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Original Article

A Randomized, Controlled Clinical Trial on the Clinical and Microbiological Efficacy of Punica granatum Linn Mouthwash

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Abstract

Objective: To evaluate the clinical efficacy of a mouthwash containing Punica granatum L as compared to 0.12% chlorhexidine digluconate on the control of dental biofilm and gingival inflammation. **Material and Methods:** A randomized, controlled, double-blind clinical trial was carried out comprising a sample of 35 students aged nine to twelve years having Simplified Oral Hygiene Index value equal to or higher than 1.6, with at least 20 teeth. The sample was divided into two groups: group A, administration of 0.12% chlorhexidine mouthwash twice a day for 14 days; and group B, administration of Punica granatum mouthwash (6.25%) following the same protocol described above. The Plaque Index (PI) and Bleeding on Probing Index (BPI) were used to evaluate biofilm control and gingival inflammation on days 0, 7 and 14. Counting of oral streptococci from saliva samples was also performed on days 0 and 14. The data were analyzed using Student's t test and Analysis of Variance (ANOVA). **Results:** The findings showed that P. granatum mouthwash reduced the mean values of PI and BPI, but with no significant difference. However, there was a significant reduction in the counting of oral streptococci. In the control group, all variables were found to be significantly reduced. **Conclusion:** Punica granatum mouthwash was not effective for the control of dental biofilm and gingival inflammation, but it was effective in reducing the counting of oral streptococci.

Keywords: Dental Plaque; Medicinal Plants; Gingivitis.

Introduction

The mechanical removal of dental biofilm, through proper use of brushes and dental floss, is considered to be the main resource for good oral hygiene [1,2]. However, clinical experience and population-based studies have shown that such methods are not being employed as accurately as they should be by a large number of people. Therefore, several chemotherapeutic agents have been developed to control bacterial plaque, aiming at improving the efficacy of daily hygiene control measures [3].

Among the chemotherapeutic agents, surfactants, essential oils and antimicrobials, bisbiguanids, salts of quaternary ammonium, fluorides and iodine derivatives were formulated to help the potential biofilm control [4,5]. However, some of these products have some use restrictions and cannot be employed for long periods. Furthermore, the increased resistance of synthetic antimicrobials has encouraged research on natural products as an alternative in the search for an ideal agent [2].

Some plants traditionally used in folk medicine were evaluated for their biological activity in vitro and in vivo [6]. In dentistry, herbal medicines have been used as anti-inflammatory agents, antibiotics, analgesics, sedatives, as well as endodontic irrigants [7].

Among the plants studied, pomegranate tree (Punica granatum Linn.) has been studied by being rich in bioactive compounds such as tannins and flavonoids, which have anti-carcinogenic, antimicrobial and anti-inflammatory activity already proven by in vitro and in vivo studies [8-10]. Previous in vitro studies have shown that the fruit peel has antibacterial activity against microorganisms present in the dental biofilm, and therefore it is suggested to be used in oral hygiene formulations [2,11,12].

In vivo studies have investigated the inhibitory effects of P. granatum on oral biofilms. Previous study [2] found that the fruit extract was effective in the growth inhibition of biofilm onto teeth previously cleaned with a prophylaxis procedure, prior to execution of a 4-day study. Another study [13] evaluated the effects of pomegranate fruit extract on relevant salivary indicators for oral health, including the risk of gingivitis in healthy subjects diagnosed with mild gingivitis. The authors observed a reduction in the number of total salivary proteins, in the activity of aspartate aminotransferase and salivary α-glucosidase, as well as an increase in the activity of ceruloplasmin. These results demonstrate the biological effects of the extract against gingivitis, suggesting that it can be incorporated into oral care products.

The purpose of this study was to evaluate and compare the clinical efficacy of a mouthwash containing the fruit peel of Punica granatum L. (pomegranate), compared with 0.12 % chlorhexidine mouthwash, in order to assist in the mechanical control of dental biofilm in children through the application of oral health indices (Plaque Index and Bleeding on Probing Index) and counting of oral streptococci.

Material and Methods

Study Design, Sample Characterization and Clinical Evaluation

We conducted a prospective double-blind randomized clinical trial with students of public schools for a period of 14 days. This research was conducted in accordance with the Resolution number 466/12 of the Ministry of Health that regulates human research and was approved by the Ethics Committee of the State University of Paraíba (UEPB), under the protocol number 0676.0.133.000-11. All children's parents or legal guardians authorized their participation by signing an informed consent form.

The sample was obtained using a non-probabilistic method and comprised children aged nine to twelve years regularly attending public schools in the city of Campina Grande, PB, Brazil. Subjects had good general health and poor oral hygiene, as detected by a Simplified Oral Hygiene Index [14] value equal to or higher than 1.6; with at least 20 teeth; who had not made use of antibiotic or antiseptic in the previous two months (two months prior to study execution).

The hydroalcoholic extract of the fruit peel of Punica granatum was acquired from a Brazilian company of flowers and herbs. Chlorhexidine digluconate 0.12% was used as positive control. The mouthwashes were prepared and coded by the Dilecta Manipulation Pharmacy© (Joao Pessoa, PB, Brazil), as follows: chlorhexidine gluconate was coded with the letter "A" and the mouthwash of Punica granatum with the letter "B".

The participants included were allocated into two groups by drawing of sealed and opaque envelopes containing the codes "A" and "B". Data collection was conducted in three stages. On day 0, we measured the Plaque Index (PI) [15] and the Bleeding on Probing Index (BPI) [16], and collected non-stimulated saliva samples into a sterile container for counting of oral streptococci in the laboratory. On the seventh day, we measured the PI and the BPI; and on the fourteenth day, we carried out the second saliva collection and measured the clinical indices used.

All examinations were performed by a single trained examiner. A pilot study was conducted with four students in the same age group in order to calibrate examiners as to the Bleeding on Probing Index (BPI) and Plaque Index (PI), obtaining a kappa value of 0.79. Clinical examinations were made in a classroom, provided by the schools' directors, under natural and artificial light (hands-free flashlight, LED-type light, Rayovac, China). The students and researchers (examiner and annotator) remained seated in chairs during all examinations, which were always made in the brunch period to avoid biases in the outcomes of the indices. The intra and extraoral examinations were made using masks, gloves, cap, goggles, gauze, medical tray, dental mirror and WHO probe. All materials were packaged in sterilizations wraps and autoclaved, following the required biosafety standards.

The rinses were performed twice a day. The first rising was performed on day 0 in the morning at school, under supervision of the researchers, after evaluation of oral health indices and saliva collection. Patients were instructed to rinse 10 mL of the undiluted mouthwash for one minute after unsupervised brushing, as usual.

The rinses performed at home, at the night and in the weekends, were supervised by parents or legal guardians previously instructed. Kits containing toothpaste and brush were provided, so that students used the same toothpaste, avoiding any interference with the results.

Microbiological Evaluation

Microbiological analysis of saliva was performed in the laboratory of Development and Testing of Drugs (LABDEM) of UEPB. Saliva was transported in a cooler with ice to maintain the microorganisms' viability. The sampling time and beginning of microbiological procedures should not exceed three hours. The microbiological evaluation technique used was that proposed by previous study [17] with adaptations. In the laboratory, 1mL of collected saliva was successively diluted in 9 mL of 0.9 % sterile saline solution in decimal series of 10-1 to 10-4. Then, 0.1 mL saliva was plated by the Spread Plate technique on the surface of mitis salivarius agar culture medium (Difco®, Detroit / MI, USA) supplemented with 20% sucrose (Synth®, Diadema / SP, Brazil), 0.2 International Units of Bacitracin Purex (Inlab®, São Paulo, Brazil) and 0.1 g potassium tellurite (Vetec®, Rio de Janeiro / RJ, Brazil). The plates were incubated in a bacteriological incubator at 37 °C for 48 h. After this period, we conducted the counting of colonies with Streptococcus-like morphology. The value obtained was multiplied by the dilution factor, obtaining the final result of CFUs/mL of saliva. The values of CFUs/mL were logarithmically (base 10) transformed to be submitted to statistical analysis.

Statistical Analysis

The data from this study were tabulated in SPSS (Statistical Package for Social Sciences) for Windows®, version 17.0, and analyzed by descriptive procedures such as mean (M) and standard deviation (SD). Analysis of Variance (ANOVA) was used to evaluate differences between control and experimental groups as to the PI and BPI at baseline and on the days 7 and 14. Students' t-test was used to compare differences between the counting of streptococci at baseline and on day 14. To evaluate the inter-group differences, we used the Student's t-test. A $p<0.05$ was considered for all statistical analyses.

Results

A total of 71 students were examined, from whom 36 of both genders were selected and allocated into two groups. Of the 36 subjects included, 35 completed the study. One subject was unable to complete the trial due to illnesses requiring drug prescription,which was defined in the protocol as one of the exclusion criteria (Figure 1).

Of the 35 children analyzed, 16 (15.1%) were included in the positive control group (grupo A, chlorhexidine) and 19 (54.3%) in the experimental group (group B, Punica granatum).

There was a reduction in the mean values of the PI index between the first and the second assessments in both groups. However, no variation was observed between the second and the third

assessments. This reduction was statistically significant in the 0.12% chlorhexidine digluconate group (Table 1).

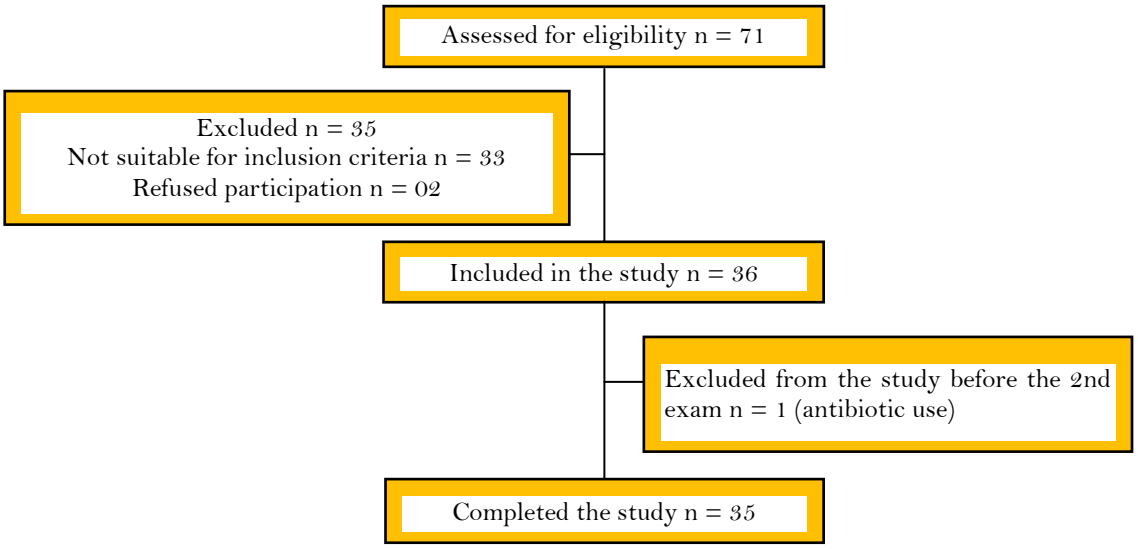

Figure1: Flow diagram of the study design.

Table 1. Plaque Index values. Campina Grande, Paraíba, Brazil, 2012.

Variable/Checkpoint	*Group A (Chlorhexidine)*	*Group B (P. granatum)*	*P value*
	Mean ± SD (Median)	Mean ± SD (Median)	
Baseline	0.77 ± 0.35 (0.79)$^{(A)}$	0.74 ± 0.28 (0.83)	$p^{(1)} = 0.740$
7 days	0.24 ± 0.18 (0.21)$^{(B)}$	0.63 ± 0.27 (0.63)	$p^{(1)} < 0.001$*
14 days	0.27 ± 0.24 (0.21) $^{(B)}$	0.69 ± 0.32 (0.63)	$p^{(1)} < 0.001$*
P value	**$p^{(2)} < 0.001$***	**$p^{(2)} = 0.169$**	

(*): Significant difference at a 5.0% significance level; (1): According to Student's t test with equal variances; (2): According to F (ANOVA) test for repeated measurements; Obs.: If all the letters in parentheses are distinct, a significant difference is confirmed between the corresponding measurements by Bonferroni pairwise comparisons.

There was a reduction in the mean values of the BPI in both groups. Nevertheless, this reduction was statistically significant only in the control group using 0.12% chlorhexidine digluconate (Table 2).

Table 2. Bleeding on Probing Index (BPI). Campina Grande, Paraíba, Brazil, 2012.

Variable/Checkpoint	*Group A (Chlorhexidine)*	*Group B (P granatum)*	*P value*
	Mean ± SD (Median)	Mean ± SD (Median)	
Baseline	11.08 ± 5.11 (10.50)	8.07 ± 4.02 (8.30)	$p^{(1)} = 0.060$
7 days	6.38 ± 3.10 (6.39)	6.65 ± 4.81 (5.95)	$p^{(1)} = 0.852$
14 days	6.01 ± 3.85 (6.06)	5.71 ± 3.74 (5.43)	$p^{(1)} = 0.815$
P value	**$P^{(2)} < 0.001$***	**$p^{(2)} = 0.060$**	

(*): Significant difference at a 5.0% significance level; (1): According to Student's t test with equal variances; (2): According to F (ANOVA) test for repeated measurements; Obs.: If all the letters in parentheses are distinct, a significant difference is confirmed between the corresponding measurements by Bonferroni pairwise comparisons.

In the microbiological evaluation of saliva, the group that used Punica granatum mouthrinse showed reduced countings of streptococci (p=0.009), while the group using chlorhexidine showed a more significant reduction (p <0.001) (Table 3).

Table 3. Mean streptococci* counting by treatment and time. Campina Grande, Paraíba, Brazil, 2012.

Time	*Group A (Chlorhexidine)* Mean ± SD (Median)	*Group B (P granatum)* Mean ± SD (Median)	*P*
Baseline	5,24 ± 1,38 (4,53)	4,98 ± 1,73 (5,22)	0,630**
14 days	2,56 ± 2,45 (2,35)	3,85 ± 2,21 (4,78)	0,110**
P	0,001***	0,009***	
Difference in means	2,68 ± 2,37 (2,28)	1,13 ± 1,69 (0,89)	0,030**
% Reduction	51,14	22,69	

(*) Log10 CFU/ml transformed counts; (**) Student's t test; (***) Students' paired t test.

Discussion

The action of mouthwashes on formed dental biofilm was analyzed using the plaque index in three stages. The reduction in the mean plaque index between baseline and final data was not significant. In contrast with that, the antimicrobial potential of P. granatum was confirmed in a previous in vitro study [11] using standard strains of Streptococcus mutans, Streptococcus mitis and Streptococcus sanguis. In addition, another in vitro study2 pointed out the antimicrobial activity of the fruit extract on strains of Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis and Prevotella. Such antimicrobial action is probably associated with the ability of tannins in inhibiting extracellular microbial enzymes; interfering with the availability of receptors present on the cell surface of microorganisms essential for the co-adhesion of other microorganisms; or acting directly on the microbial metabolism by inhibiting oxidative phosphorylation [18].

The bacteria may have their susceptibility to chemical agents changed when they become part of biofilms [19]. Thus, the antimicrobial efficacy of the mouthwash is dependent on its microbicide action coupled with its ability to penetrate alive and well organized microbial communities, as it can be observed in dental biofilms. This ability makes the difference in the in vivo action of an agent [1]. It is emphasized that no instructions on prophylaxis or dental hygiene and diet were performed prior to the present study or during analysis. Hence, this study confirmed the strong antimicrobial potential of chlorhexidine against structured microbial communities, as seen in previous reports [2,20,21].

The action obtained with the use of chlorhexidine mouthwash corroborates the data from an in vitro study that evaluated the susceptibility of Streptococcus mutans obtained from children's saliva, noting the susceptibility of these microorganisms to the antiseptic [22].

The gingival inflammation in children was assessed by means of the Bleeding on Probing Index. As noted in this study, there was a non-significant reduction in the percentage of bleeding sites in both groups. These findings are in disagreement with those of a previous study [23] including patients diagnosed with gingivitis or chronic periodontitis. The authors observed an

effective reduction in the percentage of bleeding sites in subjects that used P. granatum extract for 15 days. The extract of this plant fruit is rich in phenolic compounds such as tannins and flavonoids with possible anti-inflammatory action [13]. Another study [24] emphasize that pomegranate hydrolysable tannins inhibit the synthesis of several pro-inflammatory mediators, which explains the anti-inflammatory effects of Punica granatum.

In the present study, as well as in a previous study [25] that evaluated a P. granatum gel in adult subjects, only the presence or absence of bleeding on probing was taken into consideration. Other features of the inflammatory process were not considered, such as edema, gingival contour alterations and tissue attachment loss. Therefore, we agree with the authors that the possible effect of P. granatum in controlling the severity of gingivitis should not be discussed. We also point out that the present study was carried out with children aged nine to twelve years. The results might have been influenced by the lack of compliance when following the instructions for administration of the mouthwash at home under supervision of parents or legal guardians.

When comparing the action of the two mouthwashes on the counting of CFU/ml of Streptococcus in saliva in two collections, we observed a significant reduction in the mean CFU/ml of these microorganisms in both groups. The data obtained with the mouthwash of Punica granatum on Streptococcus are consistent with the results shown in a clinical study evaluating the action of the hydroalcoholic extract of this plant on the counting of oral biofilm microorganisms obtained from patients with braces. The authors found 84% inhibition promoted by the mouthwash in the amount of microorganisms [12].

Despite the effectiveness observed, the chlorhexidine mouthwash had possible side effects as we observed biofilm calcification in one student (on the buccal surfaces of the maxillary right and left first molars) and two others reported altered taste. These data corroborate those found in the literature regarding the side effects associated with the use of this antimicrobial agent [1,20,26]. The American Dental Association (ADA) [27] evaluates the antiplaque and anti-gingivitis products by means of a Seal of Acceptance Program, through which it should be evident the efficacy and safety of the product for human use. Hence, in vivo long-term studies are now encouraged to further evaluate the clinical efficacy of P. granatum in the control of oral biofilm and gingivitis. Future research should focus on different concentrations of the extract as well as on adverse effects such as tooth staining, taste changes, among others, associated with its use.

Conclusion

P. granatum mouthwash (6.25%) was not effective in reducing dental biofilm and gingival inflammation. Nevertheless, it showed an inhibitory effect in the counting of oral streptococci.

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