Effectiveness of Pomegranate Mouthrinse in Reducing Bacterial Plaque, Gingival Inflammation and Total Salivary Proteins over a Period of 90 Days: A Double-Blind Randomized Trial

Jeevika Chandrasekhar Deepak and Srinivasan Raj Samuel

Thai Moogambigai Dental College and Hospital, Dr. M.G.R. Educational and Research Institute University, Chennai, Tamil Nadu, India

Abstract

Objective: Pomegranate is proven to possess effective antibacterial and anti-inflammatory properties. Hence, we evaluated the efficacy of pomegranate mouthrinse on plaque accumulation, gingivitis and total salivary proteins among adolescents aged 15 to 19 years over a period of three months.

Methods: A double-blind, single-center, controlled clinical trial was conducted among 40 adolescents. The participants were randomly allocated into two groups, pomegranate and placebo, and were requested to use the mouthrinse twice daily. Plaque and gingival indexes were recorded at baseline, 30, 60 and 90 days; total salivary proteins were measured at baseline and after 9 months. Repeated measure ANOVA and post-hoc tests were used for within group comparisons; independent *t*-tests were used for between group comparisons.

Results: Pomegranate mouthrinse reduced the mean plaque and gingival index scores significantly at 3, 6 and 9 months follow-up compared to placebo, and it significantly reduced total salivary proteins from baseline until 90 days compared to placebo.

Conclusion: Pomegranate extract mouthrinse was effective in reducing plaque accumulation, gingival inflammation and total salivary protein count at 30, 60 and 90 days of follow-up and can be considered an effective alternative to chemotherapeutics in treating gingivitis without any side effects.

Key words: Pomegranate, plaque, gingivitis, salivary proteins

Introduction

Gingivitis is one of the most common oral diseases, affecting all age groups irrespective of age and sex, although a high incidence is found among children and adolescents (Hugar *et al*., 2011). It is a chronic inflammatory condition exhibiting the earliest clinical sign of bleeding from gums. As a result of the inflammatory process, plasma protein leaks into the gingival crevicular fluid (GCF), thereby increasing salivary albumin, cystatins C and amylase, and GCF ultimately drains into the oral cavity. Therefore, total protein count in saliva increases in conditions such as gingivitis (Sharma *et al*., 2004; Shaila *et al*., 2013) and it can be an effective diagnostic marker. As a diagnostic fluid, saliva offers distinctive advantages over serum because it is non-invasive and requires no formal training for collection and storage.

Gingivitis is prevented by regular practice of mechanical plaque control activities such as toothbrushing and use of other interdental aids. Effective mechanical plaque removal requires manual dexterity, and hence chemotherapeutic agents such as essential oils, chlorhexidine and triclosan have been introduced as adjuncts.

Correspondence to: Srinivasan Raj Samuel, Senior Lecturer, Department of Public Health Dentistry, Thai Moogambigai Dental College and Hospital, Dr. M.G.R. Educational and Research Institute University, Chennai- 600 107, Tamil Nadu, India. Tel: +919962850690; E-mail: samuelrajsrinivasan@gmail.com

Chemotherapeutic agents, when regularly used over a period of time, have been reported to cause extrinsic stains, alteration in taste perception and microbial resistance (Adams and Addy, 1994).

Recent focus has shifted to the anti-inflammatory and antimicrobial properties of natural products, because of the widespread microbial resistance as a consequence of drug misuse. Pomegranate (*Punica granatum*), a predominant member of the *Punicaceae* family, has proven to exhibit anti-inflammatory, antimutagenic and antibacterial effects. Therapeutically beneficial constituents of pomegranate include ellagic acid, ellagitannins, punicallagins, punic acid, flavonoids, anthocyanidins, antocyanins, and estrogenic flavones (Bachoual *et al*., 2011; Zahin *et al*., 2010; Endo *et al*., 2010). Studies have demonstrated the antimicrobial activity of hydroalcoholic extract (HAE) of pomegranate fruit on dental plaque (Menezes *et al*., 2006), and it also acts against *Staphylococcus aureus*, *Streptococcus pyogenes* and *Candida albicans* (Pai *et al*., 2010). Pomegranate components have properties that could reduce the risk of gingivitis and promote overall oral health (DiSilvestro *et al*., 2009).

Research on the use of pomegranate extract on plaque accumulation and total salivary protein count is scarce, and hence we conducted a study to evaluate the effectiveness of pomegranate mouthrinse on plaque accumulation, gingivitis and total salivary proteins among adolescents aged 15 to 19 years over a period of three months in Chennai city.

Materials and methods

This study was designed as a double blind, placebo-controlled randomized clinical trial conducted in the Department of Public Health Dentistry, SRM Dental College and Hospital, Chennai from March 2013 to June 2013. The study protocol was reviewed and approved by the institutional review board (SRMU/M&HS/SRMDC/2012/M.D.S/902).

Adolescents from two random schools in our incremental care program who fulfilled the following inclusion and exclusion criteria were included in the trial. Inclusion criteria were: presence of moderate to severe gingivitis (moderate gingivitis is defined as moderate inflammation, moderate glazing, redness, edema and hypertrophy, and bleeding on probing, and severe gingivitis is severe inflammation, marked redness and hypertrophy, ulceration, tendency to spontaneous bleeding), free from systemic diseases, non-smokers. Subjects with at least 20 natural teeth (teeth that were fully crowned or extensively restored were not included) and consented (self and parent) to participate. Exclusion criteria included subjects consuming a high polyphenolic diet such as soy beans or green tea, and those who had fixed or removable orthodontic appliance. Those on medication or who took antibiotics in past six months or underwent periodontal surgical treatment in previous 6 months were also excluded.

Sample size

The number of participants required in each intervention group was estimated to be 18 based on the effect size of 1.69, power of 80% and overall error rate of 5%. To account for 20% loss to follow-up, 22 participants were allocated to each treatment arm.

Randomization

The study participants who fulfilled the inclusion criteria as identified by the principal investigator (JCD) were assembled in a room and each of them was randomly assigned using the lottery method into group A (pomegranate rinse) and group B (placebo control) by the second investigator (SR), thus ensuring an allocation ratio of 1:1. Thus the principal investigator and subjects were blinded as to which group the participants belonged.

Allocation concealment

The respective mouthrinses were packed in a plain white plastic bottle for a period of 15 days. A department assistant who did not know about the study groups distributed the mouthrinses according to the alphabet displayed by the participant. After a 15-day period the participants were recalled for refill; if they could not make the appointment, the mouthrinse was delivered to them to avoid loss of follow-up.

Preparation of the mouthrinse

Pomegranate mouthrinse: The peel of the fruit was collected and rinsed with water. It was then dried under sunlight until water droplets completely evaporated. Pericarp (peel) was then kept in a hot air oven for 3-4 days at 33°C to dry completely. Dried pericarp was then ground to powder. Five grams of ground pericarp were added to 50 mL of boiling water and left in a hot water bath for an hour at 70°C such that secondary metabolites got completely extracted. The extract was then filtered through Whatman number one filter paper and kept in a hot air oven for drying. The dried extract was dissolved in a double volume of DMSO (dimethyl sulfoxide) to a concentration of 500 mg/mL. The solution was prepared over a period of 15 days and kept away from direct sunlight (Khan and Hanee, 2011). Placebo mouthrinse: Plain water with added flavours of pomegranate was used to protect allocation concealment and avoid contamination bias.

Intervention

The participants were requested to use 10 mL of solution and rinse the mouth for a period of 60 seconds twice a day, half an hour after brushing, for a period of 90 days. They were requested to continue their regular non-supervised, self-performed oral hygiene practices.

Gingival and plaque indices were recorded to estimate the severity of gingivitis and plaque accumulation. Total salivary protein count was estimated to check for change in salivary protein levels and as a precise measure of gingival inflammation. Gingival index (Löe and Silness, 1963) and plaque index (Löe, 1967) were recorded at baseline, 30, 60 and 90 days, whereas the total salivary proteins were assessed at baseline and at the end of three months.

Unstimulated whole saliva samples were collected around 7:00 - 7.30 AM, one hour before the subjects had breakfast. This was to ensure that the variability in salivary flow and composition be minimised due to diurnal variation. The subject was asked to rinse the mouth with distilled water thoroughly to remove any food debris, and then after 10 minutes directed to spit into a sterile plastic container (sterile 50 mL ASTRA Scientific (P) Ltd, Kerala). Three mL of saliva were centrifuged for 10 minutes at 800 x *g* to obtain a clear supernatant fluid, which was assayed immediately. Salivary total protein level was estimated based on the biuret method (Sapan *et al*., 1999) at the department of biochemistry.

Statistical analysis

The normality of the data was assessed using the Shapiro Wilk test and was found to be normally distributed; hence, parametric tests were used for analysis. The within-group differences at different time frames were analysed using repeated measures analysis of variance (ANOVA), and the between-group differences were analysed using independent Student's *t*-test. All the statistical tests were two-sided; $\alpha = 0.05$ was used as an overall experiment error rate.

Results

Forty-four subjects consented to participate (22 in pomegranate and 22 in placebo group) and at the end of 90 days 20 participants remained in each group. Of the 40 subjects, 55% were males (22) and 45% were females (18), and the mean age of participants was 17 ± 1.8 years.

There was a statistically significant reduction in the mean plaque and gingival index scores from baseline to all subsequent follow-up visits in both the pomegranate and placebo group ($p < 0.05$). Post-hoc analysis revealed a significant reduction in mean plaque and gingival index scores from baseline - 30 days, 30 - 60 days and 60 - 90 days in both the pomegranate and placebo group (*Table 1*). The between-group analysis of pomegranate and placebo is presented in *Table 2*. Independent *t*-tests elicited significant differences in the mean plaque and gingival index scores between the pomegranate and placebo group at 30 days, 60 days and at 90 days follow-up. The pomegranate group had more significant reduction in the mean scores as compared to the placebo group at all follow-up visits. Total salivary protein was found to be reduced significantly in both groups in the within-group analysis, but pomegranate rinse caused statistically significant reductions compared to placebo at the 90-day follow-up compared to baseline (*Table 2*).

Table 1. Within-group comparison of plaque, gingival index scores and total salivary protein counts in pomegranate and placebo groups.

Index	Time point	Pomegranate[a] Mean (SD)	Placebo[b] Mean (SD)	*p* value[c]	Pairwise comparison[d]
Plaque index	Baseline	1.80 (0.09)	1.76 (0.07)	< 0.001[a,b]*	Baseline < 30 days[c,d]* < 60 days[c,d]* < 90 days[c,d]*
	30 days	1.53 (0.03)	1.71 (0.03)		
	60 days	1.22 (0.07)	1.60 (0.05)		
	90 days	0.88 (0.23)	1.58 (0.1)		
Gingival index	Baseline	1.76 (0.6)	1.76 (0.06)	< 0.001[a,b]*	Baseline < 30 days[c,d]* < 60 days[c,d]* < 90 days[c]*
	30 days	1.59 (0.05)	1.71 (0.07)		
	60 days	1.22 (0.08)	1.61 (0.05)		
	90 days	0.99 (0.08)	1.57 (0.05)		
Total salivary proteins	Baseline	1.40 (0.9)	1.40 (0.9)	< 0.001[a]*	
	90 days	0.23 (0.12)	1.34 (0.7)		

[a]Within-group analysis for pomegranate group; [b]Within-group analysis for placebo group; [c]Results of repeated measures ANOVA; *$p < 0.05$ considered significant; [d]Results of post-hoc test; *$p < 0.05$ considered significant; SD, standard deviation.

Table 2. Summary of baseline, 30, 60 and 90 days mean plaque index (MPI), gingival index (GI) scores and total salivary protein (TSP) levels after use of mouthrinse.

Evaluation		MPI Mean (SD)	*p* value	GI Mean (SD)	*p* value	TSP Mean (SD)	*p* value
Baseline	Pomegranate	1.8 (0.09)	0.15	1.7 (0.6)	0.82	1.4 (0.9)	0.98
	Placebo	1.7 (0.07)		1.7 (0.7)		1.4 (0.9)	
30 days	Pomegranate	1.5 (0.03)	< 0.001	1.5 (0.05)	< 0.001	-	-
	Placebo	1.7 (0.03)		1.7 (0.06)		-	
60 days	Pomegranate	1.2 (0.07)	< 0.001	1.2 (0.08)	< 0.001	-	-
	Placebo	1.6 (0.05)		1.6 (0.05)		-	
90 days	Pomegranate	0.8 (0.2)	< 0.001	0.9 (0.08)	< 0.001	0.2 (0.1)	< 0.001*
	Placebo	1.5 (0.1)		1.5 (0.04)		1.3 (0.7)	

Student's independent *t*-test; *$p < 0.05$ considered significant.

Discussion

Gingivitis is a widely prevalent condition and affects all individuals irrespective of age, sex or race. It can be prevented by practising meticulous oral hygiene and use of adjuncts such as chemotherapeutic agents. These agents inhibit the formation of dental plaque, but have several side effects: staining of teeth, altered taste perception, gingival desquamation and painful mucosa are reported in literature (Kocaka *et al*., 2009). Natural products like pomegranate (*P. gratum*) are considered to be safe, without side effects and exhibit effective antioxidant and antimicrobial properties. Pomegranate juice and cold pressed seed oil have been reported to have antioxidant properties comparable to that of butyrated hydroxyanisole (BHA), green tea, and superior to that of red wine (Shubert *et al*., 1999).

Pomegranate mouthrinse reduced the plaque and gingivitis score significantly as compared to placebo over a period of 90 days, and this finding is consistent with results previously reported in the literature. Bhadbhade *et al.* (2011) conducted a trial to determine the amount of plaque accumulation over a period of five days after the use of pomegranate, chlorhexidine and placebo mouthrinse. At the end of five days, the pomegranate group had significantly less plaque accumulation than the placebo group, and it prevented as much plaque as the chlorhexidine rinse (Bhadbhade *et al*., 2011). Our results, in accordance with the existing reports, showed reduced the plaque scores at all follow-up visits compared to placebo (Salgado *et al*., 2006). The gingival index scores elicited a gradually decreasing trend throughout, which was statistically significant as compared to placebo group. Similar to the study reported by DiSilvestro *et al.* (2009), pomegranate mouthrinse compared to placebo used over a period of four weeks three times a day reduced plaque-forming bacteria, reduced cell injury and increased ceruloplasmin activity.

Gingival crevicular fluid is a physiological as well an inflammatory exudate arising from the blood vessels of the gingival plexus, thus acting as a carrier of biological markers finally eluted into the oral cavity. Periodontal microbes are considered to trigger the inflammatory process and thereby increase the total salivary protein levels. *Porphyromonas gingivalis* and *Treponema denticola* counts are known to be high in gingivitis patients, and thereby increase the level of total proteins (Hollman *et al*., 1999). Hence, controlling the microbes in turn decreases the inflammatory response, which in turn decreases the plasma leakage in saliva through GCF; this statement could be supported by the overall reduction in the total salivary protein levels in the pomegranate group at the 90 days follow up compared to baseline.

DiSilvestro *et al.* (2009) reported significant reductions in total salivary protein count after rinsing with pomegranate extract. This study suggests the positive correlation between salivary proteins and plaque-forming bacteria, and the increased levels in people with gingival problems, and is in concordance with previous reports (Rudney *et al*., 1993; Narhi *et al*., 1994). To the best of our knowledge this is the first study to evaluate the potential of pomegranate extract on plaque accumulation, gingivitis and total salivary protein levels over a period of 90 days. The advantages of the study include the double-blind design, longer period of study, and limited loss to follow-up. All the subjects were evaluated by a single trained examiner (JCD) who was blind as to the group allocation, the participants did not know their group allocation, and the statistician was blinded, thereby ensuring the double-blind design. This study can be generalized towards all those who are suffering from gingivitis. No adverse effect was reported by any of the study participants throughout the trial duration. The reduction in plaque accumulation, gingivitis and total salivary protein counts was significant in both study and control groups.

This could be attributed to the Hawthorne effect, as the subjects and their parents knew they were participating in a trial and their oral health was monitored. Reduction in the salivary microorganism assessment would have improved the quality of the study, but was not feasible as a result of time and financial constraints, which could be considered a limitation. The subjective nature of plaque index and the very knowledge of participating in a trial may have influenced the results in an insignificant way, but still could be considered a limitation.

In conclusion, both pomegranate extract mouthrinse and placebo rinse were effective in reducing the plaque accumulation and gingival inflammation at 30, 60 and 90 days of follow-up, but the reduction in total salivary protein counts was found only in the pomegranate group. The overall reduction of all clinical parameters was greater in the pomegranate group compared to placebo; hence, we recommend the use of pomegranate extract rinse, effectively devoid of any side effects, as adjunct to oral hygiene practices in treating gingivitis.

Acknowledgement

We would like to thank all the subjects who participated selflessly. There are no conflicts of interest to declare, and no external source of funding was obtained for this study.

References

Adams D and Addy M. Mouthrinses. *Advances in Dental Research* 1994*;* **8:** 291-301.

Bachoual R, Talmoudi W, Boussetta T, Braut F and El-Benna J. An aqueous pomegranate peel extract inhibits neutrophil myeloperoxidase *in vitro* and attenuates lung inflammation in mice. *Food and Chemical Toxicology* 2011; **49:**1224-1228.

Bhadbhade SJ, Acharya AB, Rodrigues SV and Thakur SL. The antiplaque efficacy of pomegranate mouthrinse. *Quintessence International* 2011; **42:**29-36.

DiSilvestro RA, DiSilvestro DJ and DiSilvestro DJ. Pomegranate extract mouth rinsing effects on saliva measures relevant to gingivitis risk. *Phytotherapy Research* 2009; **23:**1123-1127.

Endo EH, Cortez DA, Ueda-Nakamura T, Nakamura CV and Dias Filho BP. Potent antifungal activity of extracts and pure compounds isolated from pomegranate peels and synergism with fluconazole against *Candida albicans*. *Research in Microbiology* 2010; **161:**534-540.

Hollman R and Van Der Hoeven HJ. Inability of intact cells of *Treponema denticola* to degrade human serum proteins IgA, IgG and albumin. *Journal of Clinical Periodontology* 1999; **26:**477-479.

Hugar SM, Deshpande SD, Shigli A and Reddy PV. An overview of gingival and periodontal diseases in 12 to 15 years using gingivitis and periodontitis site prevalence index (WHO, 1978). *World Journal of Dentistry* 2011; **2:**175-181.

Khan JA and Hanee S. Antibacterial properties of *Punica granatum* peels. *International Journal of Applied Biology and Pharmaceutical technology* 2011; **2:**23-27.

Kocaka MM, Ozcanb S, Kocakb S, Topuzc O and Ertend H. Comparison of the efficacy of three different mouthrinse solutions in decreasing the level of *Streptococcus mutans* in saliva. *European Journal of Dentistry* 2009*;* **3:**57-61.

Löe H and Silness J. Periodontal disease in pregnancy. I. Prevalence and severity. *Acta Odontologica Scandinavica* 1963; **21:**533-541.

Löe H. Plaque index. *Journal of Periodontology* 1967; **38:**610-616.

Menezes SM, Cordeiro LN and Viana GS. *Punica granatum* (pomegranate) extract is active against dental plaque. *Journal of Herbal Pharmacotherapy* 2006; **6:**79-92.

Narhi TO, Tenovuo J, Ainamo A and Vilja P. Antimicrobial factors, sialic acid, and protein concentration in whole saliva of the elderly. *Scandinavian Journal of Dental Research* 1994; **102:**120-125.

Pai MB, Prashant GM, Murlikrishna KS, Shivakumar KM and Chandu GN. Antifungal efficacy of *Punica granatum*, *Acacia nilotica*, *Cuminom cyminum* and *Foeniculum vulgare* on *Candida albicans*: An *in vitro* study. *Indian Journal of Dental Research* 2010; **21:**334-336.

Rudney JD, Krig MA and Neuvar EK. Longitudinal study of relations between human salivary antimicrobial proteins and measures of dental plaque accumulation and composition. *Archives of Oral Biology* 1993; **38:**377-386.

Salgado ADY, Maia JL, Pereira SL, Lemos TL and Mota OML. Antiplaque and anti-gingivitis effects of a gel containing *Punica granatum* Linn extract. A double-blind clinical study in humans. *Journal of Applied Oral Science* 2006; **14:**162-166.

Sapan CV, Lundblad RL and Price NC. Colorimetric protein assay techniques. *Biotechnology and Applied Biochemistry* 1999; **29:**99-108.

Sharma U, Jain RL and Pathak A. A clinical assessment of the effectiveness of mouthwashes in comparison to toothbrushing in children. *Journal of the Indian Society of Pedodontics and Preventive Dentistry* 2004; **22:**38-44.

Shaila MG. Prakash Pai and Shetty P. Salivary protein concentration, flow rate, buffer capacity and pH estimation: A comparative study among young and elderly subjects, both normal and with gingivitis and periodontitis. *Journal of the Indian Society of Periodontology* 2013; **17:**42-46.

Shubert YS, Lansky EP and Neeman I. Antioxidant and eicosanoid enzyme inhibition properties of pomegranate seed oil and fermented juice flavonoids. *Journal of Ethnopharmacology* 1999; **66:**11-17.

Zahin M, Aquil F and Ahmed I. Broad spectrum antimutagenic activity of antioxidant active fraction of *Punica granatum L.* peel extracts. *Mutation Research* 2010; **703:**99-107.