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Is pomegranate peels infusion effective for disinfection of toothbrushes?

A infusão de cascas de romã é efetiva na desinfecção de escovas dentais?

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Resumo

Introdução: Os métodos de descontaminação ou desinfecção de escovas dentais têm sido questionados. **Objetivo:** Este estudo avaliou a eficácia da infusão de cascas de romã como um desinfetante de escovas dentais contra *Streptococcus mutans*. **Material e método:** Uma amostra de 16 escolares com idade entre 7 e 9 anos realizou escovação dentária cuidadosa, uma vez ao dia por 5 dias/semana durante 4 semanas. Após cada dia de escovação, as escovas foram lavadas e pulverizadas com uma solução desinfetante. Este procedimento foi repetido por 4 semanas utilizando uma das diferentes soluções por semana: água destilada (G1; grupo controle), infusão de casca de romã (*Punica granatum Linn*) (G2), hipoclorito de sódio a 1% (G3) e digluconato de clorexidina a 0,12% (G4). Após o quinto dia, as escovas foram coletadas para análise laboratorial. As cabeças das escovas foram agitadas em solução salina diluída em 10^{-1}, 10^{-2},10^{-3}, e 25µL de cada diluição foi semeada em meio de cultura agar mitis salivarius para contagem de unidade formadora de colônias (UFC) de *S. mutans*. Um examinador calibrado (Kappa = 0,91) realizou a contagem de UFC $mL^{-1} \times 10^4$. Os testes de Kruskal-Wallis e de Comparações Múltiplas de Dunn foram usados em um nível de significância de 5%. **Resultado:** G1 apresentou o maior número de UFC (3,9 ± 8,4), seguido de G2 (3,2 ± 4,0). Não foi observado crescimento de *S. mutans* em G3 e G4. Não houve diferença estatisticamente significante entre G1 e G2 e entre G3 e G4 (p>0,05). **Conclusão:** A infusão de romã foi completamente ineficaz para a desinfecção de escovas dentais contra *S. mutans* quando comparada às soluções de hipoclorito de sódio a 1% e digluconato de clorexidina a 0,12%.

Descritores: Desinfecção; escovação dentária; *Punica granatum*; *Streptococcus mutans*.

Abstract

Introduction: Methods of decontamination or sanitization of toothbrushes have been questioned. **Objective:** This study assessed the effectiveness of pomegranate peels infusion as a disinfectant of toothbrushes against *Streptococcus mutans*. **Material and method:** A sample of 16 schoolchildren aged between 7 and 9 years performed brushing 5 days/week, with a careful brushing once a day. After each day of brushing, the toothbrushes were washed and sprayed with one disinfectant solution. This procedure was repeated for 4 weeks using one of the different solutions per week: distilled water (G1; negative control), pomegranate (*Punica granatum Linn*) peels infusion (G2), 1% sodium hypochlorite (G3) and 0.12% chlorhexidine digluconate (G4). After the fifth day, toothbrushes were collected for laboratory analysis. Toothbrushes heads were subjected to agitation in saline dilution of 10^{-1}, 10^{-2},10^{-3}, and 25 µL of each dilution were seeded in mitis salivarius agar culture medium for *S. mutans* colony-forming unit (CFU) counting. One calibrated examiner (Kappa = 0.91) performed the CFU ($mL^{-1} \times 10^4$) counts. Kruskal-Wallis and Dunn Multiple Comparison tests were used at a significance level of 5%. **Result:** G1 presented the highest number of CFU (3.9 ± 8.4), followed by G2 (3.2 ± 4.0). No *S. mutans* growth was observed in G3 and G4. There was no statistically significant difference between G1 and G2 and between G3 and G4 (p>0.05). **Conclusion:** Pomegranate infusion was completely ineffective for the disinfection of toothbrushes against *S. mutans* when compared with 1% sodium hypochlorite and 0.12% chlorhexidine digluconate solutions.

Descriptors: Disinfection; toothbrushing; pomegranate; *Streptococcus mutans*.

INTRODUCTION

The toothbrush is the most effective tool to remove dental biofilm. The mechanical action of its bristles on the tooth surfaces promotes breakdown and removal of microorganisms adhered to these surfaces, enabling the maintenance of a healthy oral microbiota. Although it is important to maintain control of the *Streptococcus mutans* levels, toothbrushes become sites of colonization by microorganisms because they offer ideal conditions for bacteria, viral, fungal and parasitic growth[1]. A single use is sufficient to contaminate the bristles, and microorganisms are capable of surviving from 24 hours up to 7 days[2].

In recent years, the issue of toothbrush disinfection has become progressively important[3]. Methods of decontamination or sanitization of toothbrushes have been questioned. The literature has demonstrated that the use of chemical agents, such as sodium hypochlorite, chlorhexidine gluconate, hydrogen peroxide, cetylpyridinium chloride, sodium perborate, essential oils, Brushtox® (40% activated ethanol with biocide parabens), is considered an efficient and inexpensive method of decontamination[2,4-6]. In this context, phytoplants have demonstrated to be a good alternative to synthetic chemical antimicrobial agents, because they do not produce side effects and acquire antimicrobial resistance[7,8].

Punica granatum Linn (pomegranate) is native to the region extending from northern India to Iran and it is also widely cultivated in parts of America and Africa[9,10]. It is composed of tannins and alkaloids that are antimicrobial substances[11]. Pomegranate has many potential effects, such as bactericidal, antifungal, antiviral, immune modulation, vermifuge, stimulant, refrigerant, astringent, stomachic, stypic, laxative, diuretic and anthelmintic[9,12].

The capacity of the pomegranate extract to prevent dental biofilm adherence and dental bacterial growth may be due to the effect of its flavonoid components, which have antiglycosyltransferase activity, and polyphenols may affect the bacterial cell wall, inhibit enzymes by oxidized agents, interact with proteins and disturb co-aggregation of microorganisms[13,14]. Its methanolic extract has been shown to be effective against some common oral pathogens, such as *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Actynomyces viscosus*, *Candida* spp, *Lactobacillus acidophilus, S. mutans, Streptococcus sanguinis, Streptococcus* salivarius and *Streptococcus aureus*[7,12-15]. Pomegranate peels infusion is widely used by the Brazilian population, especially in Northeastern Brazil, as an antiseptic in throat infections; it is easily accessible, low cost and its extract has proven potential antimicrobial effect[16]. However, to our knowledge, no study has evaluated its antimicrobial power to disinfect toothbrushes.

Therefore, the aim of this comparative study was to assess the effectiveness of pomegranate peels infusion as a toothbrush disinfectant against *S. mutans*. The null hypothesis is that there is no difference among the pomegranate peels infusion comparing to different solutions (1% sodium hypochlorite and 0.12% chlorhexidine digluconate) for the disinfection of toothbrushes.

MATERIAL AND METHOD

This study was approved by the local ethical Review Board for Human Studies. All the responsibles for the children signed a term of free and informed consent allowing their participation.

Manufacture of Pomegranate Peel Infusion

Fresh pomegranates (500 g) were obtained from a public market in the city of João Pessoa, Paraíba, Brazil. The pomegranate peels were separated and oven dried at 33 °C for 7 days to eliminate the moisture and stabilize the enzyme content. Water was boiled to a temperature of 100 °C and then poured over the pomegranate peels (20 g pomegranate peels/200 mL water), which were allowed to steep in the liquid in a closed glass container for a period of 10 minutes. The liquid was strained and stored in a sterile spray bottle for 5 days, while it was being tested as disinfectant solution.

Sample

The sample comprised 16 schoolchildren, aged between 7 and 9 years, who met the following inclusion criteria: children whose parents consented to their participation in the study; children who had not been using antimicrobial substances such as mouthwashes and antibiotics or immunosuppressants for at least three months before the study began; children who attended classes during the week of collection, and those without systemic disorders.

Groups and Experimental Periods

A sample of 16 schoolchildren aged between 7 and 9 years performed brushing 5 days/week during 4 weeks. The children carefully brushed their teeth once a day for two minutes at school under supervision. After each day of brushing, the toothbrushes were washed and sprayed with one of the following solutions: distilled water (G1; negative control), pomegranate (*Punica granatum Linn*) peels infusion (G2), 1% sodium hypochlorite (G3) and 0.12% chlorhexidine digluconate (G4).

The researcher visited the school daily and supervised the toothbrushing performed by the schoolchildren in the classroom, in first period of the morning. Each child received in the first day of the experiment a new toothbrush (Condor Trip®, Condor, São Bento do Sul – Santa Catarina, Brazil) and the dentifrice (about 0.5 grams) was placed on the child's toothbrush by the transverse technique (Colgate Tripla Ação®,1450 ppm fluoride, São Bernardo do Campo – São Paulo, Brazil). The child was provided with a glass of water to perform a mouth rinse and wash the brush. After brushing, the toothbrushes were collected and washed in tap water for one minute. Then the disinfection procedure was performed using the disinfectant substance, which was sprayed six times on the bristles, maintaining a distance of approximately five centimeters between the spray nozzle and brush bristles. This procedure was performed 5 days a week, with the same toothbrushes and the same disinfectant substance.

After the fifth day, toothbrushes were collected for laboratory analysis. This procedure was repeated for 4 weeks using one of the different solutions per week. New toothbrushes were provided

when a new disinfection solution had been used. There were 54 toothbrushes used in the entire experiment.

After collecting all toothbrushes, microbiological analysis were performed. Each toothbrush was then decapitated and the head transferred to a tube containing 7.5 mL of sterile phosphate-buffered saline (PBS). The tube containing the toothbrush head was refrigerated (cooler with ice) and immediately taken to the laboratory. The tubes were submitted to sonication in an ultrasonic apparatus (Ultrasonic Cleaner®, Odontobrás, Ribeirão Preto – São Paulo, Brazil) for five minutes to release the bacteria adhered to the bristles. The brush head was removed and the resulting suspension was serially diluted (10^{-1}, 10^{-2}, 10^{-3}; and 25 µl of each dilution were seeded in Mitis salivarius agar (Difco Laboratories, Detroit, MI, USA) with 30U/mL bacitracin[17] for *S. mutans* CFU (Colony Forming Units) counting. The plates were incubated at 37 °C, for 48 h under microaerophilic conditions (jar with candle system). A single trained investigator performed the CFU counts, manually in a counter (Phoenix CP 608®, Phoenix Industry and Trade of Scientific Equipment Ltd., Araraquara – São Paulo, Brazil). For this step, calibration had previously been performed, and an excellent intra-examiner agreement (Kappa = 0.91) had been observed.

Statistical Analysis

The results of CFU counts ($mL^{-1} \times 10^4$) for each disinfectant solution were analyzed by GraphPad Prism 3.4 for Windows (GraphPad Software®, San Diego, California, USA) software program. The Kolmogorov-Smirnov test was used to test the normal distribution of data. The nonparametric Kruskal-Wallis test was used to compare the number of *S. mutans* CFU among the four disinfectant solutions. The Dunn Multiple Comparison was applied to verify whether there was significant difference between groups tested. The significance level was set at 5%.

Statistical test power for the sample size was calculated online using the website OpenEpi[18].

RESULT

The negative control G1 (distilled water) and G2 (pomegranate peels infusion) groups presented the highest number of CFU of *S. mutans*. No bacteria growth was observed in the samples treated with 1% sodium hypochlorite (G3) and 0.12% chlorhexidine digluconate (G4) solutions. There was no statistically significant difference between G1 and G2 and between G3 and G4. Statistically significant difference ($p<0.001$) was found between the groups: G1 and G3, G1 and G4, G2 and G3, and G2 and G4 (Table 1).

Considering the difference of 3.2 ± 4.0 for the pomegranate peels infusion, a sample size of sixteen schoolchildren provided a statistical power of 92.1%.

DISCUSSION

Currently, there are no protocols on toothbrush disinfection for healthy individuals, and the contamination with microorganisms increases with repeated use[19]. In this context, it is important to mention the antimicrobial properties of the pomegranate[9]. To our knowledge, this is the first study evaluating the effectiveness of pomegranate peels infusion on the disinfection of toothbrush bristles.

Pomegranate peels are constituted by phenolic punicalagins; gallic acid and other fatty acids; catechin; EGCG (Epigallocatechin gallate); quercetin, rutin, and other flavonols; flavones, flavonones; anthocyanidins[9]. The pomegranate tannins are capable of crossing the cell wall composed of several polysaccharides and proteins, and bind to its surface. Polyphenols may affect the bacterial cell wall, inhibit enzymes by oxidized agents, interact with proteins and disturb co-aggregation of microorganisms[11-13].

According to the results of the present study, the pomegranate peels infusion showed no antimicrobial activity on *S. mutans*, being similar to the negative control group (distilled water). It can be suggested that the inefficacy of the pomegranate to disinfect toothbrushes bristles might be due to the formulation of *Punica granatun Linn* prepared as peels infusion. Previous studies have shown effectiveness of pomegranate hydroalcoholic extract[8,9,12,20] or in the form of phytotherapeutic gel[13] on the growth of dental biofilm microorganisms. When compared with methanol extracts, none of the aqueous pomegranate peel extracts exhibited good antibacterial activity at the highest screening concentration against Gram-positive (*Bacillus subtitlis* and *S. aureus)* and Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*)[21].

It should be emphasized that there are different results regarding the use of the pomegranate against dental biofilm, depending on its preparation, such as dry powders, leaves, peels, fruits or seeds. A recent study tested the dry powders of pomegranate and extracts in propylene glycol (200 mg/mL). The authors observed that the *Punica granatum Linn* extract completely inhibited the growth of *S. mutans*, *Staphylococcus* spp. and *Candida* spp. Thus, *S. mutans* showed high sensitivity to the pomegranate extract[6]. Another study tested the *Punica granatum Linn* mesocarp (middle layer of the pericarp of a fruit) aqueous extract and observed its effectiveness against *Candida* spp. and the cariogenic bacteria (*S. mitis* and *S. mutans*)[15].

Table 1. CFU ($ml^{-1} \times 10^4$) counts (mean ± SD) of *S. mutans* among different disinfectant solutions used in the study

Counting of *S. mutans*	Group			
	G1	G2	G3	G4
	Distilled water (control)	Pomegranate peels infusion	1% Hypochlorite solution	0.12% Chlorhexidine digluconate
CFU (mean ± SD)	3.9 ± 8.4 [A]	3.2 ± 4 [A]	0.0 ± 0.0 [B]	0.0 ± 0.0 [B]

Same letter does not differ statistically by Kruskal-Wallis and Dunn multiple comparison tests ($p<0.05$).

Another possible explanation for the ineffectiveness of pomegranate infusion might be the time of exposure to the solution and its concentration, because the bactericidal action is dose and time dependent[21]. It should be mentioned that the period between the last disinfection and laboratory examination did not exceed 4 hours. Devatkal et al.[8] verified that pomegranate peel extracts (1%, 5% and 10%) significantly reduced the growth of bacterial cell right from the fourth hour of incubation, and the concentration of 1 g/10 mL of pomegranate peel to water may not be enough to release a sufficient amount of antibacterial compounds.

It was also found that all the samples sprayed with distilled water were infected by *S. mutans*. Contamination by *S. mutans* after use in samples sprayed with distilled water has also been observed in previous *in vivo* studies[2,6,22,23], which justifies the use of this solution as negative control in this study.

On the other hand, the toothbrushes sprayed with 1% sodium hypochlorite presented no *S. mutans* growth. This result is in agreement with the findings of previous studies[22,24], in which there was absence or low percentage of *S. mutans* growth on toothbrushes after using this solution to disinfect the bristles. Sodium hypochlorite has bactericidal and fungicidal effect on the surface and depth of biofilm. The chlorine released by hypochlorous acid in contact with the tissue proteins, produces nitrogen, formaldehyde and acetaldehyde, which break the sequence of peptides and dissolve proteins. The hydrogen of the amino groups (–NH) is replaced by chlorine (–NCL) forming chloramines, highly toxic compounds, which interfere in cellular metabolism[23]. In spite of the excellent results of disinfection with sodium hypochlorite, its daily use without rinsing the brush properly, could lead to irritation of the oral mucosa, and development of stomach problems, therefore, it is not indicated for use by unsupervised children[25].

In the present study excellent results were found in samples sprayed with 0.12% chlorhexidine digluconate. No bacterial growth was observed and this result corroborates the results shown in the literature[2,22]. Chlorhexidine is a broad-spectrum antimicrobial agent, whose action is related to its cationic bisbiguanide molecular structure that has substantivity and low-grade toxicity. At low concentration it is bacteriostatic, while at higher concentration it is bactericidal as it brings about coagulation and precipitation of cytoplasm[26]. Chlorhexidine digluconate inhibited biofilm formation of *S. mutans* on the toothbrush bristles[22,25,27]. The 0.12% chlorhexidine has been widely used as a mouthwash and is the most effective solution to disinfect toothbrushes contaminated with *S. mutans, Streptococcus pyogenes, S. aureus, Candida albicans*[28].

The limitations of the present study were not considering other specific microorganisms responsible of biofilm-related oral diseases and only six times of solution were sprayed on the bristles. Thus, other oral microorganisms should also be evaluated by counting of total microorganisms. Additionally, the higher standard deviation of CFU counts could be explained by a heterogeneous sample. No initial *S. mutans* screening was performed in schoolchildren in order to select them to participate in this study according to their dental caries risk. Further studies should include children with high count of *S. mutans* in their saliva with the presumption the brushes used by them predominately contain *S. mutans*.

Herbal medicines in particular in emerging countries are commendable due the availability of the flora, the low income population and the possibility of using products with low environmental risk. *Punica granatum Linn* can be cultivated locally and its extract can be obtained locally by public for therapeutic use. Further studies on this product and its extracts are recommended, perhaps testing different periods of exposure (immersion period or solution sprayed), the type of compounds responsible for its antibacterial effect and the development of its formulation, such as ethanol extract, different concentrations, infusion of the leaves, extract of fruit mesocarp, pulp extract gel and dry powders.

CONCLUSION

In conclusion, the pomegranate peels infusion was completely ineffective for the disinfection of toothbrushes against *S. mutans* when compared with 1% sodium hypochlorite and 0.12% chlorhexidine digluconate solutions, which were capable of inhibiting bacterial growth. The null hypothesis was accepted.

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CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

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