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Research

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The Antimicrobial Activity of Pomegranate Polyphenol Extract (POMx) Lozenges in a Saliva-Derived Biofilm Model System

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ABSTRACT

The ellagitannin type polyphenols present in Pomegranate extracts (POMx) have been associated with numerous health benefits including antibacterial activities. Despite their antibacterial potency, however, these purified pomegranate polyphenol extracts need to be incorporated into a delivery system for convenient human treatments. In this study, we performed a detailed investigation of the antimicrobial activity of pomegranate polyphenol fortified lozenges against oral bacteria including the major cariogenic species *Streptococcus mutans*. Since oral bacteria exert their cariogenic effects when they are attached as biofilms on the tooth surface, we developed a stationary saliva-derived biofilm model for high throughput parallel screening to assess effects on bacteria in their biologically relevant mode. We found strong antibacterial activities (up to >99% killing of biofilm cells) for the POMx lozenges at exposure times (15-20 min) relevant for lozenge consumption. Most interestingly, *S. mutans* appeared to be even more sensitive to these products than the general biofilm population. Furthermore, consistent with data derived for polyphenols extracted from other plants, the POMx lozenges completely prevented bacterial surface adherence as determined by live imaging. In summary, our data show that the strong antibacterial and anti-adherence activities of pomegranate extracts are maintained after processing in products such as the POMx lozenges that can be easily consumed and are therefore excellent candidates for prevention and possibly treatment of oral disease.

KEYWORDS: Pomegranate extract; Antimicrobial activity; Adherence; Biofilm; *Streptococcus mutans.*

ABBREVIATIONS: POMx: Pomegranate extracts; HBP: Hop Bract Polyphenols; GAE: Gallic Acid Equivalents; Scr: Sucrose; BHI: Brain Heart Infusion; MIC: Minimum Inhibiting Concentration; PBS: Phosphate-Buffered Saline.

INTRODUCTION

Oral plaque (biofilm) control is essential to oral disease prevention due to its strong association with dental caries and periodontitis incidence.[1] Typical approaches to oral biofilm reduction include treatment with antibacterial synthetic chemical compounds such as chlorhexidine or triclosan.[2] In recent years, the search for effective antimicrobials lead to a renewed interest in traditionally used natural substances in medical as well as the dental applications. An increasing body of research explores the antibacterial potential of especially plant extracts such as polyphenols in search for more effective and readily available biofilm control agents.[3-14] A considerable portion of these reports focuses on the efficacy against *Streptococcus mutans,*[3,5-9,11,15-24] an oral pathogen which has been recognized as the primary etiological agent for dental caries.[25]

Polyphenols extracted from various plants were reported to display a broad spectrum

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of antimicrobial, antiviral and antifungal activities,[26] which include a positive effect on oral health.[27] More detailed mechanistic studies revealed that green and oolong tea extract containing catechines and other polymeric polyphenol inhibit glucosyltransferase activity in different species of mutans streptococci, consequently reducing glucan synthesis.[5,16,19] Diminished glucan synthesis was reported to decrease *S. mutans* surface attachment *in vitro*[28] as well as caries incidence in *S. mutans* infected rats.[29] Similar to the polyphenols found in tea leafs, polyphenols isolated from Hop Bract Polyphenols (HBP) exhibited inhibition on *S. mutans* glucan synthesis and adherence *in vitro*.[9] More recently, a clinical study revealed that HBP can suppress plaque re-formation during a three day period after complete plaque removal.[22]

Another botanical product with a natural high content of polyphenols are pomegranates.[30,31] These fruits have been cultivated since ancient times and were early on linked to numerous health benefits which include strong antibacterial activities.[26] A number of clinical trials have confirmed the health promoting effect of pomegranate based products in reducing low-density lipoprotein oxidation and plaque build up in atherosclerotic lesions,[32-34] and most recently, a clinical study indicated that an alcoholic pomegranate extract is active against dental plaque bacteria.[27]

In this study, we evaluated the antimicrobial effect of lozenges containing a pomegranate extract (POMx) that differs in composition from a previously reported alcoholic extract.[27] We developed an *in vitro* saliva-derived biofilm model that enables assessment of effects against oral biofilms and the primary causative agent of dental caries, *S. mutans* in particular. Inhibition of oral bacterial surface adherence was monitored using time-lapse video microscopy. The finding that the POMx containing lozenges exhibit very strong antibacterial as well as anti-adherence activities is very promising, since these products are ready for human consumption and can be easily applied for therapeutic treatment of oral diseases.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

S. mutans strain JM11 (UA140::*pldh-luc*, Spc^r)[35] was grown anaerobically (N_2 85%, H_2 10%, and CO_2 5%) at 37 °C in BHI medium or on BHI agar plates supplemented with 800 µg/ml spectinomycin (Sigma, St Louis, MO, USA).

Treatment Solutions

Pomegranate extract (POMx) containing lozenges were obtained from POM Wonderful, LLC (Los Angeles, CA, USA). Lozenges were manufactured according to standard hard lozenge sugar process (Yummy Earth, LLC) containing two levels of purified pomegranate polyphenol extract powder (POMx). Lozenge-50 and lozenge-100 contained 1.44% for 10,000 ppm (50 mg/lozenge) and 3.09% for 20,000 ppm (100 mg/lozenge) polyphenol Gallic Acid Equivalents (GAE), respectively. The mean lozenge weight was 5 grams with the hard lozenge base mainly comprised of Sucrose (Scr). The lozenges were dissolved in 10 ml microorganism-free saliva per one lozenge since 10 ml is the average amount of saliva produced by a person eating one lozenge considering an average whole saliva production of about 0.6 ml/min and a dissolving time of about 15-20 min.[36] To address the effect of sugar in the lozenge base, 5 grams of Scr were dissolved in 10 ml microorganism-free saliva (resulting in a 50% Scr solution) as a placebo control. Microorganism-free saliva was prepared by collecting unstimulated saliva from 6 different subjects followed by a high speed spin (10 min at 16,000×*g*) and filter-sterilization (0.22 µm Millipore Corp. Billerica, MA, USA) for removal of debris and bacteria. Treatment with microorganism-free saliva served as a negative control to adjust for "carrier" and wash solution effects, whereas 70% EtOH was used as a positive microbiocidal control.

Determination of the Minimal Inhibitory Concentration (MIC)

An overnight culture of *S. mutans* strain JM11 was grown anaerobically (N_2 85%, H_2 10%, and CO_2 5%) in Brain Heart Infusion (BHI) at 37 °C and diluted to 10^5 cells/ ml. Hundred µl of this bacterial culture were placed into the necessary number of wells of a 96-well culture plate. A series of 1:2 dilutions of the treatment solutions was prepared, 100 µl portions thereof were added to each well and incubated overnight in an anaerobic chamber (N_2 85%, H_2 10%, and CO_2 5%) at 37 °C. Minimum Inhibiting Concentration (MIC) was determined to be the highest dilution at which no viable cells were observed as evaluated by both microscopic examination using cell viability stains (Invitrogen) and plating on BHI plates.

Biofilm growth

Biofilms were grown in sterile 48-well cell culture plates that had been coated with about 100 µl of 50% microorganism-free saliva in Phosphate-Buffered Saline (PBS) (pH 7.0) per well (see treatment solutions for preparation of microorganism-free saliva). After air-drying the saliva solution completely, the wells were UV sterilized for 1 hour. Biofilms were then seeded into the coated wells as follows: Unstimulated saliva was collected (in the late afternoon) from 6 subjects who had refrained from cleaning their teeth for about 8 hrs. This saliva was pooled at equal proportions, diluted 1:4 into BHI supplemented with 1% glucose, 1% mannose and 1% sucrose and subjected to a low speed centrifugation (10 min at 600×*g*) to remove eukaryotic cells and large debris. For specific evaluation of antibacterial effects on *S. mutans* in a saliva-derived biofilm setting, this cariogenic oral pathogen was added to the salivary bacteria as follows: An overnight culture of thespectinomycin-resistant *S. mutans* strain JM11 was grown in unsupplemented BHI, diluted 1:3 in BHI and further grown into the logarithmic growth phase.

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About 1.25×10^6 of these *S. mutans* cells were added together with 400 µl of the saliva mixture into each well of the saliva-coated 48-well cell culture plate. The inoculated plates were incubated anaerobically (N_2 85%, H_2 10%, and CO_2 5%) at 37 °C overnight (16 to 18 hrs for the treatments up to 30 min of duration and 24 hrs for the overnight treatment) to allow biofilm formation. This procedure would typically yield saliva-derived biofilms containing about 0.5 to 1×10^8 total biofilm cells with an *S. mutans* proportion of 5 to 10%.

Biofilm treatments

Prior to exposure to the above treatment solutions, the growth medium was removed from the biofilms and they were washed twice with 800 µl PBS. Residual PBS was carefully removed and 100 µl (200 µl for overnight treatment) of the respective treatment was added. The biofilms were incubated with the treatment solutions for the indicated time periods and the treatment was stopped by addition of 800 µl PBS. All liquid was then removed immediately and the biofilms were washed two more times with PBS. Exposure to PBS does not interfere with biofilm viability (data not shown). Treatment efficiency was evaluated by determining colony forming units (cfu). Biofilms were vigorously resuspended by pipetting in 1 ml BHI to break up the biofilms. Serial dilutions were prepared and plated for enumeration of surviving biofilm cells. To determine the effect of the treatment solution on *S. mutans*, an aliquot of the serial dilutions was plated onto BHI plates containing 800 µg/ml spectinomycin, which only allows growth of the *S. mutans* derivative JM11 that was added to the saliva-derived biofilms. Total biofilm survival was evaluated as mentioned above by plating an aliquot of the same serial dilutions onto BHI plates without antibiotic. The plates were incubated anaerobically o/n and colony forming units were counted. Treatments were typically performed in triplicate.

Assessment of bacterial adherence by video microscopy

Additionally, biofilm formation was monitored by video microscopy to address the effect of above treatments solution on the ability of bacteria to attach to the surface as well as their growth. Saliva was collected and processed as described above for preparation of biofilms. The microorganisms present in 500 µl salivary solution were collected *via* high speed centrifugation (10 min@16,000×*g*), the supernatant was removed and the pellet resuspended in 1 ml treatment solution, followed by an additional 1:5 dilution. Four hundred µl of the diluted saliva sample were placed into a well of a 24-well cell culture plate. The bacterial cells were allowed to settle for 30 min prior to recording. The plate was positioned on an inverted microscope (Nikon Eclipse TE300) fitted with a temperature stage set to 37 ºC. Biofilm development was monitored over a 7.5 hr time period with a live imaging system (SPOT camera/software-Diagnostic Instruments, Inc).

RESULTS

Effect of pomegranate polyphenol based POMx lozenges on the cariogenic oral bacterium *Streptococcus mutans*

First, the potential impact of the pomegranate extract-based POMx lozenges on oral health was evaluated by determining their MICs for *S. mutans*, an oral species that has been strongly associated with the development of dental caries. The *S. mutans* killing efficacy was directly correlated with the polyphenol content of the POMx lozenges and displayed significant antimicrobial effects at 1:4 and 1:8 final dilutions for lozenge-50 and Lozenge-100, respectively, which corresponds to about 1.25 mg/ml GAE for each lozenge (Table 1). In contrast, the 50% Scr in microorganisms-free saliva lozenge base control did not seem to have any negative effect on *S. mutans* growth despite the high osmolarity of the solution.

Treatment	MIC *Streptococcus mutans*
Microorganism-free saliva	No effect
50% Scr	No effect
Lozenge-50	1:4dil (1.25 mg/ml GAE)
Lozenge-100	1:8dil (1.25 mg/ml GAE)

The 50% Scr solution and the lozenges were prepared in microorganism-free saliva as indicated in the Materials and Methods section.

Table 1: MICs of POMx containing lozenges for *Streptococcus mutans*.

Effect of pomegranate polyphenol based POMx lozenges on the cariogenic oral bacterium *Streptococcus mutans* grown in a saliva-derived biofilm environment

MICs are typically obtained with planktonic cells. Most oral microorganisms including *S. mutans*, however, reside in complex oral biofilm communities also referred to as dental plaque. Since biofilm cells are more resilient to antimicrobial agents and have different surface properties compared to planktonic cells,[37] the effect of pomegranate polyphenol based products tested in this study on *S. mutans* was evaluated in the context of a saliva-derived biofilm environment. Addition of 1.25×10^6 *S. mutans* cellsto the saliva biofilm inoculum resulted in a proportion of *S. mutans* comprising 5-10% of the total biofilm cells (Figures 1, 2 and 3-saliva treated control). The biofilms were incubated overnight anaerobically at 37 ºC in the presence of the different treatment solutions. A striking reduction (more than 4 orders of magnitude) of total biofilm cell viability similar to EtOH treatment was observed for POMx lozenges when compared to the microorganism-free saliva control. Exposure to 50% Scr did not reduce the overall biofilm population but surprisingly resulted in a 10-fold reduction in the proportion of *S. mutans* (Figure 1).

To narrow down the effective time scale of the POMx lozenges tested in this study, overnight grown *S. mutans*-spiked

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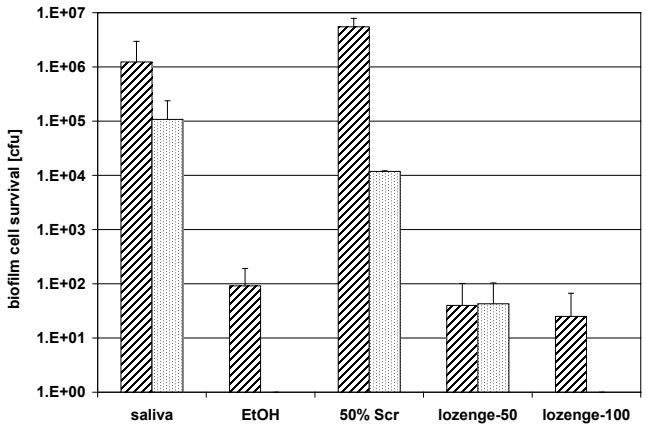

Figure 1: Survival of S. *mutans*-spiked saliva-derived biofilms after overnight exposure with treatment solutions. Shown are the colony forming units (cfu) on BHI (striped columns to indicate total biofilm cell survival) and on BHI supplemented with spectinomycin (dotted columns to indicate specific S. mutans survival) after biofilms were treated with the respective treatment solution including negative (saliva) and positive (70% EtOH) controls overnight. A minimum of three experiments was performed for each treatment solution.

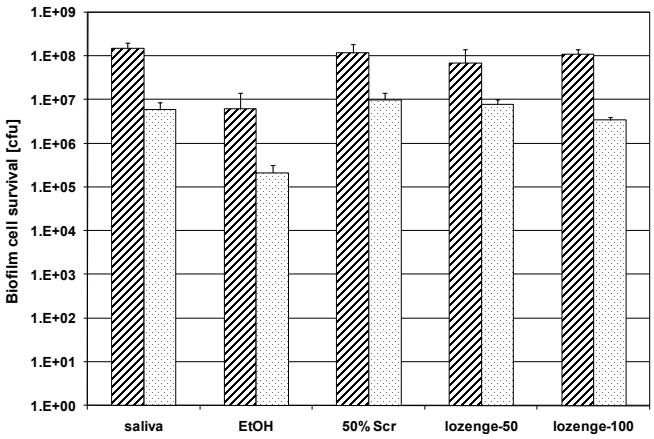

Figure 2: Survival of S. *mutans*-spiked saliva-derived biofilms after 1 min exposure with treatment solutions. Shown are the colony forming units (cfu) on BHI (striped columns to indicate total biofilm cell survival) and on BHI supplemented with spectinomycin (dotted columns to indicate specific *S. mutans* survival) after biofilms were treated with the respective treatment solution including negative (BHI, PBS, saliva) and positive (70% EtOH) controls for 1 min. A minimum of three experiments was performed for each treatment solution.

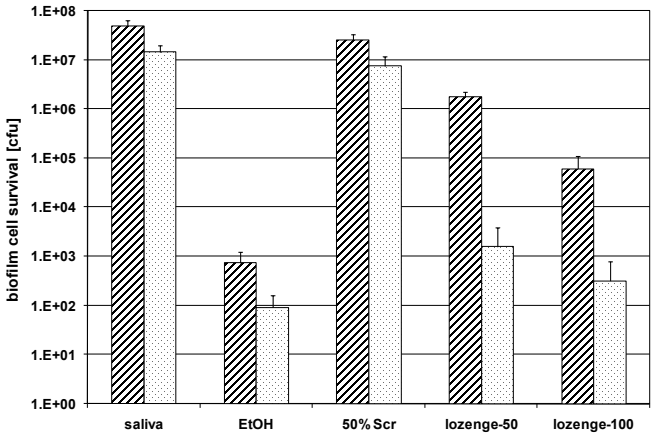

Figure 3: Survival of S. *mutans*-spiked saliva-derived biofilms after 30 min exposure with treatment solutions. Shown are the colony forming units (cfu) on BHI (striped columns to indicate total biofilm cell survival) and on BHI supplemented with spectinomycin (dotted columns to indicate specific *S. mutans* survival) after biofilms were treated with the respective treatment solution including negative (BHI, PBS, saliva) and positive (70% EtOH) controls for 30 min. A minimum of three experiments was performed for each treatment solution.

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saliva-derived biofilms were exposed to the different treatment solutions for 1 min or 30 min, respectively. At the relatively short treatment time of 1 min, a slight reduction of *S. mutans* viability was observed for treatments with POMx lozenge-100 compared to the negative control treatment with microorganism-free saliva (Figure 2-dotted bars). The overall saliva-derived biofilm population, however, did not appear to be affected by any of the treatment solution at this exposure time (Figure 2-striped bars). Upon longer exposure (30 min), a different picture emerged. Both POMx lozenges produced a striking killing effect of 3 to 4 orders of magnitude on *S. mutans* (Figure 3-dotted bars). A less pronounced but nonetheless significant reduction in overall biofilm viability (more than one order of magnitude for lozenge-50 and almost three orders of magnitude for lozenge-100) was observed (Figure 3-striped bars). For both exposure times the 50% Scr control was indistinguishable from the microorganism-free saliva treated controls.

Detailed evaluation of the antibacterial effect of POMx lozenges on saliva-derived biofilms

Lozenges constitute a well-accepted and easy to use delivery vehicle that allows for extended exposure since they are dissolved slowly in the mouth. Since the POMx lozenges displayed very striking bacterial killing during the 30 min treatment, their antibacterial activities were further examined on a more detailed timescale by exposing *S.mutans*-spiked saliva-derived biofilms to the test solutions for 1, 5, 10, 15, 20 and 30 min (Figure 4). POMx lozenge-100 started to exhibit significant antibacterial activity after only 5 min of exposure, eliminating about 50% of the biofilm population (Figure 4-striped bars). At the 20 min time point, lozenge-100 had eliminated more than 99% of the biofilm cells. Consistent with the previous observations (Figures 1 and 3) the antimicrobial activity of the POMx lozenges on the cariogenic species *S. mutans* (dotted bars) was even more pronounced than on the overall biofilm population. The killing curve for both the total biofilm population as well as *S. mutans* fits best a logarithmic decline with R^2-values of 0.984 and 0.9324, respectively. Consistent with the data obtained for 1 min, 30 min and overnight exposures, lozenge-50 was less effective than lozenge-100 but followed a similar trend and also resulted in a logarithmic decline in bacterial survival, while exposure to 50% Scr did not exhibit any discernible effects (data not shown).

Evaluation of anti-adherence effect of POMx lozenges based treatment solutions

The effect of POMx lozenges on the ability of bacteria to adhere to a surface was tested using the live imaging approach described in Material and Methods. This approach enables distinction between growth and adherence. During the 7.5 hr observation period the salivary bacteria that were suspended in the microorganism-free saliva used as a control solution grew to almost confluence even though no additional carbohydrate source was added (Figure 5-upper panel). In the presence of either one of the POMx lozenges bacterial adherences was greatly reduced and no growth occurred during the observation period (Figure 5-lower panel: POMx lozenge-100 is shown as an example).

DISCUSSION

The search for new sources of biologically active components has triggered a renewed interest in the health benefits of natural products that have been historically used in many

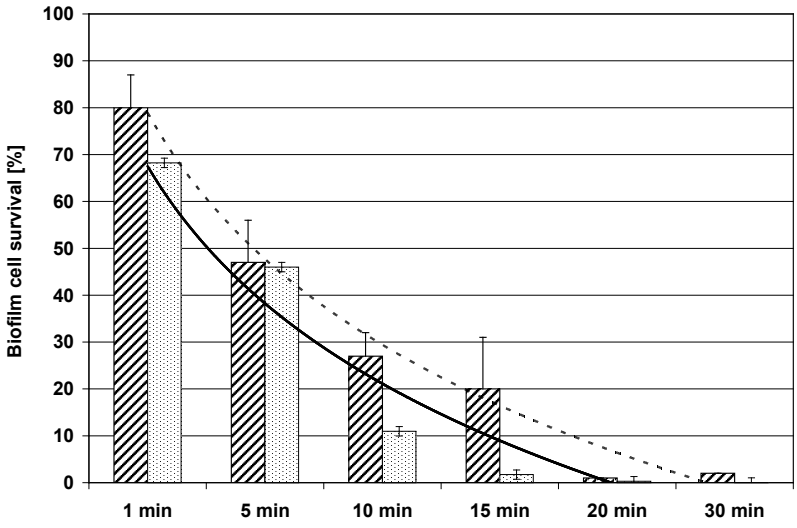

Figure 4: Detailed time course of antibacterial activities of POMx lozenge-100. Shown is the survival of all bacterial cells grown as a saliva-derived overnight biofilm (diagonally striped bars) and the *S. mutans* population (dotted bars) within these biofilms after treatment for 1, 5, 10, 15, 20 and 30 min withPOMxlozenge-100 containing 100 mg GAE/piece. The percentage survival was calculated relative to the negative control samples that were treated with microorganism-free saliva. The decline in survival over time was best fitted by logarithmic trend lines with R^2 values of 0.984 for the killing of the total biofilm population (solid line) and 0.9324 for the *S. mutans* cells (dashed line).

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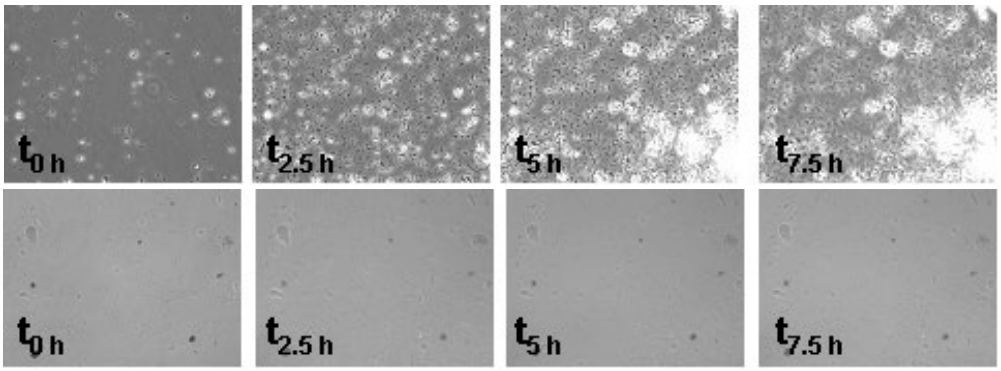

Figure 5: Effect of POMx lozenges on surface adhesion of oral bacteria. Shown are selected frames from movies taken over a7.5 hour time period. Large debris visible in the images originate from particles present in the different treatment solutions and do not correspond to bacteria.

cultures to treat a variety of ailments. Especially ellagitannin polyphenols, a main ingredient of pomegranate and a number of other plant extracts, have been studied extensively for their antibacterial activities. The effect of polyphenols extracted from a variety of plants on oral health in general and especially on the highly cariogenic species *S. mutans* has been the subject of numerous studies.[3,5-9,11,17,19-24,38] The data presented in this study revealed that the previously described health benefits of pomegranate polyphenol extract (POMx) can be extended to the oral cavity. Consistent with the findings in a limited clinical trial, which demonstrated oral biofilm reduction upon treatment with a hydroalcoholic pomegranate extract,[27] the pomegranate extracts based lozenges (which are different in composition from the hydroalcoholic extracts used by Menezes and co-workers) evaluated in this study displayed significant antibacterial activity against oral biofilm bacteria and especially the cariogenic species *S. mutans*.

Due to its role as an important oral pathogen *S. mutans* has been the major focus of a number of reports that examine the antibacterial activities of plant extracts including tea, cacao, grapesamong others. MICs between 0.5 and 32 mg ml^{-1} (4 mg ml^{-1} pomegranate skin extract) for *S. mutans* have been described for polyphenols from different sources.[8] The MICs for the pomegranate extract based lozenges tested in this study was found to be 1.25 mg/ml, which puts their activity towards the more effective end of the polyphenol extract spectrum. Even though MICs can provide a good general idea on antibacterial activities of the test components, the use of planktonic cells does not always allow drawing conclusions for bacteria grown in a biofilm setting. In particular, up to 1000-fold increased resistance against antimicrobial agents has been observed.[37] Since the majority of persistent infections including oral diseases such as caries are based on pathogenic species present in resident biofilms, the search for novel antibacterial agents with anti-biofilm activities has become an important task, which increasingly focuses on naturally occurring products. To address this important aspect, we expanded the assessment of the antibacterial activities of the POMx lozenges from standard MIC determination (Table 1) to efficacy testing against relevant oral biofilms containing the major cariogenic species *S. mutans* at a final proportion of about 5-10% (Figure 1). Most interestingly, *S. mutans* appeared to be even more sensitive to these POM products than the general biofilm population. The concern that the high osmolarity of the dissolved lozenges (50% Scr) could affect biofilm viability was not substantiated and the observed antimicrobial activities can therefore be fully attributed to pomegranate polyphenols present in the POMx based lozenges. Very importantly for a potential application as a clinically relevant and easy to administer prophylactic measure, the effective anti-biofilm and anti-adherence concentration and time frame of exposure fit very well real life lozenge consumption (Figures 3 and 4).

In addition to the antibacterial activities observed against biofilm grown bacteria the POMx lozenges drastically inhibited the surface attachment of salivary bacteria as monitored over a 7.5hr time period with video microscopy (Figure 4). This suggests that their high carbohydrate content, which would typically promote bacterial adherence and growth[39] is superseded by the antibacterial and anti-adherence activities of the polyphenols present. The effect of polyphenols on the surface attachment of *S. mutans* has been the focus of numerous mechanistic studies and revealed that a major factor is the inhibitory effect on glycosyltransferases (Gtfs).[5,11,16,19,20,29,40-42] Glycosyltransferases play a key role in biofilm formation since they are the major enzymes involved in glucan production which comprises the matrix material that *S. mutans* needs for surface attachment and biofilm build-up.[43,44] The enzymatic activity of Gtfs is greatly reduced in the presence of various polyphenols, resulting in significant decrease in surface attachment.[5,8,11] This inhibitory effect of polyphenols on one of the key enzymes for sucrose-dependent surface attachment of *S. mutans* could play in the observed effect of POMx lozenges that attachment is inhibited despite the high Scr content of the test solutions. Substitution of the high sucrose content in the lozenge base with a biological inert substitute could potentially further enhance the antibacterial activity of the POMx lozenges in oral applications.

CONCLUSION

The pomegranate polyphenol extract based lozenges tested in this study displayed antibacterial activities consistent

with their polyphenol content against saliva-derived oral biofilms and *S. mutans* in particular. The most striking effects were observed for lozenge-100, which exhibited antibacterial activities comparable to other oral care products. In addition to antibacterial activities, anti-adherence activities consistent with earlier studies on the effects of polyphenols were found. The POMx lozenges incorporate these two important polyphenol mediated anti-biofilm into a delivery vehicle (lozenge) that is generally more easily accepted than traditional oral care products such as tooth pastes or mouth rinses. Furthermore, lozenges are typically slowly dissolved in the oral cavity thereby enabling longer exposure times to the active ingredients compared to especially mouth rinses. Due to these distinguishing features the POMx lozenges provide a promising new perspective on oral care and therapy especially of children, elderly or disabled populations which are often less perceptive to traditional oral care.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Authors Contributions

Lina Li and Lihong Guo performed the experiments and analyzed the data. Lawrence Wolinsky, Wenyuan Shi and Renate Lux designed and supervised the study. All authors participated in writing the manuscript.

CONSENT

No consent is required for publication of this article.

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