International Journal of Scientific Advances
ISSN: 2708-7972
Volume: 7 | Issue: 6 | Nov – Dec 2026 Available Online: www.ijscia.com
DOI: 10.51542/ijscia.v7i6.24

Inhibitory Effects of *Syzygium jambos* (L.) Leaf Extract, *Citrus sinensis* (L.) Peel Extract, and the Inducers HMF and Naringenin on Biofilm Formation of *Streptococcus mutans* and *Candida albicans*

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ABSTRACT

In this *in vitro*, post-test only control group study, *Streptococcus mutans* ATCC 25175 and *Candida albicans* ATCC 10231 were incubated for 24 h in 96-well microplates together with *Syzygium jambos* (L.) leaf extract (0.125, 0.25, 0.5 mg/mL), *Citrus sinensis* (L.) peel extract (15, 20, 25 mg/mL), 5-hydroxymethylfurfural (HMF; 25, 50, 100 mg/mL) or naringenin (25, 50, 100 mg/mL). Quercetin served as positive control. Biofilm biomass was quantified by crystal violet staining (OD_{590}) in three replicates, and percentage inhibition was calculated. Data were analysed descriptively. All materials reduced biofilm biomass in a concentration-dependent manner. Against S. mutans, naringenin 100 mg/mL (83.58%), S. *jambos* 0.5 mg/mL (82.96%), and C. *sinensis* 25 mg/mL (82.35%) gave numerically higher inhibition than quercetin (79.77%), whereas HMF gave 75.65%. Against C. albicans, C. *sinensis* 25 mg/mL was the most active (91.19%), followed by S. jambos (85.05%), naringenin (84.70%), and HMF (81.92%); none exceeded quercetin (92.88%). All four materials inhibited biofilm formation by S. mutans and C. albicans *in vitro* in a concentration-dependent manner and may be promising candidates for further development as natural antibiofilm agents against oral pathogens, pending statistical confirmation and testing in multispecies models and biocompatibility studies.

Keywords: *Syzygium jambos (L.); Citrus sinensis (L.); 5-hydroxymethylfurfural; Naringenin*; Antibiofilm; *Streptococcus mutans; Candida albicans*

INTRODUCTION

Dental and oral diseases remain a significant global health burden. According to the World Health Organization, oral diseases affect nearly 3.5 billion people worldwide, with dental caries among the most prevalent conditions [1]. In Indonesia, national survey data indicate that the prevalence of dental caries is close to 90%, highlighting the urgency of effective preventive and therapeutic strategies. Dental caries is a multifactorial disease characterised by progressive demineralisation of dental hard tissues, initiated by microbial activity within dental biofilms [5].

Streptococcus mutans is widely recognised as the primary cariogenic bacterium because of its strong ability to adhere to tooth surfaces, produce extracellular polysaccharides, and generate organic acids through carbohydrate fermentation. These properties allow *S. mutans* to form highly structured and resilient biofilms that contribute to enamel demineralisation and caries progression. Once established, biofilms show increased resistance to antimicrobial agents, which makes infection control more challenging [3,5]. Fungal organisms also play a role in oral infections. *Candida albicans* is the most frequently isolated fungal species in the oral cavity

and the main causative agent of oral candidiasis. This opportunistic pathogen can shift from a commensal to a virulent form under favourable conditions, such as immunosuppression or microbial imbalance, and its ability to form biofilms further enhances its pathogenicity and resistance to antifungal treatment [6–8].

Biofilm formation is a complex and dynamic process regulated by microbial communication systems known as quorum sensing. Through quorum sensing, microorganisms coordinate gene expression related to adhesion, extracellular matrix production and virulence factor secretion. Disruption of quorum sensing has emerged as a promising approach to inhibit biofilm formation without exerting bactericidal or fungicidal pressure, which may reduce the risk of antimicrobial resistance [9,10].

Natural products have attracted considerable interest as alternative antibiofilm agents because of their availability and diverse bioactive compounds. *Syzygium jambos* (L.) leaves contain phenolic compounds and triterpenoids with antimicrobial and anti-inflammatory properties [12,19], and their ethanolic extracts have shown antibiofilm and anti-quorum activity [11]. *Citrus sinensis* (L.) peels are rich in flavonoids such as naringin and hesperidin [14], and citrus flavonoid-rich extracts have been reported to possess quorum sensing inhibitory and antibiofilm activities [13,25,27,28]. In addition, 5-hydroxymethylfurfural (HMF) and naringenin have been identified as potential quorum sensing modulators [15,16,24] and are referred to in this study as inducers, i.e. bioactive compounds proposed to modulate quorum sensing. However, much of this evidence comes from Gram-negative models such as *Pseudomonas aeruginosa*, and comparative studies of plant extracts and bioactive compounds against both bacterial and fungal oral biofilms remain limited.

Therefore, this study aimed to evaluate the antibiofilm activity of *S. jambos* leaf extract, *C. sinensis* peel extract, HMF, and naringenin against *S. mutans* and *C. albicans* using an *in vitro* crystal violet biofilm assay. We hypothesised that each material would reduce biofilm formation in a concentration-dependent manner.

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. Plant extracts were prepared at the Testing Service Unit (ULP), Universitas Airlangga, Surabaya, and all biofilm work was carried out at the Research Center, Faculty of Dental Medicine, Universitas Airlangga, between July and November 2025.

Microorganisms

Streptococcus mutans ATCC 25175 and *Candida albicans* ATCC 10231 were obtained from the culture collection of the Research Center, Faculty of Dental Medicine, Universitas Airlangga. *S. mutans* was cultured on Mueller-Hinton agar and suspended in brain heart infusion broth (BHIB), while *C. albicans* was cultured on Sabouraud dextrose agar (SDA) and suspended in Sabouraud dextrose broth (SDB). The turbidity of each suspension was adjusted to a 0.5 McFarland standard (approximately 1×10^8 CFU/mL) before use.

Preparation of Extracts and Inducers

Fresh *Syzygium jambos* (L.) leaves and *Citrus sinensis* (L.) peels were collected from Batu, East Java, Indonesia [authentication details]. The leaves were washed with sterile water, air-dried away from direct sunlight at room temperature (±28 °C) for 48 h, and ground into a fine powder [23]. The peels were washed, dried at room temperature (±30 °C) for two days, and blended into a fine powder [14,21]. Fifty grams of leaf powder was macerated in 250 mL of 96% ethanol, and 10 g of peel powder in 100 mL of 96% ethanol, each for 3 days. The mixtures were filtered through Whatman No. 1 filter paper, and the filtrates were concentrated using a rotary vacuum evaporator at ≤40 °C. The leaf extract was weighed and stored in a tightly closed container at room temperature until use. The peel extract was weighed, sterilised under UV light for 24 h, and stored in a tightly closed container at 4 °C until use.

The inducers were used as supplied by the manufacturer: 5-hydroxymethylfurfural (HMF; 99% purity) and naringenin (≥ 95% purity), both from Sigma-Aldrich. Quercetin (≥ 95% purity, Sigma-Aldrich) served as the positive control. HMF, naringenin and quercetin were each dissolved in dimethyl sulfoxide (DMSO).

Treatment Groups

For each microorganism, a negative control (broth with the microorganism and sterile distilled water) and a positive control (broth with the microorganism 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. *S. mutans* groups were prepared in BHIB and *C. albicans* groups in SDB. Each group was assayed in three replicates.

Biofilm Formation and Treatment

Biofilms were grown in sterile 96-well microplates. Standardised suspensions of *S. mutans* (in BHIB) and *C. albicans* (in SDB) were inoculated into the wells simultaneously with the respective treatment and incubated at 37 °C for 24 h [atmosphere] to allow biofilm formation [Li 2019]. Negative controls received sterile distilled water, and positive controls received quercetin (100 mg/mL). The same control wells served as the reference for all test materials within each microorganism.

Crystal Violet Assay

Biofilm biomass was quantified by crystal violet staining [6]. The adherent biofilm was stained with 0.1% crystal violet (200 µL per well) for 15 min. The

stain was removed by pipette, and the wells were washed twice with running water. The plate was air-dried at room temperature, and the bound dye was solubilised with 95% ethanol (200 µL per well). The plate was covered to reduce evaporation and left for 30 min. Absorbance (optical density, OD) was read at 590 nm on a microplate reader.

Data Analysis

The mean OD_{590} of the three replicates was calculated for each group. TThe percentage of biofilm inhibition relative to the negative control was calculated as [7]:

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

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 *S. mutans* and *C. albicans* relative to the negative control, and inhibition increased with concentration. Against *S. mutans*, naringenin at 100 mg/mL gave the highest inhibition (83.58%), followed closely by *S. jambos* leaf extract at 0.5 mg/mL (82.96%) and *C. sinensis* peel extract at 25 mg/mL (82.35%). All three were numerically higher than the quercetin positive control (79.77%), whereas HMF (75.65%) was lower. Against *C. albicans*, *C. sinensis* peel extract at 25 mg/mL gave the highest inhibition (91.19%), and none of the test materials reached the level of quercetin (92.88%).

Inhibition of *S. mutans* biofilm

For *S. mutans*, the lowest biofilm biomass (highest inhibition) within each test material was reached at the top concentration tested. The quercetin positive control gave a mean OD_{590} of 0.526 (79.77% inhibition). Among the test materials, naringenin gave the lowest OD (0.427), followed by *S. jambos* leaf extract (0.443), *C. sinensis* peel extract (0.459), and HMF (0.633).

TABLE 1: Biofilm inhibition of *S. mutans* by different test materials.

Group	Material (Best Concentration)	Mean OD590	Inhibition (%)
K (-)	Negative Control	2.600	0
K (+)	Quercetin 100 mg/ml	0.526	79.77
P6	*S. jambos* leaf extract 0.5 mg/ml	0.443	82.96
P3	*C. sinensis* peel extract 25 mg/ml	0.459	82.35
P9	HMF 100 mg/ml	0.633	75.65
P12	Narigenin 100 mg/ml	0.427	83.58

Inhibition of *C. albicans* biofilm

For *C. albicans*, the highest inhibition within each test material was again reached at the top concentration. The quercetin positive control gave the lowest OD (0.177; 92.88% inhibition). *C. sinensis* peel extract was the most active test material (OD 0.219), followed by *S. jambos* leaf extract (0.371), naringenin (0.380), and HMF (0.450).

TABLE 2: Biofilm inhibition of *C. albicans* by different test materials.

Group	Material (Best Concentration)	Mean OD590	Inhibition (%)
K (-)	Negative Control	2.486	0
K (+)	Quercetin 100 mg/ml	0.177	92.88
P15	*S. jambos* leaf extract 0.5 mg/ml	0.371	85.05
P18	*C. sinensis* peel extract 25 mg/ml	0.219	91.19
P21	HMF 100 mg/ml	2.486	0
P24	Narigenin 100 mg/ml	0.177	92.88

Concentration-dependent inhibition

Inhibition rose with each step in concentration for all materials against both microorganisms (Table 3).

TABLE 3: Biofilm inhibition (%) at each concentration tested.

Material	Concentration (mg/mL)	*S. mutans*	*C. albicans*
S. jambos leaf extract	0.125	41.69	66.49
	0.25	66.96	71.08
	0.5	82.96	85.05
C. sinensis peel extract	15	53.54	39.79
	20	66.65	62.84
	25	82.35	91.19

Material	Concentration (mg/mL)	*S. mutans*	*C. albicans*
HMF	25	52.00	52.13
	50	65.00	67.21
	100	75.65	81.92
Naringenin	25	53.15	57.61
	50	66.96	65.88
	100	83.58	84.70
Quercetin (K+)	100	79.77	92.88

Comparison across materials

Within each plant pathway, the purified compound did not consistently outperform its crude extract. HMF gave lower inhibition than *S. jambos* leaf extract against both *S. mutans* (75.65% vs 82.96%) and *C. albicans* (81.92% vs 85.05%). Naringenin gave slightly higher inhibition than *C. sinensis* peel extract against *S. mutans* (83.58% vs 82.35%) but lower inhibition against *C. albicans* (84.70% vs 91.19%). On a mass basis, *S. jambos* leaf extract achieved inhibition comparable to or higher than *C. sinensis* peel extract at a 50-fold lower concentration (0.5 vs 25 mg/mL), and comparable to or higher than HMF at a 200-fold lower concentration (0.5 vs 100 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 *S. mutans* and *C. albicans in vitro*. All four materials reduced biofilm biomass of both microorganisms in a concentration-dependent manner. Against *S. mutans*, naringenin, *S. jambos* leaf extract and *C. sinensis* peel extract gave numerically higher inhibition than quercetin at their top concentrations, whereas against *C. albicans* none of the materials reached the level of the positive control. Antimicrobial activity of *S. jambos* leaf extract against *S. mutans* [22] and of *C. sinensis* peel extract against caries-related bacteria [21] has been reported previously; the present findings extend these observations to biofilm formation.

The reduction in crystal violet signal reflects a decrease in total biofilm biomass rather than direct evidence of cell killing. Because each treatment was added together with the inoculum, the results describe inhibition of biofilm formation, not disruption of an established biofilm. *S. mutans* biofilm formation is regulated by cell-to-cell signalling, including the competence-stimulating peptide and LuxS/AI-2 systems [5,9,10], whereas *C. albicans* biofilm development depends on surface adhesion, the yeast-to-hypha transition and quorum-sensing molecules such as farnesol [8,10]. Interference with these processes is a plausible explanation for the reduced biofilm. Naringenin has been reported to inhibit *S. mutans* biofilm formation [16], and other flavonoids, such as myricetin, act partly through anti-adhesion effects [17]; *S. jambos* leaf extracts have shown antibiofilm and anti-quorum properties [11]. However, the evidence for quorum-sensing inhibition by HMF, naringenin and citrus peel extracts comes mainly from Gram-negative models such as *Pseudomonas aeruginosa* and *Yersinia enterocolitica* [15,24,25,27], so extrapolation to *S. mutans* and *C. albicans* remains speculative. The crystal violet assay also cannot distinguish quorum-sensing inhibition from growth inhibition or simple anti-adhesion effects, so any proposed mechanism requires direct confirmation.

At the highest concentrations, *C. albicans* showed higher inhibition than *S. mutans* for all four test materials, although the differences were small for naringenin (84.70% vs 83.58%) and *S. jambos* leaf extract (85.05% vs 82.96%). At lower concentrations, the pattern was mixed. *C. sinensis* peel extract at 15 and 20 mg/mL inhibited *S. mutans* more than *C. albicans* (53.54% vs 39.79%, and 66.65% vs 62.84%), whereas *S. jambos* leaf extract at 0.125 mg/mL was far more active against *C. albicans* (66.49% vs 41.69%). Compounds with different molecular targets have been shown to differ in efficacy against *C. albicans* and *S. mutans* [18]. These cross-species comparisons should be made cautiously, since the two organisms differ in cell structure, growth medium (BHIB vs SDB) and baseline biofilm density, and quercetin itself was also more effective against *C. albicans* (92.88% vs 79.77%), which suggests the yeast assay may have been more sensitive overall.

The purified compounds did not consistently outperform the crude extracts. Naringenin was slightly more active than *C. sinensis* peel extract against *S. mutans* but less active against *C. albicans*, and HMF was less active than *S. jambos* leaf extract against both microorganisms. This may indicate that the activity of a crude extract arises from several constituents acting together rather than from a single marker compound [19,20,28]. In addition, flavonoids in sweet orange peel occur largely as glycosides such as naringin and hesperidin [14], whereas naringenin is the aglycone of naringin, so the extract and the pure compound are not chemically equivalent.

The comparison between materials should not be read as potency on an equal footing, because the materials were tested at very different concentrations. *S. jambos* leaf extract reached 82.96% and 85.05% inhibition at 0.5 mg/mL, whereas *C. sinensis* peel extract, HMF and naringenin required 25 to 100 mg/mL for comparable effects. On a mass basis, *S. jambos* leaf extract was therefore considerably more potent, which is relevant for any future formulation work. Mass-based comparison between a crude extract and a pure compound is also only approximate, since the extract is a mixture of unknown composition.

This study has several limitations. The monospecies biofilm model does not capture the polymicrobial complexity of dental plaque. The crystal violet assay quantifies total biomass only and does not assess cell viability, matrix composition or gene expression. The negative control contained distilled water, while quercetin, HMF and naringenin were dissolved in DMSO, so a matched vehicle control and reporting of final in-well concentrations are needed to exclude a solvent contribution. Replicate variation was notable in some groups (for example, *C. sinensis* peel extract at 15 mg/mL against *C. albicans*, with OD values from 1.086 to 1.971), and the small number of replicates, single reference strains and descriptive analysis limit the strength of statistical inference. Biofilm formation was assessed with a single reference strain per species and with replicates whose independence from separate experiments should be confirmed. The medium was used as described and no sucrose supplementation was applied, which may limit extrapolation to sucrose-dependent plaque formation. Further work using multispecies biofilm models, inferential statistics, growth and viability assays, gene expression analysis 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 *S. mutans* and *C. albicans in vitro* in a concentration-dependent manner. Naringenin was the most active material against *S. mutans* (83.58%), and *C. sinensis* peel extract against *C. albicans* (91.19%), while *S. jambos* leaf extract was the most potent on a mass basis. These materials are promising candidates for further development as natural antibiofilm agents against oral pathogens; however, confirmation with statistical analysis, multispecies biofilm models and biocompatibility testing is needed before clinical application.

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