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Research Article

# Eradication of Dual-Species Cariogenic Biofilms by Punicalin and Punicalagin: Microbiological and Gene Expression Analyses

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**ABSTRACT**

This in vitro study evaluated the biofilm eradication potential of two pomegranate-derived compounds, punicalin (PUN) and punicalagin (PUG), against dual-species biofilms comprising *Streptococcus mutans* and *Streptococcus gordonii*. Antimicrobial activity was assessed through minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays, while the minimum biofilm eradication concentration (MBEC) was determined using the crystal violet assay. At 100 µg/mL ($MBEC_{50}$), PUN and PUG were further evaluated in a glass beads biofilm model for their effects on bacterial viability, biomass, biofilm morphology (via SEM) and gene expression (via RT-qPCR). Both compounds significantly reduced biofilm biomass and *S. mutans* population, indicating selective targeting of the cariogenic species. PUN exerted its effects predominantly through downregulation of *gtf* and *gbp* genes, which are involved in EPS production and biofilm stability. In contrast, PUG demonstrated greater biofilm disruption and *S. mutans* reduction, accompanied by significant upregulation of *sodA* and *lytF*, suggesting a dual mechanism involving both oxidative stress and autolysin-mediated dispersal. SEM analysis further supported these findings, showing more extensive matrix disintegration in PUG-treated biofilms. These results highlight the therapeutic potential of PUN and PUG as selective antibiofilm agents for managing cariogenic biofilms, with PUG exhibiting both intrinsic and extrinsic modes of action.

**Key words:** Biofilm, dual-species, punicalagin, punicalin, *Streptococcus mutans*, *Streptococcus gordonii*

## INTRODUCTION

Dental caries is one of the most widespread chronic diseases globally, arising from the prolonged activity of cariogenic biofilms that form on tooth surfaces. These biofilms consist of structured microbial communities encased in a self-produced extracellular polymeric substance (EPS) matrix. Dental caries develops when a susceptible tooth surface is colonised by cariogenic bacteria in the presence of dietary sucrose or refined sugars (Yadav & Prakash 2016). Among these bacteria, *Streptococcus mutans* is recognised as a primary contributor due to its strong adhesion to the enamel surface, efficient fermentation of carbohydrates into lactic acid, and remarkable acid tolerance (Lemos *et al*., 2019).

*Streptococcus mutans* promotes biofilm development through the synthesis of extracellular glucans by glucosyltransferase (Gtf) enzymes, which facilitate bacterial aggregation and enhance the EPS matrix. The EPS includes exopolysaccharides, extracellular DNA (eDNA), and lipoteichoic acid (LTA), all of which promote microbial accumulation on the tooth surface and create a diffusion-limiting barrier that protects bacteria from external threats (Castillo Pedraza *et al*., 2017). Over time, this biofilm environment becomes increasingly acidic, contributing to the demineralisation of tooth enamel and the progressive destruction of the tooth structure. Because this process occurs gradually, it often goes unnoticed until significant damage has occurred.

Beyond its local effects, untreated dental caries can have a significant impact on overall health. It may cause chronic pain, difficulty in eating, and impaired nutrition, particularly affecting vulnerable groups such as children (Innes & Robertson, 2018). If left unmanaged, caries-related infections can progress from pulpitis to deeper oral tissues, allowing bacteria to breach the bone and cause severe complications such as facial cellulitis (Słotwińska-Pawlaczyk *et al*., 2023). In some cases, the infection may spread systemically, leading to conditions like bacteremia or even endocarditis, especially in immunocompromised individuals. (Uyghurturk *et al*., 2022).

Current strategies for dental caries prevention primarily include mechanical plaque removal through tooth brushing and flossing, the use of fluoride-containing toothpaste and mouth rinses, dietary modifications, and professional interventions such as sealants and fluoride varnishes (Almoharib *et al*., 2018; Meyer *et al*., 2024). Additionally, antimicrobial agents such

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as chlorhexidine and triclosan have been incorporated into oral care products to reduce microbial load and biofilm formation. However, these conventional approaches present several limitations. Long-term use of chemical antimicrobials may lead to adverse effects. Some self-reported adverse drug reactions of CHX included tooth staining, altered taste sensation, mucosal irritation, dry mouth and tooth discolouration (Haydari *et al*., 2017; Deus & Ouanounou, 2022). The rise of microbial resistance and the limited efficacy of conventional agents in disrupting mature biofilms or selectively targeting cariogenic species highlight the need for alternative strategies. Natural products offer a promising solution, with broad-spectrum, multi-targeted actions and lower risk of resistance. An ideal antibiofilm approach should eliminate pathogenic biofilms while preserving beneficial oral microbes, supporting a balanced oral ecosystem. Previous in vitro studies have shown the effectiveness of pomegranate peel extract against oral microorganisms (Ferrazzano *et al*., 2017; Abd-El & Sallam, 2020), leading to its evaluation in subsequent clinical trials, where it also demonstrated notable antiplaque activity (Pinni *et al*., 2018; Jacob *et al*., 2021; Mahd *et al*., 2023). However, reported effective concentrations of extract have varied significantly, largely due to differences in fruit cultivar, geographical origin, and the type of solvent used for extraction. While these findings confirm the potential of pomegranate peel extract in biofilm control, such variability poses significant challenges for reproducibility, regulatory approval, and ultimately, commercialisation for clinical use.

To overcome these limitations, focusing on isolated phytocompounds provides a more stable and consistent alternative. Phytocompounds offer improved chemical stability, reproducible bioactivity, and easier standardisation, making them ideal for more accurate and controlled investigations. Notably, some studies have identified effective single compounds such as thymol derived from thyme oil, which has been successfully commercialised and extensively patented, demonstrating the feasibility and therapeutic potential of phytocompound-based approaches in oral healthcare (Biswal & Pazhamalai, 2021). Among the major phytocompounds identified in pomegranate peel extracts are punicalagin, ellagic acid, gallocatechin, punicalin, catechin, and corilagin (Li *et al*., 2015; Man *et al*., 2022).

In this study, our objectives aim to investigate the biofilm eradication effects of punicalagin (PUG) and punicalin (PUN), two major phytocompounds from pomegranate peel, on cariogenic biofilm of *S. mutans*-*S. gordonii* and its mode of action, intending to evaluate its potential use as a drug to prevent dental caries.

## MATERIALS AND METHODS

### Compounds

Punicalin (PUN, CAS 65995-64-4) and punicalagin (PUG, CAS 65995-63-3), both ≥98% purity (HPLC), were purchased from Chengdu Biopurify Phytochemicals Ltd (China). Compounds were dissolved in 10% DMSO to prepare 20 mg/mL stock solutions and stored at –20°C. For each assay, stocks were diluted to ensure that the final DMSO concentration was ≤1%.

### Bacterial strain

*Streptococcus gordonii* ATCC 51656 and *S. mutans* UA159 were obtained from ATCC, and 25% glycerol stocks were stored at the Faculty of Dentistry, UiTM. Single colonies grown on BHI agar (Merck) were cultured in BHI broth and incubated overnight at 37°C with 5% $CO_2$. After 18 hr, late exponential-phase cells were harvested, washed with PBS (pH 7.2), and resuspended in BHI for MIC/MBC assays or in BHI with 1% sucrose (BHIS) for MBEC and biofilm eradication assays.

### Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The antimicrobial activities of PUN and PUG were determined using the broth microdilution method outlined by the Clinical and Laboratory Standards Institute (CLSI) with minor modifications (CLSI, 2018). PUG and PUN were diluted in BHI broth in two-fold serial dilutions to obtain final concentrations from 12.5–3200 µg/mL at a total volume of 100 µL per well in 96-well microtiter plates. Each tested strain (100 µL) at a final concentration of $1.5 \times 10^6$ CFU/mL was added to each well and incubated for 24 hr at 37°C in a 5% CO2 atmosphere. The BHI broth, 0.12% (w/v) CHX and 1% DMSO were used as the sterility, positive and negative controls, respectively. After incubation, the MIC was determined by visually inspecting each well for the presence of bacterial growth. MIC was identified as the lowest concentration of compound at which no visible growth was observed, which was indicated by the absence of turbidity in the well. For MBC, a 10 µL aliquot from each well with no visible bacterial growth was transferred onto agar plates. These plates were incubated overnight at 37°C. The MBC was defined as the lowest concentration of the antimicrobial compound at which no bacterial growth was observed on the agar plate.

### Preparation of clarified human saliva

In all experiments, clarified human saliva was used to precoat surfaces, forming an acquired pellicle that mimics the natural salivary pellicle. In biofilm models, precoating surfaces with an acquired pellicle provides a more physiologically relevant substrate, closely mimicking in vivo conditions and enabling more accurate evaluation of microbial adherence and colonisation dynamics.

Stimulated whole saliva was collected from a healthy female donor (40 years old) with written consent, following exclusion criteria and ethical approval (REC/01/2023 PG/MR/27). Saliva collection followed the method by Rahmani-Badi *et al*. (2015) using Parafilm M for stimulation. The saliva was mixed at a 1:1 ratio with an adsorption buffer (50 mM KCl, 1 mM phosphate buffer, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$, pH 6.5) and supplemented with 0.1 M phenylmethylsulfonyl fluoride (PMSF). After centrifugation (5500 g, 10 min, 4°C), the supernatant was filtered (0.22 µm PES filter) to obtain clarified saliva. To preserve enzymatic activity, saliva was processed and used on the same day, collected consistently simultaneously (Lemos *et al*., 2010).

### Minimum Biofilm Eradication Concentration (MBEC) using Crystal Violet (CV) assay

The Minimum Biofilm Eradication Concentration (MBEC) assay determines the lowest concentration of PUG and PUN required to eradicate established biofilms. At this stage, the crystal violet (CV) assay was conducted to quantify total biofilm

biomass, providing an initial estimation of effective concentrations for downstream analyses. The experiment used 96-well flat-bottom polystyrene plates (Corning® Costar®), following Folliero *et al*. (2022) with minor modifications, including the formation of an acquired pellicle on the wells. To establish the acquired pellicle, 250 µL of clarified human whole saliva was applied to the wells and left for 120 min at 37°C. During the acquired pellicle formation (120 min), saliva was exchanged three times at 30-min intervals. In each exchange, the previous saliva was removed, and a fresh 250 µL sample was added immediately (PelÁ *et al*., 2020). After the pellicle formed, excess saliva was carefully removed by blotting the plate on clean absorbent paper. A dual-species biofilm was formed by mixing *S. gordonii* and *S. mutans* suspensions ($10^6$ CFU/mL each) in a 1:1 (v/v) ratio. The plates were incubated at 37°C for 48 hr. After incubation, nonadherent cells were removed by washing twice with sterile phosphate-buffered saline (PBS). Test compounds (12.5–1600 µg/mL) were then applied to preformed biofilms and incubated for 4 hr. Wells treated with 1% DMSO served as the negative control, while wells treated with 0.12% CHX served as the positive control.

After treatment, wells were washed with PBS to remove residual compounds and planktonic cells. Biofilms were stained with 100 µL of 0.1% crystal violet for 30 min, washed, and the bound dye was solubilised with 33% acetic acid. Absorbance was measured at 570 nm. The MBEC was defined as the lowest concentration showing a significant reduction in biofilm biomass compared to the control. Biofilm eradication (%) was calculated using the following formula:

$$\text{Biofilm eradication } \% = \left[1 - \left(\frac{\text{OD test}}{\text{OD negative control}}\right)\right] \times 100$$

The minimum biofilm eradication concentrations at 50% and 90% ($MBEC_{50}$ and $MBEC_{90}$) represent the concentrations of a compound required to eliminate at least 50% and 90% of an established biofilm, respectively (Zhong *et al*., 2019). In this study, the $MBEC_{50}$ values of PUG and PUN were utilised to assess biofilm eradication using the bead assay.

**Biofilm eradication using the bead assay**

Based on the bead biofilm assay by Konrat *et al*. (2016), we adapted this method to establish dual-species biofilms of *S. mutans* and *S. gordonii* under cariogenic conditions. The assay was conducted in a 24-well plate, with one glass bead in each well. Conditions were optimised to promote a low-pH, highly acidogenic environment characteristic of cariogenic plaque, favouring the dominance of aciduric organisms such as *S. mutans* within the biofilm community. The acquired pellicle was formed using clarified human saliva that had been prepared above. Sterile glass beads (5 mm radius) were immersed in 1 mL clarified human whole saliva and incubated for 120 min at 37°C to allow pellicle formation. During pellicle formation (120 min), saliva was refreshed every 30 min by replacing it with 1000 µL of fresh clarified saliva. Afterwards, the beads were rinsed with sterile distilled water to remove excess saliva. Each bead was placed in a well, and 0.5 mL of each bacterial strain (in a 1:1 ratio) was added. The plates were incubated at 37°C with 5% $CO_2$ for 48 hr, with media refreshed at 24 hr to maintain nutrient availability.

**Determination of microbial population, biomass, biofilm morphology and gene expression**

After incubation, nonadherent cells were removed by washing the beads twice with sterile PBS. Mature biofilms were treated with PUG and PUN at 100 µg/mL ($MBEC_{50}$) for 4 hr at 37°C. Control groups included 1% DMSO (negative) and 0.12% chlorhexidine (positive). Beads containing biofilm cultures were transferred to new wells, rinsed with PBS to remove residual compounds, and processed for further analysis, including bacterial quantification by CFU count, biomass measurement using the crystal violet (CV) assay, biofilm architecture assessment via scanning electron microscopy (SEM), and gene expression analysis using RT-qPCR.

For the bacterial population, three beads were sonicated in an ultrasonic bath for 10 min to dislodge biofilms, and the resulting suspensions were serially diluted and plated on Mitis-salivarius and BHI agar. Dual-species colonies were differentiated based on morphology, compared to single-species controls, and confirmed using commercial biochemical test kits: Rapid ID 32A for *S. gordonii* and API 20 Strep for *S. mutans* (BioMérieux Vitek, Marcy l'Etoile, France).

To complement the bacterial population, total biofilm biomass was quantified using the CV staining. Following treatment and PBS washing, each glass bead was transferred to a new plate and well and fully submerged in 1 mL of 0.1% crystal violet solution. The plate was incubated at room temperature for 15 min to allow staining of the biofilm biomass. After staining, the crystal violet solution was removed, and the beads were gently washed three times with sterile distilled water to eliminate excess stain. The beads were then entirely air-dried for approximately 1 hr within a biosafety cabinet. Once dried, each bead was transferred to a new well and incubated with 1 mL of 33% acetic acid for 15 min at room temperature with gentle shaking to solubilise the bound dye. From the resulting destained solution, 200 µL was transferred into a 96-well microplate in triplicate, and the absorbance was measured at 595 nm using a microplate reader to quantify total biofilm biomass.

The expression of virulence factors associated with *S. mutans* was assessed through real-time quantitative PCR. Beads were gently rinsed in 1 mL sterile $ddH_2O$ using sterile tweezers to remove loosely attached bacteria, then transferred to 2 mL microcentrifuge tubes. Biofilms were detached by vortexing and sonication for 10 min in a BactoSonic® ultrasonic bath (Bandelin, Germany). A 0.5 mL aliquot of the bacterial suspension was mixed with 1 mL RNAprotect Bacteria Reagent (Qiagen, Germany), centrifuged at 5,000× g for 10 min at 4°C, and the pellet was stored at –80°C. No RNA was obtained from CHX-treated samples due to the bactericidal effect. RNA was extracted using the RNeasy Minikit and purified with the RNeasy MinElute Cleanup Kit (Qiagen). RNA concentration and purity were assessed via NanoDrop (Thermo Scientific, USA). DNA contamination was checked using RNA samples without reverse transcriptase. cDNA synthesis was performed with the QuantiTect Reverse Transcription Kit (Qiagen). qPCR was performed using the StepOne Real-Time PCR System (Life Technologies) with gene-specific primers. Table 1 lists the forward (F) and reverse (R) primer sequences targeting specific *S. mutans* genes along with the reference gene 16S rRNA. Each 20 µL reaction included 10 µL SYBR Green Master Mix, 1 µL of each primer, 4 µL nuclease-free water, and 4 µL of 20× diluted cDNA. Cycling conditions were: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 55–57°C for 15 s, and 72°C for 20 s (Kaur *et al*., 2017). Each RNA preparation was analysed in triplicate from five independent experiments. Negative controls included nuclease-free water in place of cDNA. Relative gene expression was calculated using the 2^−ΔΔCT method

with 16S rRNA as the reference gene. The experiments followed MIQE guidelines (Bustin *et al*., 2009).

**Table 1.** Primer sequences used for quantitative gene expression analysis of *S.mutans*

| *S.mutans* gene | | Primer sequence (5'-3') | References |
|---|---|---|---|
| *16S rRNA* | F | CCTACGGGAGGCAGCAGTAG | Sun *et al*., 2021 |
| | R | CAACAGAGCTTTACGATCCGAAA | |
| *gtfB* | F | AGCAATGCAGCCAATCTACAAAT | |
| | R | ACGAACTTTGCCGTTATTGTCA | |
| *gtfC* | F | GGTTTAACGTCAAAATTAGCTGTATTAGC | |
| | R | CTCAACCAACCGCCACTGTT | |
| *gbpD* | F | TTGACTCAGCAGCCTTTCGT | |
| | R | CTTCTGGTTGATAGGCGGCA | |
| *gbpB* | F | ATGGCGGTTATGGACACGTT | |
| | R | TTTGGCCACCTTGAACACCT | |
| *lytF* | F | GAATGCTGGCTGTTAGTTCTTATAC | Nagasawa *et al*., 2023 |
| | R | CTGTACAGCGTTGACATCAAAAG | |
| *sodA* | F | GCAGTGCTAAGACTCCCGAATC | Wolfson *et al*., 2023 |
| | R | TTGCGGAAGTGTGAGATTGGC | |

**Scanning electron microscopy (SEM)**

Three beads were fixed in 4% glutaraldehyde overnight, then washed with 0.1 M sodium cacodylate buffer (pH 7.4) and post-fixed in 2% osmium tetroxide overnight. The following day, the samples were rinsed twice with distilled water (15 min each) and then dehydrated through a graded ethanol series (15%, 30%, 50%, 70%, 90% & 100%, each for 15 min), followed by two 15-min washes in 100% ethanol. Dehydration was continued using ethanol-acetone mixtures (3:1, 1:1, 1:3) and followed by three final rinses in pure acetone (15 min each). Samples were then critically point dried, mounted on aluminium stubs with copper tape, and gold-coated using an iron sputter coater. SEM imaging was performed at 10,000× magnification using a Quanta™ FEG 450 scanning electron microscope (Netherlands).

**Statistical analysis.**

All quantitative experiments were conducted with five independent biological replicates, each measured in technical triplicate. Results are expressed as mean ± standard deviation (SD). Normality was assessed using the Shapiro–Wilk test, and parametric tests were applied to normally distributed data. All statistical analyses and graph generation were performed using GraphPad Prism v10. A $p<0.05$ was considered statistically significant.

## RESULTS

**Antimicrobial activity of PUN and PUG**

The MIC and MBC values of PUN and PUG against *S.gordonii* and *S.mutans* are shown in Table 2. The antimicrobial activity of PUN and PUG was evaluated against *S. gordonii* and *S. mutans* using MIC and MBC assays. Both compounds exhibited inhibitory effects, with PUG demonstrating a lower MIC value (100 µg/mL) compared to PUN (200 µg/mL) for both bacterial strains. The MBC of PUG against *S. mutans* was also lower (200 µg/mL) than that of PUN (400 µg/mL), indicating stronger bactericidal activity. For *S. gordonii*, the MBC was 400 µg/mL for both compounds. The MBC/MIC ratio ranged from 2 to 4, suggesting that both compounds possess bactericidal potential. Although MIC and MBC assays are performed on planktonic cells and may not directly reflect clinical conditions where oral bacteria predominantly exist in biofilms, these results still provide valuable insight into the inherent antimicrobial sensitivity of the tested organisms. Notably, PUG and PUN exhibited greater inhibitory and bactericidal activity against *S. mutans* than *S. gordonii*, suggesting a degree of selectivity. This is particularly promising, as *S. mutans* plays a key role in cariogenic biofilm formation, while *S. gordonii* is more commonly associated with a healthy oral microbiome.

**Table 2.** Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and MBC/MIC ratios of punicalagin (PUG) and punicalin (PUN) against *S. gordonii* and *S. mutans*

| | *S. gordonii* | | | *S. mutans* | | |
|---|---|---|---|---|---|---|
| | MIC | MBC | MBC/MIC ratio | MIC | MBC | MBC/MIC ratio |
| PUN | 200 | 400 | 2 | 200 | 400 | 2 |
| PUG | 100 | 400 | 4 | 100 | 200 | 2 |

**$MBEC_{50}$ and $MBEC_{90}$**

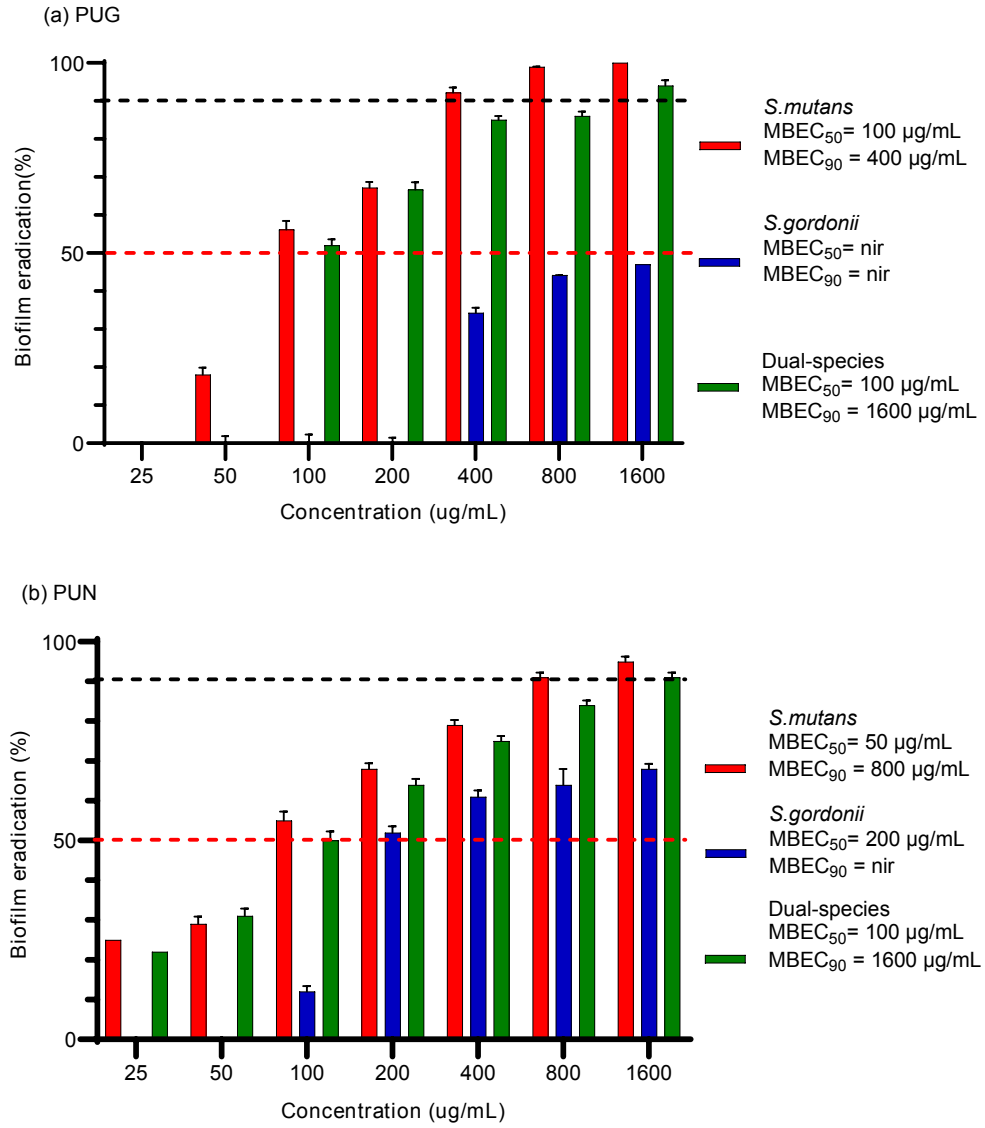


**Fig. 1.** Biofilm eradication activity of (a) PUG and (b) PUN, against *S. mutans*, *S. gordonii*, and dual-species biofilms. Biofilms were treated with increasing concentrations of each compound (25–1600 µg/mL) for 4 hr, and residual biomass was quantified using the crystal violet assay. The $MBEC_{50}$ and $MBEC_{90}$ values are indicated in the legends. "nir" denotes "not in range" when eradication thresholds were not reached within the tested concentration range. Data are presented as mean ± SD from five independent determinations, each performed in triplicate.

Figure 1 shows the biofilm eradication (%) of *S. mutans*, *S. gordonii*, and dual-species biofilms at increasing concentrations (25–1600 µg/mL) of (a) PUG and (b) PUN. For PUG, *S. mutans* shows a dose-dependent increase in biofilm eradication, reaching 50% eradication at 100 µg/mL ($MBEC_{50}$) and 90% eradication at 400 µg/mL ($MBEC_{90}$). *S. gordonii* shows a minimal response, failing to achieve 50% eradication at any concentration tested ($MBEC_{50}$ and $MBEC_{90}$ are not within the tested range). Dual-species biofilms reach $MBEC_{50}$ at 100 µg/mL and $MBEC_{90}$ at 1600 µg/mL. For PUN, *S. mutans* is more sensitive, with $MBEC_{50}$ achieved at 50 µg/mL and $MBEC_{90}$ at 800 µg/mL. *S. gordonii* responds with $MBEC_{50}$ at 200 µg/mL, while dual-species biofilms also require higher concentrations, with $MBEC_{50}$ at 100 µg/mL and $MBEC_{90}$ at 1600 µg/mL. A concentration of 100 µg/mL was selected for downstream analysis, as it corresponds to the $MBEC_{50}$ for dual-species biofilms. At this concentration, both PUN and PUG achieved (~50%) reduction of *S. mutans* biofilm while having minimal effect on *S. gordonii* in single-species assays, thereby preserving the commensal population. This working concentration enabled the evaluation of both intrinsic (gene regulation) and extrinsic (matrix disruption) effects under biologically relevant conditions without complete eradication.

**Biofilm eradication against dual-species biofilm**

*Biomass of the biofilm after treatment*

Figure 2 demonstrates the effects of PUG, PUN, and CHX on dual-species biofilm biomass. The 1% DMSO control group exhibited the highest biomass. Both PUG and PUN significantly reduced biomass compared to the control ($p$<0.05). Among the test compounds, PUG produced a greater reduction than PUN ($p$<0.05), though both were less effective than CHX, which yielded the lowest biomass and the strongest eradication effect.

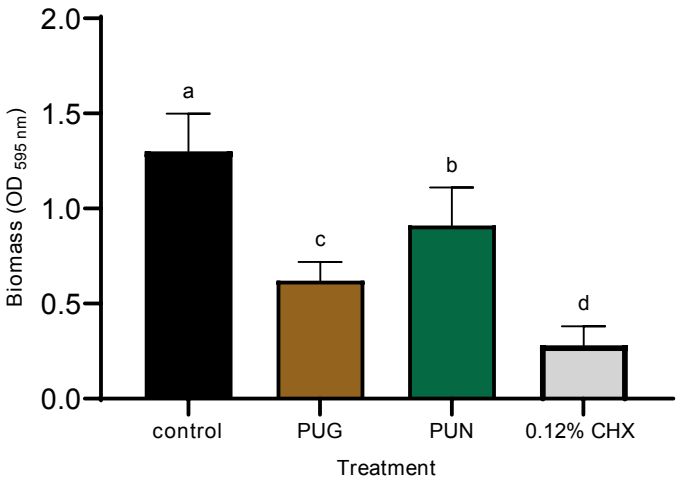


**Fig. 2.** Quantification of biofilm biomass following 4-hr treatment on 48-hr dual-species biofilms of *S. mutans* and *S. gordonii*, assessed using crystal violet staining. Bars represent the mean optical density ($OD_{595}$ nm) ± SD. Different letters (a–d) indicate statistically significant differences between groups ($p$<0.05).

*Bacterial population on the remaining biofilm after treatment*

$Log_{10}$ CFU/bead (Figure 3) revealed that treatment with punicalagin (PUG) and punicalin (PUN) significantly reduced the viable bacterial population of *S. mutans* in the remaining biofilm compared to the control ($p$<0.0001). Among the two compounds, PUG achieved a significantly greater reduction in *S. mutans* CFU counts than PUN ($p$<0.0001). In contrast, *S. gordonii* CFU counts in the residual biofilm remained statistically unchanged across the control, PUG, and PUN groups (ns), suggesting that both compounds selectively target *S. mutans* while sparing *S. gordonii*. This selective effect is beneficial in preserving beneficial oral commensals. As expected, chlorhexidine (CHX) significantly reduced the CFU counts of both species ($p$<0.0001), reflecting its broad-spectrum antimicrobial activity but lack of selectivity.

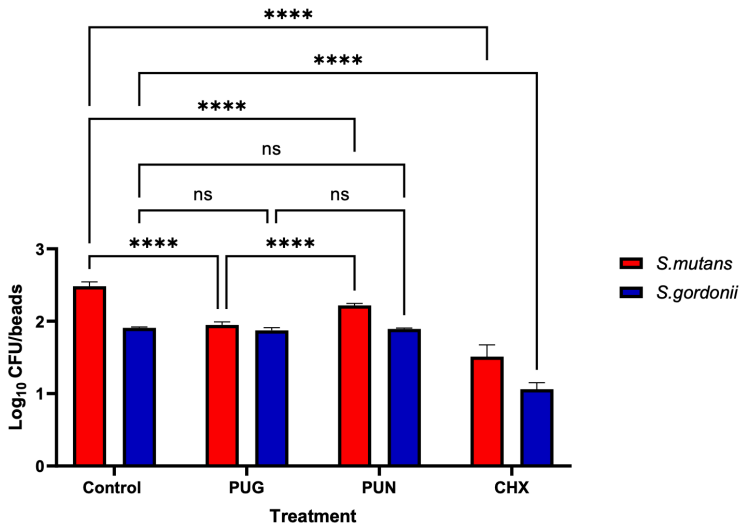


**Fig. 3.** Viable bacterial counts ($Log_{10}$ CFU/bead) of *S. mutans* and *S. gordonii* in dual-species biofilms following 4 hr treatment with punicalagin (PUG), punicalin (PUN), and chlorhexidine (CHX). CFU enumeration was performed to assess the bacterial population remaining in the biofilm after treatment. Both PUG and PUN significantly reduced *S. mutans* counts compared to the untreated control (****$p$<0.0001), while *S. gordonii* counts were not significantly affected (ns), indicating selective activity of *S. mutans*. In contrast, CHX significantly reduced CFU counts of both species (****$p$<0.0001). Data represent mean ± SD of five independent determinations performed in triplicate. Significance annotations denote pairwise comparisons with the respective control group as well as between treatments within the same species.

### Scanning electron microscopy (SEM)

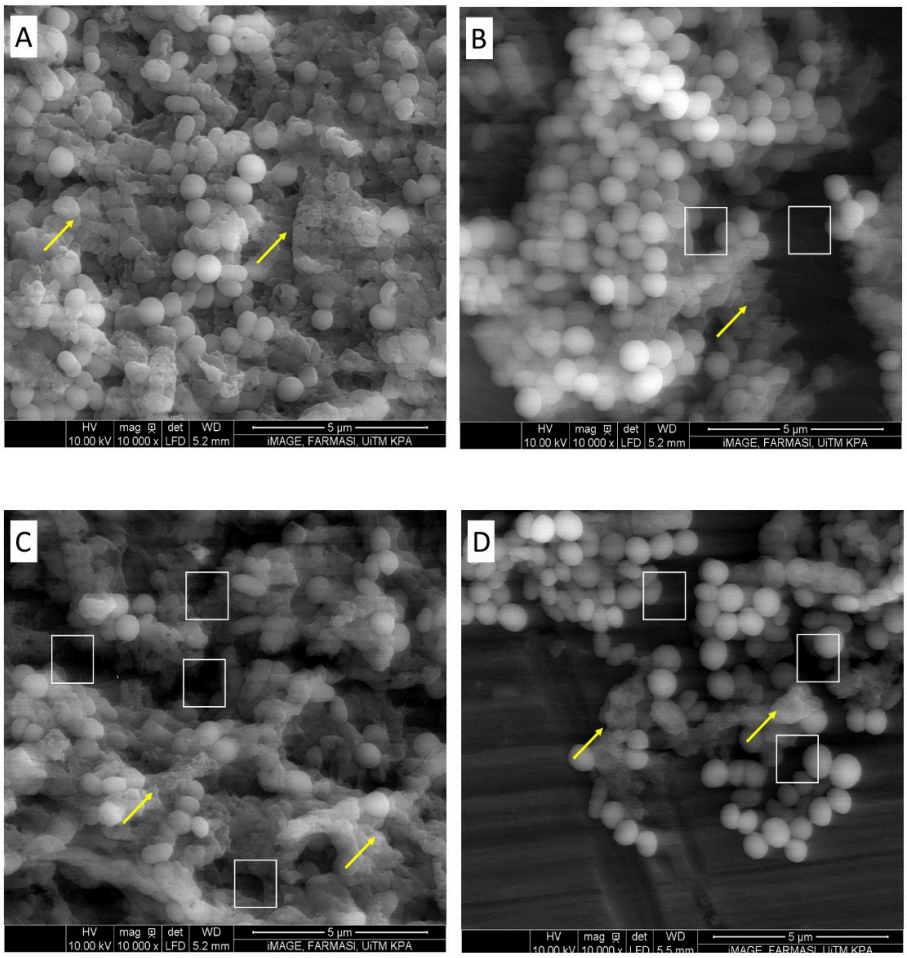


**Fig. 4.** Scanning Electron Micrographs (SEM) of dual-species biofilms formed by S. mutans and S. gordonii after 48 hr of incubation and 4 hr treatment with a) 1% DMSO (negative control), b) PUG, c) PUN and d) 0.12% CHX. Yellow arrows indicate residual EPS, and white boxes highlight regions of matrix disruption or bacterial clearance. Magnification: 10,000×.

The SEM micrograph of the negative control group (Figure 4A) revealed extensive biofilm formation by the dual-species culture of *S. mutans* and *S. gordonii*. A substantial amount of extracellular polymeric substance (yellow arrow) was present, forming a thick, amorphous matrix encapsulating the bacterial cells. This EPS layer appeared sponge-like and contributed to the structural cohesion and protection of the biofilm. In PUG-treated biofilms (Figure 4B), notable matrix disintegration was observed. The extracellular polymeric substance (EPS) appeared irregular and discontinuous (yellow arrows), with visible voids and disrupted areas within the biofilm structure (white boxes), suggesting a partial collapse of the matrix. The EPS appeared thinner and less cohesive, and many bacterial cells were exposed and not encapsulated by the protective EPS, further indicating weakened biofilm integrity and reduced matrix-mediated protection. Biofilms treated with PUN (Figure 4C) showed a denser and more continuous EPS matrix (yellow arrows), with fewer and smaller voids (white boxes) compared to PUG. While signs of disruption were present, the overall biofilm architecture remained relatively intact, with more bacterial clusters embedded within the matrix. Biofilms treated with CHX (Figure 4D) exhibited extensive structural collapse, with large voids in the EPS matrix and numerous scattered or partially lysed bacterial cells. The overall cell density appeared to be reduced, and several bacteria were seen as being loosely adhered or detached from the biofilm surface. The white-boxed areas indicated regions of complete matrix loss, suggesting CHX's strong extrinsic effect through disruption of cell membranes and solubilization of EPS components. These findings align with CHX's known ability to act as a cationic surfactant and biocide, leading to rapid biofilm disintegration.

### RT-qPCR

In this study, we investigated genes associated with biofilm formation (*gtf* & *gbp* genes), redox and oxidative stress response (*sodA*), and cell division biofilm dispersal (*lytF*). The relative gene expression levels for *S. mutans* are illustrated in Figure 5.

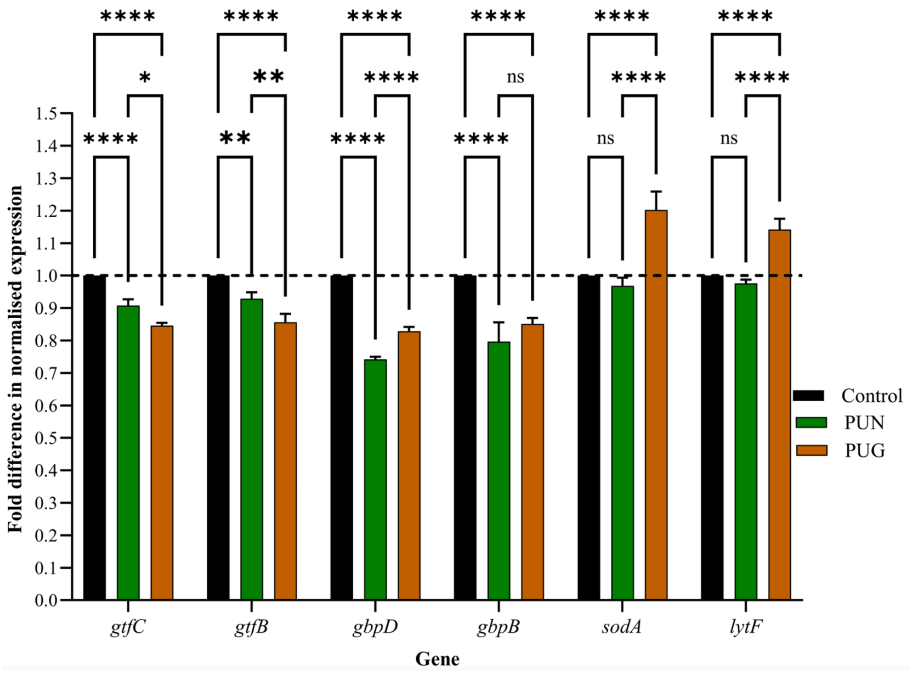


**Fig. 5.** Relative expression of virulence genes in *S. mutans* dual-species biofilm following 4-hr treatment with punicalagin (PUG) and punicalin (PUN) at 100 µg/mL ($MBEC_{50}$). Gene expression was normalised to the 16S rRNA reference gene and compared to the respective untreated controls. Bars represent mean fold change ± SD from five independent determinations, each performed in triplicate. Statistical analysis was performed using one-way ANOVA and Tukey's post hoc test with comparisons made between the control and each treatment (PUG & PUN), and additionally between the two treatments (PUG vs. PUN). Asterisks indicate statistical significance levels (**, $p$<0.01; ***, $p$<0.001; ****, $p$<0.0001), while "ns" denotes non-significant differences.

A 4-hr exposure was chosen to capture both EPS disruption and gene expression changes by PUG and PUN. This duration allows sufficient time for matrix destabilisation and the measurable transcriptional downregulation of key biofilm-associated genes. Both PUG and PUN significantly downregulated EPS-associated genes (*gtfC*, *gtfB*, *gbpD* & *gbpB*), all of which are crucial for extracellular polysaccharide synthesis and the stability of the biofilm matrix. This downregulation indicates that both compounds interfere with matrix formation, contributing to reduced biofilm integrity. Importantly, PUG significantly upregulated *lytF* ($p$<0.0001), which encodes an autolysin associated with cell separation and biofilm dispersal. This suggests that PUG may actively trigger a dispersal response through enhanced autolytic signalling, promoting cell detachment from the biofilm structure without necessarily causing widespread lysis, as SEM images showed intact cells. In addition, *sodA* was also significantly upregulated by PUG, indicating the presence of oxidative stress. This upregulation supports the notion that PUG induces a dual mechanism that disrupts the biofilm matrix, both intrinsically via gene suppression (*gtf*, *gbp*) and extrinsically through oxidative stress and autolysin-mediated dispersal. In contrast, PUN downregulated the EPS-associated genes but did not affect *lytF* or *sodA*, suggesting a more matrix-focused inhibitory mechanism without substantial involvement of dispersal or oxidative pathways.

## DISCUSSION

This study provides evidence of the selective antibiofilm activity of purified ellagitannins, PUG and PUN, against a dual-species model of *S. mutans* and *S. gordonii*, integrating microbiological, structural and gene expression analyses. While previous work has documented the general antimicrobial and antibiofilm effects of pomegranate extract activity (Ferrazzano *et al*., 2017; Pinni *et al*., 2018; Abd-El & Sallam, 2020; Jacob *et al*., 2021), the current study addresses variability issues by employing purified compounds, enabling precise mechanistic insights. Both PUG and PUN demonstrated significant eradication of mature biofilms, as evident from biomass reduction, CFU quantification, and SEM imaging. Notably, this eradication occurred at concentrations below the minimum bactericidal concentration (MBC), indicating that these compounds disrupt the biofilm structure without necessarily inducing cell death, an important feature that could mitigate the development of resistance. The differential impact of PUG and PUN on biofilm eradication can be attributed to distinct mechanisms. Both compounds significantly downregulated *gtfB*, *gtfC*, *gbpB*, and *gbpD* genes critical for glucan synthesis and matrix stability. The *gtf* and *gbp* genes are among the most commonly studied targets in antibiofilm research on *S. mutans*, as they are central to EPS synthesis and initial biofilm formation (Ren *et al*., 2016; Gabe *et al*., 2019; Wu *et al*., 2020; Kim *et al*., 2025). Inhibition of *S. mutans* glucosyltransferases (GTFs) can occur via several mechanisms: disrupting upstream signalling pathways that regulate *gtf* expression, downregulating *gtf* gene transcription, or directly inhibiting GTF enzymatic activity, thereby reducing extracellular polysaccharide synthesis and weakening the biofilm matrix (Zhang *et al*., 2021). This confirms their intrinsic antibiofilm effect by targeting the EPS biosynthetic machinery. However, biofilm eradication, particularly in mature or established biofilms, may involve additional mechanisms beyond EPS production. In this study, PUG exhibited a more profound effect on biofilm clearance, which coincided with the upregulation of *sodA* and *lytF*, suggesting that oxidative stress induction and autolysin-mediated dispersal act as complementary mechanisms to suppress EPS. Upregulation of *sodA*, which encodes superoxide dismutase, reflects an intracellular oxidative stress response likely triggered by elevated reactive oxygen species (ROS). Such oxidative stress has been shown to destabilise the EPS matrix and enhance bacterial susceptibility to environmental stressors. Similar findings were reported by Khan *et al*. (2020),

where exposure to *Salvadora persica* essential oils induced *sodA* expression in *S. mutans*, supporting the role of ROS in biofilm disruption. Concurrently, *lytF* upregulation, a gene encoding a murein hydrolase, suggests activation of autolytic pathways, enabling controlled cell separation and detachment from the matrix, a hallmark of regulated biofilm dispersal rather than random lysis. SEM micrographs reinforced this finding by showing intact bacterial morphology amid disintegrated EPS, supporting a dispersal mechanism without overt cell destruction. This dual action, involving EPS gene suppression and enhanced dispersal, confers PUG with superior efficacy over PUN, which primarily exhibits transcriptional interference without triggering stress or dispersal responses. The structural basis for this difference may lie in the hydroxylation pattern of PUG, known to enhance its chelation potential and interaction with biofilm components. According to Nijampatnam *et al*. (2016), hydroxyl groups at specific positions, particularly position three on ring A, are critical for antibiofilm efficacy. PUG, with extensive hydroxylation at these key sites, aligns well with this structure-activity relationship model, likely enhancing its ability to disrupt EPS and downregulate biofilm-associated genes. These findings highlight the potential of hydroxyl-rich polyphenols as targeted therapeutics for biofilms. Moreover, the selectivity of both compounds toward *S. mutans*, with minimal impact on *S. gordonii*, highlights their potential for targeted modulation of oral microbiota. This selectivity is clinically relevant, as *S. gordonii* plays a protective role in oral biofilm ecology and may even antagonise cariogenic species through hydrogen peroxide production and competition for adhesion sites (Rostami *et al*., 2022). However, a key limitation of this study is the use of only two bacterial species, which does not fully capture the complexity of the oral biofilm community. While the selective targeting of *S. mutans* over *S. gordonii* is promising, future studies should expand the model to include additional pioneer and commensal species such as *Streptococcus sanguinis* or *Streptococcus mitis* to better mimic in vivo biofilm ecology and assess the broader microbiome selectivity of these compounds.

## CONCLUSION

PUG and PUN effectively eradicated *S. mutans*-dominant dual-species biofilms, with PUG showing greater efficacy through both gene suppression and potential biofilm dispersal mechanisms. Their selective action against *S. mutans* while sparing *S. gordonii* suggests potential for targeted oral biofilm control using purified phytocompounds

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## ETHICAL STATEMENT

This study was approved by the Research Ethics Committee, Universiti Teknologi MARA, approval number REC/01/2023 (PG/MR/27)

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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