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Effect of *Punica granatum* on the virulence factors of cariogenic bacteria *Streptococcus mutans*

Zandiswa Gulube, Mrudula Patel*

Division of Oral Microbiology, Department of Oral Biological Sciences, School of Oral Health Sciences, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

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ABSTRACT

Dental caries is caused by acids produced by biofilm-forming *Streptococcus mutans* from fermentable carbohydrates and bacterial byproducts. Control of these bacteria is important in the prevention of dental caries. This study investigated the effect of the fruit peel of *Punica granatum* on biofilm formation, acid and extracellular polysaccharides production (EPS) by *S. mutans*. Pomegranate fruit peels crude extracts were prepared. The Minimum bactericidal concentrations (MBC) were determined against *S. mutans*. At 3 sub-bactericidal concentrations, the effect on the acid production, biofilm formation and EPS production was determined. The results were analysed using Kruskal-Wallis and Wilcoxon Rank Sum Tests. The lowest MBC was 6.25 mg/mL. *Punica granatum* significantly inhibited acid production ($p < 0.01$). After 6 and 24 h, it significantly reduced biofilm-formation by 91% and 65% respectively ($p < 0.01$). The plant extract did not inhibit the production of soluble EPS in either the biofilm or the planktonic growth. However, it significantly reduced the insoluble EPS in the biofilm and the plantktonic ($p = < 0.01$) form of *S. mutans*. The crude extract of *P. granatum* killed cariogenic *S. mutans* at high concentrations. At sub-bactericidal concentrations, it reduced biofilm formation, acid and EPS production. This suggests that *P. granatum extract* has the potential to prevent dental caries.

1. Introduction

The oral cavity contains a wide variety of commensal microflora that exists in harmony with the host. *Streptococcus mutans* is one of the major flora and it exist in the form of biofilm.

It has ability to rapidly ferment dietary carbohydrates, particularly sucrose, produce strong acids and cause demineralization of enamel called dental caries. Dental caries is a disease of the mineralized tooth that affects the majority of people worldwide. In addition, *S. mutans* also causes root canal infections, odontogenic abscesses and endocarditis. The acidic environment also stimulates growth and virulence of *Candida* in the oral cavity [1,2]. *Streptococcus mutans* also produce soluble and insoluble extracellular polysaccharides (EPS) which serve as nutrients and adherence moieties [3]. Therefore, biofilm formation, production of acids and EPS are considered virulence properties of *S. mutans*. Inhibition of acid, EPS and biofilm formation is important in the prevention of dental caries [4]. Many oral hygiene products containing antimicrobial compounds such as fluoride, triclosan, chlorhexidine and iodine, have been used for many years [3].

Medicinal plants have attracted attention due to their antimicrobial effects against oral bacteria. For example propolis, *Mikania species*, *Nidus vespae* have shown antiplaque properties [5–7]. *Punica granatum* (pomegranate) has been used traditionally for treatment of infections throughout the world, including South Africa [8]. Parts of pomegranate fruit such as juice, seeds and peel have been found to have antibacterial properties [9,10]. The peel of the pomegranate fruit also has antifungal property against unicellular fungi, *Candida albicans* [11]. At high concentrations, the fruit juice and whole fruit reduces oral plaque and periodontal pathogens [12,13]. However, studies have shown that the peel extracts have antibacterial effect against oral pathogens particularly cariogenic bacteria [14,15]. This study investigated the effect of *P. granatum* fruit peel on virulence properties such as biofilm formation and, acid and extracellular polysaccharides production of cariogenic *S. mutans*.

* Corresponding author. Division of Oral Microbiology, Department of Oral Biological Sciences, School of Oral Health Sciences, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, 2050, South Africa.
E-mail address: Mrudula.patel@wits.ac.za (M. Patel).

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2. Materials and methods

2.1. Cultures and inocula

Ethical clearance was obtained from The Committee for Research on Human Subjects (Medical), University of the Witwatersrand (Clearance number 10,205). Saliva samples from patients attending the Dental clinic at Charlotte Maxeke Teaching hospital, Johannesburg were collected and cultured on Mutans Bacitracin agar to isolate *Streptococcus mutans* (clinical isolates). Five clinical isolates of *S. mutans* were used in the study. These cultures were identified using API 20 Strep auxanogram (Bio Mèrieux) and additional biochemical reactions.

Fresh inocula with optical density of 0.2 (405 nm) containing approximately 10^5–10^6 organisms per millilitre were prepared and used throughout the study.

2.2. Plant material and extract preparation

Punica granatum fruits were collected from Zanddrift Farm in Cape Town, South Africa. The peel was harvested, dried, milled and stored at 4 °C until required. One gram of fine powder was extracted in 10 mL methanol with vigorous shaking and centrifugation. The procedure was repeated three times. The solvents were removed under a cold air stream which produced a yield of 0.15 g dried extract. The crude dry extract was weighed and dissolved in 10% DMSO to obtain a solution containing 50 mg of crude extract per ml of DMSO. Fresh extracts were prepared for each experiment.

2.3. Minimum bactericidal concentration (MBC)

Two-fold dilution of crude peel extract was prepared using tryptone broth in a microtitre plate. Fresh inocula, with optical density of 0.2 (405 nm) containing approximately 10^5–10^6 cfu/mL were prepared and added to the wells. The plates were incubated at 37 °C under CO_2 for 48 h. Chlorhexidine gluconate was used as a positive control and water as a negative control. Effect of 10% DMSO on the bacterial growth was determined. After incubation, each well was subcultured on blood agar and incubated at 37 °C under CO_2 for 48 h. It was difficult to obtain MIC results because the peel extract precipitated with the culture medium. Therefore, MBC results were obtained, which were more suitable in determining subinhibitory concentrations for the subsequent experiments. The lowest concentration, that had no growth was recorded as MBC for that test organism. Each experiment was performed in triplicate. Three sub-bactericidal concentrations were selected for the subsequent experiments.

2.4. Acid production by S. mutans

Thirty milliliters tryptone broth containing three sub-bactericidal concentrations, (1.56, 3.125 and 6.25 mg/mL) of methanolic crude peel extract was inoculated with 100 μL of inoculum containing 10^7 cfu/mL of *S. mutans* and incubated for 16 h. The pH of the media was measured at 0 and after 10, 12, 14 and 16 h [16]. Water was used as a control. The effect of DMSO was also determined. *Streptococcus mutans* count was also measured at 0 and after 10, 12 and 14 h using microdilution technique. The experiments were performed in triplicates on the five strains of *S. mutans*. The results were compared using Kruskal-Wallis test.

2.5. Biofilm formation

The effect of crude peel extract on the biofilm formation by *S. mutans* was also studied using a modified technique described by Naidoo et al., in 2012 [17]. Each strain of *S. mutans* was grown on glass slides in a beaker containing tryptone broth and 3 methanol extracts (1.56, 3.125 and 6.25 mg/mL) at 37° C under CO_2 for 30 h. The medium was changed after 24 h. A slide with biofilm was removed after 6, 24 and 30 h. Biofilms were washed with PBS, the attached cells were scrapped off the slides, suspended in PBS, vortexed and quantified using serial dilution technique. Briefly, one hundred microliters of tenfold dilutions (1:10, 1:100, 1:1000, 1:10,000) were spread on blood agar plates, incubated under CO_2 at 37 °C for 48 h. Colony counts were performed and the bacterial counts were calculated. Negative controls with water and positive controls with 0.2% CHX, instead of plant extracts, were included in each experiment. The experiments were repeated three times and the results were compared using Wilcoxon Rank Sum Test.

2.6. Soluble extracellular polysaccharide production (SEPS)

The effect of *P. granatum* on the EPS production was studied using the technique described by Koo et al. (2003) and Dubois et al. (1956) [18,19]. *Streptococcus mutans* strains were grown in 5% sucrose broth for 48 h. Five millilitres of the culture was centrifuged at 10,000 g for 10 min (repeated three times). Supernatant was collected and placed in a clean centrifuge tube. Three volumes of absolute ethanol were added and placed at −20 °C for 30 min to precipitate polysaccharides. The mixture was centrifuged, pellet was washed with 70% alcohol and the precipitate was resuspended in 5 mL NaOH. Two ml of 5% aqueous solution of phenol and 5 mL of concentrated sulphuric acid was added to the mixture, which was allowed to stand at room temperature in the dark for 10 min. Thereafter, it was vortexed for 30 s, placed in a water bath at 25 °C for 20 min and the absorbance was read at 490 nm. Each experiment was repeated three times on the five strains of *S. mutans*.

2.7. Insoluble extracellular polysaccharide production (IEPS)

Five ml of 1 M sodium hydroxide was added to the pellet. The suspension was vortexed for 30 s, agitated for 15 min and centrifuged for 5 min at 10000 g. The supernatant was transferred to a beaker. This step was repeated three times. The supernatant was pooled together and three times volume of cold ethanol was added. The polysaccharides were allowed to precipitate at −20 °C for 30 min. The mixture was centrifuged and the pellet was washed with 70% ethanol. The precipitate was then resuspended in 5 mL of NaOH. Two ml of 5% aqueous solution of phenol and 5 mL of concentrated sulphuric acid was added rapidly to the mixture and was left to stand at room temperature in the dark for 10 min. Thereafter, it was vortexed for 30 s, placed in water bath at 25 °C for 20 min and the absorbance was read at 490 nm. These experiments were repeated three times for 5 strains of *S. mutans*. The results were compared using Wilcoxon Rank Sum Test.

3. Results

3.1. MBC

DMSO had no effect on bacterial growth of *S. mutans*. The positive control chlorhexidine gluconate killed the test strains. MBC for *S. mutans* ranged from 12.5 to 25 mg/mL. Based on these MBC, sub-bactericidal concentrations of 1.56, 3.125 and 6.25 mg/mL were used to determine the effect on biofilm formation, acid and extracellular production. (see Table 1).

3.2. Acid production

Punica granatum significantly inhibited acid production