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In vitro assessment of the antimicrobial effects of pomegranate (*Punica granatum* L.) peel decoction on saliva samples

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ABSTRACT

Several products have been developed to eliminate or reduce potential pathogenic microorganisms of the oral microbiome. The continuous use of these synthetic products can result in side effects such as vomiting, diarrhea, darkening of the teeth and the induction of microbial resistance. Pomegranate (Punica granatum) peel decoction was tested to assess its antimicrobial activity. In vitro analysis showed the decoction had antimicrobial activity against strains of Pseudomonas aeruginosa and Candida albicans, but none was detected against Enterococcus faecalis. When tested on saliva samples from children, the decoction showed great potential in reducing the load of microorganisms, the inhibition haloes produced with saliva samples being similar to those of the antimicrobial control (0.12% chlorhexidine). The pomegranate peel decoction in water could thus provide a promising source for developing solutions for use against oral diseases.
Keywords: Punica granatum. Pomegranate. Antibacterial activity. Medicinal plant.

INTRODUCTION

Brazil has a great potential for the development of herbal medicines, having the highest plant diversity in the world (Oliveira et al., 2007). In this context, phytochemical compounds have assumed an important role as economically viable medicines and alternative treatments in dentistry, owing to their antimicrobial properties against oral diseases, especially those arising from biofilm (Petersen et al., 2005; Pereira et al., 2006; Oliveira et al., 2007). *Punica granatum* L. is a shrub, about 3 meters high, or small tree. Its phytochemical analysis shows the presence of up to 28% gallic tannins in the stem, bark, fruit and, in lesser amounts, in the leaves (Lorenzi & Matos, 2008; Jardini et al., 2010; Moneim 2012). Fixed oils, such as punicic acid (Jardini et al., 2010), are found in the seed oil. The presence of various phenolic acids, including ellagic, gallic, salicylic and quinic acid, has been reported (Lorenzi & Matos, 2008; Jardini et al., 2010; Moneim 2012).

Dental caries is an infectious disease influenced by factors such as the relationship between diet and cariogenic microorganisms in the oral cavity and the characteristics of the host. These factors are important in the kinetics of the establishment and progression of the lesion (Petersen et al., 2005; Palombo, 2011; Klinke et al., 2009). The interaction between them can result in localized and progressive loss of the mineralized tissue of teeth (Petersen et al., 2005; Palombo, 2011; Klinke et al., 2009). Periodontal disease is caused by bacteria associated with dental plaque. Among these, *Streptococcus mutans* is one of those best adapted to the cariogenic environment and their natural habitat is tooth surface. However, many other relevant species have characteristics that may help the progress of the lesion (Marinho & Araujo, 2007), such as the lactobacilli, lactic acid producers, since they show extreme tolerance to acidic environments, and *Candida albicans*, which is associated with dental caries in all age groups (Klinke et al., 2009). This yeast is associated with periodontal disease and caries, owing to its presence at sites such as the tongue mucosa, plaque, beneath the gums and caries and accompanying false teeth. The genera *Pseudomonas* and *Enterococcus* have virulence factors capable of aggravating the periodontal disease and have been implicated in refractory cases of periodontal disease. These microorganisms hinder conventional periodontal treatments, damaging the lives of debilitated patients who are elderly or suffer from immunosuppression. Several attempts have been made to eliminate or reduce potential pathogenic microorganisms of the oral microbiome. The continual use of antimicrobial substances generates undesirable effects, enhancing the search for new antimicrobial products for oral use (Palombo, 2011; Awadalla et al., 2011; Karthikeyan et al., 2011).

This environment is predominantly comprised of gram-negative anaerobic species (Souto et al., 2006). Despite the many advances that have occurred in all areas of health, the prevalence of dental caries in industrialized countries remains very high, reaching 60-90% of school-

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age children and also adults (Petersen et al., 2005; Palombo, 2011; Karthikeyan et al., 2011). Several attempts have been made to eliminate or reduce the number of pathogenic microorganisms in the oral microbiome. Given the broad spectrum of antibacterial applications already described (Menezes et al., 2008) for the tea and pomegranate extracts, this study was designed to assess the *in vitro* antimicrobial activity of the decoction of *P. granatum* L. fruit peel.

MATERIAL AND METHODS

Plant material and preparation of aqueous decoction **–** Commercial fruit peels of *P. granatum* were acquired in the Municipal Market of Juiz de Fora, Minas Gerais, Brazil. The material was dried in an oven for 7 hours and ground in an electric mill, thereby producing 30 g of powder. The decoction was carried out by adding the milled peel to 300 mL of distilled water in a flask. This suspension was heated and kept boiling for 20 minutes, after which it was left to cool and the decoction was filtered, rendering a concentration of 100 mg/mL.

Saliva samples from schoolchildren **–** Samples of saliva (n=20) were taken from children (8-11 years old), in Juiz de Fora, Minas Gerais, Brazil. Initially, 10 saliva samples were exposed to various concentrations of decoction. In a second assay, 10 new saliva samples were used to test the decoction at a single concentration: 100 mg/mL. The saliva samples were collected in clean, dry vials, under non-stimulated conditions, and were maintained under refrigeration at 4 °C. The project was approved by the Research Ethics Committee of the Federal University of Juiz de Fora (Protocol number 2543.283.2011).

Antimicrobial activity **in vitro** ***test*** - The method was tested on solid diffuson medium with standard ATCC (American Type Culture Collection) strains of *Candida albicans* (ATCC 18804), *Enterococcus faecalis* (ATCC 29212) and *Pseudomonas aeruginosa* (ATCC 1107099). The microorganisms were obtained from the Oswaldo Cruz Foundation (FIOCRUZ) collection, Rio de Janeiro, Brazil. To grow the inocula of the strains, 100 µL of BHI (Hymedia) was seeded with bacteria transferred by a sterile swab from a culture on BHI solid medium and incubated at 37 °C for 24 h under aerobic conditions. Portions of this culture were placed in test tubes containing 0.9% sterile saline solution and the turbidity was adjusted to match the 0.5 standard ($15x10^8$ cells/mL) on the McFarland scale. Aliquots of 200 µL of this microbial suspension were transferred to petri dishes containing BHI agar and spread with a sterile swab and dried. Round wells of about 6 mm were bored in the culture medium (2 per plate) and 50 µL of pomegranate decoction was introduced into each well. As a control, a solution of 0.12% chlorhexidine was also tested. The plates were incubated at 37 ºC for a period of 24 hours. The inhibition zones formed were then measured (in mm). An aliquot of each saliva sample (1 mL) was diluted in sterile 0.9% NaCl, to yield the dilutions 10^{-1}, 10^{-2} and 10^{-3}, and processed as described above for the standard strains. The results for these dilutions were similar. The halo diameters were analyzed statistically by the Kruskal-Wallis test. Ten haloes were measured for each saliva sample.

RESULTS

In the conditions used in the *in vitro* test, the pomegranate decoction did not fully inhibit the growth of *Enterococcus faecalis*. Within the inhibition zones, small isolated colonies could be seen. The chlorhexidine control produced inhibition haloes of 17.11 mm (Table 1). At concentrations of 60, 80 and 100 mg/mL, the decoction showed inhibition zones against *C. albicans*. The diameters of these haloes were similar to those produced by the control (22.81±0.952 mm), showing antimicrobial activity (Table 1). Statistical analysis revealed that the decoction of pomegranate, at 60, 80 and 100 mg/mL, produced larger growth inhibition zones ($p < 0.001$) than chlorhexidine against *P. aeruginosa* (Table 1).

The saliva samples analyzed showed sharply defined inhibition haloes, when assayed with the decoction or the control (Table 2). The chlorhexidine solution inhibited the growth of microorganisms within an average zone diameter of 21.39 mm. The largest inhibition zones were obtained at a concentration of 100 mg/mL of the decoction (Table 2). The test was repeated on 10 different saliva samples at a dilution of 10^{-1}, with 100 mg/mL of the decoction. The data showed differences in susceptibility between saliva samples. In saliva samples from two children, the growth inhibition was observed with 0.12% chlorhexidine but not with the decoction. The overall average diameter of the inhibition haloes for the decoction in this second experiment was 24.49±12.81 mm, statistically higher than the average for the control (21.26 mm; p=0.0014; Table 3).

Table 1: Antimicrobial potential of pomegranate decoctions assessed at several concentrations (20-100 mg/mL) against type strains of *Enterococcus faecalis*, *Candida albicans* and *Pseudomonas aeruginosa*. Values are mean diameters of inhibition zones ± standard deviation.

Microorganism/ Pomegranate decoction	*E. faecalis*	*C. albicans*	*P. aeruginosa*
Control	17.11±0.703	22.81±0.952	20.03±0.891
20 mg/mL	-	14.50±1.175^{ns}	13.65±0.906^{ns}
40 mg/mL	-	18.32±0.766^{ns}	20.97±1.370^{ns}
60 mg/mL	-	20.36±0.993^{ns}	22.88±1.175$^{\#}$
80 mg/mL	-	19.87±1.478^{ns}	22.70±0.369$^{\#}$
100 mg/mL	-	21.90±0.762^{ns}	23.91±1.280$^{\#}$

Control - Chlorhexidine solution (0.12%). ns - not significant . (#) $p<0.01$.

Table 2: Average inhibition zone diameters obtained with saliva samples from children at a dilution of 10-1 (200 µL) on BHI plates, with various concentrations of pomegranate peel decoction in wells.

Saliva sample	Diameter of inhibition zones (mm)					Control
	20 mg/mL	40 mg/mL	60 mg/mL	80 mg/mL	100 mg/mL	Chlorhexidine 0.12%
1	21.98	23.81	27.85	28.22	30.76	19.62
2	0	24.95	26.91	26.65	26.56	20.66
3	0	0	0	30.49	32.67	25.70
4	18.04	21.19	22.13	23.56	24.43	22.00
5	0	0	0	0	0	20.88
6	21.08	22.29	24.12	26.36	28.78	22.22
7	19.595	21.15	22.22	23.70	25.59	21.38
8	18.73	21.23	22.24	25.56	27.68	20.61
9	0	0	0	0	0	20.83
10	20.29	21.21	23.07	24.11	28.22	20.01

Table 3: Inhibition of growth of bacteria from saliva samples incubated with pomegranate peel decoction at a concentration of 100 mg/mL. Values represent mean diameters of haloes in duplicate experiments.

Individual saliva sample	Diameter of inhibition zones (mm) around pomegranate decoction at 100 mg/mL	Chlorhe-xidine 0.12%
1	0	26.02
2	35.93	19.36
3	0	21.10
4	29.35	22.26
5	32.13	21.35
6	29.67	19.11
7	27.84	19.07
8	32.25	20.63
9	30.81	21.16
10	26.86	21.89

DISCUSSION

There are reports in the literature about the ability of pomegranate extracts to show bacteriostatic and bactericidal activity (Pereira et al., 2005, Vasconcelos et al., 2006; Siddiqi et al., 2007). The action of the ethanolic extracts against the microorganisms present in the supragingival biofilm revealed the potential of *P. granatum* to control the population of aerobic microorganisms (Souto et al., 2006; Jardini et al., 2010). However, it should not be used in direct contact with mucous membranes. The continuous use of alcoholic extracts may be sufficient to stimulate the expression of hyper-keratinized injury and genotoxic effects (Marinho & Araújo, 2007).

The decoction of pomegranate, while showing no bacteriostatic or bactericidal activity against *E. faecalis*, gave highly relevant results against *C. albicans* and *P. aeruginosa*. In the case of *P. aeruginosa*, the results were very promising, since the haloes were equivalent to or better than the control, at concentrations of 60 mg/mL or more. The decoction of pomegranate, prepared in an aqueous vehicle, could be developed into an antimicrobial solution to be applied to the control of organisms of relevance to oral pathology.

Chlorhexidine, used as the positive control, was effective for the *in vitro* trials, against both the ATCC strains and the saliva samples. This antiseptic was introduced for many years as a broad spectrum antimicrobial mouthwash. Its action is associated with the disrupting of the cell membrane of the microorganism, resulting in the loss of vital cell constituents. However, despite its high antimicrobial efficacy, prolonged use is limited by side effects that may arise, such as reversible desquamation of oral mucosa, staining of teeth and changes in taste (Hofer et al., 2011). The decoction uses water as the extracting medium. This preparation of pomegranate peel extracted a large quantity of tannins with antimicrobial activity, as already shown in other studies (Lorenzi & Matos, 2008; Jardini et al., 2010; Moneim 2012). Thus, the antimicrobial activity observed at several concentrations of the pomegranate decoction may be ascribed to these phenolic derivatives of high molecular weight. These results indicate that the decoction of pomegranate is a viable option for the development of products for oral use.

CONCLUSIONS

Based on the recent success of herbal medicines and the discovery of side effects of using the antiseptic chlorhexidine and mouthwashes containing ethanol, this study is intended as a preliminary study to promote the development of new aids for the prevention and treatment of cariogenic diseases. From the decoction of *P. granatum* (pomegranate), substances with antimicrobial activity were extracted, which may contribute to the composition of products for use against oral infections and to control microorganisms in the oral cavity by reducing bacterial plaque.

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RESUMO

Avaliação in vitro do potencial antimicrobibiano do decoto de cascas de romã (Punica granatum L.) sobre amostras de saliva de escolares

Existem vários esforços para o desenvolvimento de produtos capazes de reduzir ou eliminar os microrganismos patogênicos presentes na cavidade oral. A literatura relata uma série de efeitos adversos associados ao uso contínuo destes produtos, dentre eles vômitos, diarreia e o escurecimento da dentina. A indução da resistência microbiana é um dos fatores de destaque relacionado ao uso destes produtos. Neste trabalho, o decocto de romã (Punica granatum L.), obtido a partir das cascas do fruto, foi utilizado para avaliação de seu potencial antimicrobiano sobre cepas de Pseudomonas aeruginosa, Candida albicans e Enterococcus faecalis, sendo ativos contra os dois primeiros microrganismos. A aplicação do decocto sobre os microrganismos presentes em amostras de saliva de crianças mostrou halos de inibição semelhantes ao obtido com a solução de clorexidina a 0,12%. A atividade antimicrobiana do decocto de romã aponta esta preparação como uma fonte em potencial para o desenvolvimento de produtos de uso oral.

Palavras-chave: Pun ica granatum. Romã. Atividade antimicrobiana. Plantas medicinais.

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