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Inhibitory Effects of Rose Apple Leaf Extract (*Syzygium jambos* (L.)) and Sweet Orange Peel Extract (*Citrus sinensis* (L.)) as Well as HMF and Naringenin Inducers on Biofilm Formation of *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*

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ABSTRACT

Porphyromonas gingivalis and *Aggregatibacter actinomycetemcomitans* are key periodontal pathogens. Both depend on biofilm formation to persist in the subgingival niche. Each species uses LuxS-dependent autoinducer-2 (AI-2) quorum sensing (QS) to regulate biofilm development. Interfering with QS is therefore an attractive anti-virulence strategy that may reduce biofilm formation without imposing strong selective pressure for resistance. This study evaluated the inhibitory effects on *P. gingivalis* and *A. actinomycetemcomitans* biofilm formation in vitro of four agents: ethanolic rose apple (*Syzygium jambos*) leaf extract, sweet orange (*Citrus sinensis*) peel extract, and their constituent bioactive compounds 5-hydroxymethylfurfural (HMF) and naringenin. Biofilms of *P. gingivalis* ATCC 33277 and *A. actinomycetemcomitans* ATCC 29523 were grown in brain heart infusion broth in 96-well microplates for 24 h at 37 °C. For each species, wells were exposed to rose apple leaf extract (0.125, 0.25 and 0.5 mg/mL), sweet orange peel extract (15, 20 and 25 mg/mL), HMF or naringenin (25, 50 and 100 mg/mL), or quercetin (100 mg/mL) as the positive control. Biofilm biomass was quantified by crystal violet staining at OD_{570} in triplicate, and the percentage of inhibition was calculated relative to the untreated control. All agents reduced biofilm biomass of both species, and inhibition increased with concentration. Against *P. gingivalis*, maximal inhibition was 67.07% for rose apple leaf extract, 68.94% for sweet orange peel extract, 70.27% for HMF, and 70.11% for naringenin, compared with 81.77% for quercetin. Against *A. actinomycetemcomitans*, the corresponding values were 65.75%, 72.68%, 75.94% and 74.55%, compared with 79.05% for quercetin. HMF produced the highest inhibition at its top concentration against both species, and *A. actinomycetemcomitans* was generally more susceptible than *P. gingivalis*. On a mass basis, rose apple leaf extract achieved comparable inhibition at a 50-fold lower concentration than sweet orange peel extract. Rose apple leaf extract, sweet orange peel extract, HMF, and naringenin inhibit biofilm formation by *P. gingivalis* and *A. actinomycetemcomitans* in vitro. Interference with LuxS/AI-2 QS is a plausible mechanism, but it requires confirmation by AI-2 bioassays and gene expression analysis.

Keywords: biofilm; quorum sensing; *autoinducer-2*; *Porphyromonas gingivalis; Aggregatibacter actinomycetemcomitans; Syzygium jambos*; *Citrus sinensis*

INTRODUCTION

Periodontitis is a chronic inflammatory disease of the tooth-supporting tissues and a major cause of tooth loss in adults. Severe forms affect an estimated 11% of the global population [1]. The disease is initiated and sustained by dysbiotic subgingival biofilms. In these biofilms, microbial communities embedded in a self-produced extracellular matrix trigger a destructive host inflammatory response [2]. Biofilm-associated bacteria are markedly more tolerant than their planktonic counterparts to antimicrobial agents, host immune defences, and environmental stress.

This tolerance limits the long-term effectiveness of mechanical debridement and adjunctive antibiotics, and repeated antibiotic exposure further raises concerns about antimicrobial resistance [2,3].

Among the periodontal microbiota, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* are two of the most extensively studied pathogens.

P. gingivalis is a Gram-negative, obligately anaerobic rod and a keystone pathogen. It has been detected in up to approximately 86% of subgingival plaque samples from patients with periodontitis [4]. Its virulence arsenal, which enables it to subvert host immunity and promote dysbiosis, includes the cysteine proteases (gingipains) RgpA, RgpB and Kgp, fimbriae, hemagglutinins, lipopolysaccharide, and a capsule [4–6].

A. actinomycetemcomitans is a Gram-negative, facultatively anaerobic coccobacillus. It is strongly associated with rapidly progressing periodontitis in young individuals. Its main virulence factors are leukotoxin (LtxA) and cytolethal distending toxin [7,8]. The two species frequently co-occur in patients with periodontitis, which suggests possible cooperative interactions during colonisation and disease progression [9].

Biofilm formation is coordinated by *quorum sensing* (QS), a cell-to-cell communication system in which bacteria produce, release and detect diffusible signal molecules (autoinducers) to regulate gene expression in response to population density [10,11]. The main signal classes are acyl-homoserine lactones (AHLs) in Gram-negative bacteria, autoinducing peptides in Gram-positive bacteria, and autoinducer-2 (AI-2), which mediates interspecies communication across both groups [10].

AI-2 is synthesised through the activated methyl cycle. S-adenosylhomocysteine is converted by Pfs to S-ribosylhomocysteine. LuxS then cleaves S-ribosylhomocysteine to 4,5-dihydroxy-2,3-pentanedione, which spontaneously cyclises into the active AI-2 signal [10].

In *P. gingivalis*, LuxS/AI-2 signalling modulates gingipain activity, hemagglutination, hemin acquisition and stress responses, all of which contribute to biofilm development and survival within the periodontal pocket [12–14]. AI-2 signalling also supports the integration of *P. gingivalis* into multispecies communities with early colonisers such as Streptococcus gordonii [15].

In *A. actinomycetemcomitans,* inactivation of luxS markedly reduces biofilm biomass and depth. Exogenous AI-2 restores the biofilm, which indicates that AI-2 acts as a genuine regulatory signal rather than a metabolic by-product [16]. The signal is recognised by the periplasmic binding proteins RbsB and LsrB [17] and transduced through the QseBC twocomponent system to control biofilm formation and in vivo virulence [18]. Notably, AI-2 produced by *A. actinomycetemcomitans* can complement a luxS mutation in *P. gingivalis* [13]. This makes the AI-2 pathway a shared target relevant to both pathogens.

Because QS regulates virulence and biofilm formation rather than bacterial viability, QS inhibition has been proposed as an anti-virulence strategy that could attenuate pathogenicity while imposing less selective pressure than conventional bactericidal agents [11]. Several small molecules, including halogenated furanones and the sugar analogues D-ribose and D-galactose, have been shown to suppress AI-2-mediated biofilm formation by periodontal pathogens [19–21].

Plant polyphenols, particularly flavonoids, are another important source of QS inhibitors [22]. They can antagonise QS receptors and downregulate virulence-associated genes. Quercetin, for example, attenuates *P. gingivalis* virulence properties [23] and displays broad-spectrum anti-biofilm and anti-QS activity against Gram-negative bacteria [24]. In this study, the term inducer refers to such defined small bioactive molecules, which modulate QS-regulated gene expression without being primarily bactericidal.

Syzygium jambos (L.) Alston (rose apple; Myrtaceae) is a tropical plant native to Southeast Asia and widely used in Indonesian traditional medicine. Its ethanolic leaf extract attenuated QS-regulated virulence and biofilm formation in Pseudomonas aeruginosa PAO1 at sub-inhibitory concentrations of 0.0625–0.5 mg/mL [25]. In the same study, 5-hydroxymethylfurfural (HMF) and betulin were identified as major bioactive constituents, and docking analysis indicated that both bind the LasR and RhlR receptors [25]. HMF is a furan derivative, and furan and furanone scaffolds are well-documented interferers with both AHL and AI-2 signalling [19,26]. This makes HMF a plausible candidate inhibitor of AI-2-dependent biofilm formation.

Sweet orange (*Citrus sinensis (L.*) Osbeck) peel is an abundant agro-industrial by-product rich in flavonoids such as hesperidin and naringenin, as well as volatile terpenes such as limonene [27]. *C. sinensis* peel phytochemicals inhibited *P. aeruginosa* biofilms and motility, and naringenin showed the highest predicted binding affinity for LasR among the compounds tested [28]. A citrus extract rich in flavanones reduced both AHL and AI-2 production in Yersinia enterocolitica without affecting growth [29]. Sweet orange peel extracts have also shown antimicrobial activity against oral cariogenic bacteria [30].

Despite this evidence, most studies of S. jambos, C. sinensis and their constituents have been performed on P. aeruginosa and other AHL-dependent systems. Their effects on the AI-2-dependent biofilms of periodontal pathogens remain largely unexplored. Moreover, it is unclear whether a single purified constituent is as effective as, or more effective than, the crude extract from which it originates. This question is relevant to the future development of standardised oral care formulations.

This study therefore aimed to evaluate the inhibitory effects of *S. jambos* leaf extract, *C. sinensis* peel extract, HMF and naringenin on biofilm formation by *P. gingivalis* and *A. actinomycetemcomitans* in vitro, and to compare the activity of each crude extract with that of its corresponding constituent compound.

MATERIALS AND METHODS

Study design

This was a true experimental, laboratory-based in vitro study using a post-test-only control group design. The study was approved by the Health Research Ethical Clearance Commission, Faculty of Dental Medicine, Universitas Airlangga (No. 1232/HRECC.FODM/XII/2025). Plant extracts were prepared at the Testing Service Unit (ULP), Universitas Airlangga, and all biofilm work was carried out at the Research Center, Faculty of Dental Medicine, Universitas Airlangga, between July and November 2025.

Bacterial strains

Porphyromonas gingivalis ATCC 33277 and *Aggregatibacter actinomycetemcomitans* ATCC 29523 were obtained from the culture collection of the Research Center, Faculty of Dental Medicine, Universitas Airlangga. Each strain was grown in brain heart infusion broth (BHIB), and the turbidity of the suspension was adjusted to a 0.5 McFarland standard (approximately 1×10^8 CFU/mL) before use.

Preparation of extracts and test compounds

Fresh *S. jambos* leaves and *C. sinensis* peels were collected from Malang, East Java, Indonesia, and authenticated at the Materia Medica Herbal Laboratory, Batu. Both materials were oven-dried (40–60 °C), ground to a powder, and extracted by cold maceration in 96% ethanol. The filtrate was concentrated under reduced pressure to yield a crude extract, which was reconstituted in dimethyl sulfoxide (DMSO) to the required working concentrations [5].

The test compounds were used as supplied by the manufacturer: 5-hydroxymethylfurfural (HMF; 99.85% purity) and naringenin (≥ 95% purity). Quercetin (≥ 95% purity) served as the positive control. HMF, naringenin, and quercetin were each dissolved in DMSO.

Treatment groups

For each bacterial species, a negative control (BHIB with bacteria and sterile distilled water) and a positive control (BHIB with bacteria and quercetin 100 mg/mL) were run alongside the test groups: *S. jambos* leaf extract at 0.125, 0.25 and 0.5 mg/mL; *C. sinensis* peel extract at 15, 20 and 25 mg/mL; HMF at 25, 50 and 100 mg/mL; and naringenin at 25, 50 and 100 mg/mL. Each group was assayed in three technical replicates.

Biofilm formation and crystal violet assay

Biofilms were grown in sterile flat-bottomed 96-well microplates. Standardised bacterial suspensions were seeded with BHIB supplemented with 5% sucrose, the respective treatment was added, and the plates were incubated at 37 °C for 24 h [6]. Biofilm biomass was then quantified by crystal violet staining [7]. Planktonic cells were discarded, and the wells were washed gently; the adherent biofilm was stained with 0.1% crystal violet (200 µL per well) for 15 min; excess dye was

removed and the wells were washed with running water; the plate was air-dried at room temperature; the bound dye was solubilised with 95% ethanol (200 µL per well); and the absorbance (optical density, OD) was read at 570 nm on a microplate reader.

Data analysis

The mean OD_{570} of the three replicates was calculated for each group. The percentage of biofilm inhibition relative to the negative control was calculated as:

$$\text{Inhibition (\%)} = \frac{\text{OD negative control} - \text{OD treatment OD}}{\text{negative control}} \times 100\ \%$$

Data were analysed descriptively and comparatively. For each test material, the concentration giving the highest percentage inhibition was identified, and the most effective concentration of each crude extract was then compared with that of its corresponding constituent compound.

RESULTS

All four test materials reduced biofilm biomass of both *P. gingivalis* and *A. actinomycetemcomitans* relative to the negative control, and inhibition increased with concentration. Against both species, HMF at 100 mg/mL produced the highest inhibition among the four test materials, although none reached the level of the quercetin positive control.

Extraction yield

Starting from 1,500 g of fresh material each, oven-drying reduced the *S. jambos* leaves and *C. sinensis* peels to about 750 g, and cold maceration in 96% ethanol yielded approximately 50 g of crude extract per plant [5,6].

Inhibition of *P. gingivalis* biofilm

For *P. gingivalis,* the lowest biofilm biomass (highest inhibition) within each test material was reached at the top concentration tested. The quercetin positive control gave the greatest inhibition overall (81.77%).

TABLE 1: Biofilm inhibition of P. gingivalis by different test materials.

Group	Material (Best Concentration)	Mean OD570	Inhibition (%)
K (-)	Negative Control	2.5437	0
K (+)	Quercetin 100 mg/ml	0.4637	81.77
P6	*S. jambos* leaf extract 0.5 mg/ml	0.8377	67.07
P3	*C. sinensis* peel extract 25 mg/ml	0.7900	68.94
P9	HMF 100 mg/ml	0.7560	7.27
P12	Narigenin 100 mg/ml	0.7600	70.11

Inhibition of *A. actinomycetemcomitans* biofilm

For *A. actinomycetemcomitans,* the highest inhibition within each test material was again reached at the top concentration. Inhibition values were generally higher than those recorded against *P. gingivalis* for the same materials, except for *S. jambos* leaf extract.

TABLE 2: Biofilm inhibition of *A. actinomycetemcomitans* by different test materials.

Group	Material (Best Concentration)	Mean OD570	Inhibition (%)
K (-)	Negative Control	3.2037	0
K (+)	Quercetin 100 mg/ml	0.6710	79.05
P18	*S. jambos* leaf extract 0.5 mg/ml	1.0973	65.75
P15	*C. sinensis* peel extract 25 mg/ml	0.8753	72.68
P21	HMF 100 mg/ml	0.7710	75.94
P24	Narigenin 100 mg/ml	0.8153	74.55

Comparison across materials

Within each plant pathway, the purified constituent produced slightly higher inhibition than its crude extract. HMF exceeded *S. jambos* leaf extract, and naringenin exceeded *C. sinensis* peel extract, against both species. HMF gave the single highest inhibition among all test materials against both *P. gingivalis* (70.27%) and *A. actinomycetemcomitans* (75.94%). On a mass basis, *S. jambos* leaf extract achieved inhibition comparable to *C. sinensis* peel extract at a 50-fold lower concentration (0.5 vs 25 mg/mL).

DISCUSSION

This study evaluated the ability of *S. jambos* leaf extract, *C. sinensis* peel extract, HMF, and naringenin to inhibit biofilm formation by *P. gingivalis* and *A. actinomycetemcomitans* in vitro. All four materials reduced biofilm biomass of both species in a concentration-dependent manner, which is consistent with the study hypotheses.

The reduction in crystal violet signal reflects a decrease in total biofilm biomass rather than direct evidence of cell killing. Because both species rely on LuxS/AI-2 quorum sensing to coordinate adhesion, nutrient acquisition, and community maturation, interference with this signalling pathway is a plausible explanation for the reduced biofilm formed. When AI-2 signalling is disrupted, bacteria may fail to reach or interpret the quorum threshold, so that the expression of biofilm-associated genes declines and the resulting biofilm is less mature and less stable [11]. However, the crystal violet assay cannot distinguish quorum sensing inhibition from growth inhibition or simple antiadhesion effects, so the proposed mechanism remains an interpretation that requires direct confirmation.

A. actinomycetemcomitans was generally more susceptible than *P. gingivalis*, showing higher inhibition for the same material at the same concentration. One exception was *S. jambos* leaf extract at 0.5 mg/mL, which inhibited *P. gingivalis* (67.07%) slightly more than *A. actinomycetemcomitans* (65.75%). The generally greater susceptibility of *A. actinomycetemcomitans* may relate to its comparatively direct AI-2 recognition and processing route, whereas *P. gingivalis* possesses a more complex and adaptive regulatory network that may buffer the effect of quorum sensing disruption [13].

For both species, the purified constituents produced slightly higher inhibition than their respective crude extracts, and HMF was the most active material overall. The smaller molecular size and higher polarity of HMF may favour diffusion into the biofilm matrix and interaction with molecular targets, which could account for its activity [5]. The apparently stronger performance of the crude extracts and purified compounds must be interpreted with caution, because the differences between closely ranked groups (for example, HMF versus naringenin) are small and were not tested statistically.

The comparison between the two plants should not be read as crude potency on an equal footing. Although *C. sinensis* peel extract gave higher percentage inhibition than *S. jambos* leaf extract, it was used at concentrations roughly 50-fold higher. On a mass basis, S. jambos leaf extract was therefore considerably more potent, which is relevant for any future formulation work.

This study has several limitations. The monospecies biofilm model does not capture the polymicrobial complexity of subgingival biofilms. The crystal violet assay quantifies total biomass only and does not assess cell viability, matrix composition, or gene expression; the proposed involvement of LuxS/AI-2 therefore remains inferential. The test compounds were dissolved in DMSO, so a matched vehicle control and reporting of final in-well concentrations are needed to exclude a solvent contribution. Finally, the small number of replicates and the descriptive analysis limit the strength of statistical inference. Further work using multispecies biofilm models, AI-2 bioassays, quorum sensing gene expression analysis, growth and viability assays, and biocompatibility testing is required before these materials can be considered for clinical application.

CONCLUSION

S. jambos leaf extract, *C. sinensis* peel extract, HMF and naringenin inhibited biofilm formation by *P. gingivalis* and *A. actinomycetemcomitans* in vitro, and inhibition increased with concentration. HMF at 100 mg/mL produced the highest inhibition among the four test materials against both species. *A. actinomycetemcomitans* was generally more susceptible than *P. gingivalis*, and the purified constituents were slightly more active than their crude extracts; on a mass basis, S. jambos leaf extract was the most potent material.

Interference with LuxS/AI-2 quorum sensing is a plausible mechanism for these effects, but it was not measured directly in this study. Confirmation requires AI-2 bioassays, quorum sensing gene expression analysis, growth and viability testing, and multispecies biofilm models before these materials can be considered as candidate agents for adjunctive periodontal therapy.

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