its active antimicrobial ingredients on the understudy bacteria. On the other hand, interference with the adherence mechanism of oral bacteria to orthodontic wire is another suggested theory in this respect.

Conclusion

Results of the present study are indicating the potentially effects of *P. granatum*L. flower water extract on five understudy oral bacteria and reduction of their biofilm formation on orthodontic wire. This finding can raise hope for reducing oral diseases due to orthodontic appliances by the use of *P. granatum* L. flower water extract. However, complementary studies in the clinical setting are required on this subject.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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