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Anti-biofilm and Anti-adherence Effects of Soursop (*Annona muricata*) Leaf Extract Against Dental Pathogens *Streptococcus* mutans and *Streptococcus sobrinus*

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Abstract
One of the most prevalent oral issues occurring worldwide is dental caries (tooth decay), which is primarily caused by major dental pathogens *Streptococcus mutans* and *Streptococcus sobrinus*. Dental caries begins with biofilm formation, which facilitates the quorum-sensing mechanism of oral bacteria. Increasing antimicrobial resistance of oral pathogens has become a major challenge in combating the issue. This led to the discovery of a plant-based therapeutic agent for its potential therapeutic activities against oral pathogens. This study assessed the anti-biofilm and anti-adherence effects of aqueous soursop (*Annona muricata*) leaves extract (Aq-SLE) against *S.mutans* and *S.sobrinus*. LC-MS analysis of the Aq-SLE revealed the presence of several bioactive compounds, including gallic acid, quercetin, and annonacin. To assess the anti-biofilm properties, both bacterial cells were treated with varying concentrations of Aq-SLE ranging from 3.125 mg/mL to 50 mg/mL at 2-fold intervals. For the anti-adherence assay, concentrations ranged from 12.5 mg/mL to 200 mg/mL. The extract induced a dose-dependent reduction in biofilm formation, with an MBIC value determined at 50 mg/mL for both bacterial strains. In both bacterial species, adhesion to the resin surface was reduced at 200 mg/mL, but further dose-response analysis is required. These findings demonstrate that Aq-SLE exhibits promising anti-biofilm and anti-adherence properties against cariogenic pathogens, particularly in disrupting early stages of biofilm formation.
Keywords: Anti-biofilm; anti-adherence; soursop leaves; *S. mutans*; *S. sobrinus*

Introduction

There are 2.3 billion untreated caries lesions occurring in the permanent dentition, and dental caries continues to be one of the most prevalent diseases despite efforts to prevent and manage it (Giacaman et al., 2022). *S.mutans* and *S.sobrinus* are the main causative organisms that contribute to the development of dental caries (Oda et al., 2015). Both bacterial species are Mutans Streptococci that are capable of producing lactic acid, which is able to demineralize the tooth enamel. A specialized enzyme called glucosyltransferases (Gtfs) produced by these cariogenic bacteria is responsible for inducing the formation of biofilms on the teeth surfaces (Liana Mohd Zulkamal et al., 2023; Xu et al., 2018). A number of efforts to prevent and manage dental caries had been explored for the past decades, including mechanical removal of plaque biofilms and the use of anti-microbial agents (Kuang et al., 2018; Jiao et al., 2019).

In recent years, a large number of research studies have been carried out in an effort to search for new alternatives to tackle the issue of oral diseases. Consequently, there has been a growing interest in exploring natural products and phytochemicals as safe, affordable, and biocompatible alternatives to conventional oral therapeutics. Soursop (*Annona muricata*) has been extensively studied for its therapeutic benefits, where various parts of the fruit have been reported to contain bioactive compounds with many medicinal properties (Mutakin et al., 2022). While studies have examined its antibacterial and cytotoxic effects against various pathogens and cancer cells, limited research has focused specifically on its anti-biofilm and anti-adherence activities against oral pathogens such as *S. mutans* and *S. sobrinus.*

Understanding the ability of *A. muricata* leaf extract to inhibit bacterial adhesion and biofilm formation is crucial, as these early steps are fundamental to the initiation and progression of dental caries. By interfering with the mechanisms responsible for bacterial colonization and biofilm maturation, natural compounds could significantly reduce cariogenic potential without the adverse effects associated with synthetic agents.

This study was designed to evaluate the anti-biofilm and anti-adherence activities of aqueous *A. muricata* leaf extract (Aq-SLE) against *S. mutans* and *S. sobrinus*. The research also aimed to identify the active phytochemical constituents responsible for these effects using liquid chromatography-mass spectrometry (LC-MS). The findings from this study will contribute to the growing body of knowledge on natural oral therapeutics and may inform the development of novel plant-based agents for dental care applications.

Materials and methods

Soursop (*A. muricata*) leaf powder was purchased from an online shop for this study. Aqueous extraction was performed using the decoction method. Specifically, 20 g of powdered soursop leaf was boiled in 200 mL of distilled water for about 45 minutes. The mixture was cooled to room temperature, filtered through Whatman No. 1 filter paper, and then concentrated using a rotary evaporator at 40°C. The concentrated extract was then freeze-dried to obtain the powdered form of aqueous soursop leaf extract (Aq-SLE). The dried extract was stored at –20 °C until further analysis.

The identification of bioactive compounds in the Aq-SLE was performed using Liquid Chromatography–Mass Spectrometry (LC-MS) equipped with a Triple Quadrupole (TQ) detector. The LC-MS system was operated under optimized conditions to identify known compounds based on retention time and mass spectra comparison against references from previous studies.

Two bacteria of interest, *Streptococcus mutans* (ATCC 25175) and *Streptococcus sobrinus* (ATCC 33402), were used in this study. Both strains were cultured in Brain Heart Infusion (BHI) broth and incubated at 37 °C for 24 hours. For long-term storage, the bacterial cultures were preserved in 25% glycerol at –80 °C. Working cultures were prepared by adjusting the bacterial suspension to a 0.5 McFarland standard to ensure uniform cell density for all assays.

The minimum biofilm inhibitory concentration (MBIC) of Aq-SLE was determined using a crystal violet (CV) staining assay in 96-well microtiter plates. 100 µL of standardized bacterial suspension and 100 µL of Aq-SLE at concentrations ranging from 3.125 mg/mL to 50 mg/mL, prepared through 2-fold serial dilution in BHI broth, were added into each respective well of the sterile flat-bottomed 96-well plates. Negative control wells received bacteria without extract, and the positive control contained bacteria with 0.25% chlorhexidine. The plates were incubated at 37 °C for 24 hours to allow biofilm formation. Following incubation, wells were gently washed with phosphate-buffered saline (PBS) to remove non-adherent cells and dried at room temperature. Each well was then stained with 0.1% crystal violet solution for 15 minutes. Excess stain was rinsed with distilled water, and the plates were air-dried. The bound dye was solubilized with 33% acetic acid, and absorbance was measured at 595 nm using a microplate reader.

The anti-adherence activity of Aq-SLE was assessed using a modified method based on Kim et al. (2015) with synthetic tooth resin. Each resin was sterilized by using ethanol and were then exposed under UV light. Bacterial suspensions of *S. mutans* and *S. sobrinus* were first prepared by adjusting to a 0.5 McFarland standard in phosphate-buffered saline (PBS), followed by the addition of 1% sucrose-supplemented BHI broth to promote adherence. Diluted Aq-SLE at concentrations ranging from 12.50 mg/mL to 200.00 mg/mL was prepared in universal bottles, each labelled according to concentration. A total of 2 mL of the respective extract was mixed with 2 mL of standardized bacterial suspension, and a resin tooth was immersed in each mixture. The tubes were then gently agitated at 37 °C for 120 minutes to allow bacterial adhesion. After incubation, each resin tooth was transferred to a fresh universal bottle containing phosphate-buffered saline (PBS) and sonicated for 1 minute to dislodge the adherent bacteria. The resulting suspensions were serially diluted (10-fold) and plated onto BHI agar. After 24 hours of incubation, colony-forming units (CFUs) were counted. The number of adherent bacteria in treated samples was compared with a negative control to determine the percentage of adherence inhibition.

With the exception of the anti-adherence assay, all experiments will be carried out in triplicate. Data are expressed as mean + standard deviation (SD). Statistical analysis was conducted using GraphPad Prism 10. In the crystal violet assay, a one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test was used to compare the effects of different concentrations of soursop leaf extract with the untreated control. A p-value < 0.05 will be considered statistically significant for all analyses.

Results and discussion

LC-MS profiling of Aq-SLE revealed the presence of several phenolic and flavonoid compounds with known antimicrobial activities. Gallic acid and quercetin were identified as major components, both of which are associated with anti-biofilm effects. Annonacin, an acetogenin compound, was also detected and is reported to contribute to antimicrobial properties (Aguilar-Hernández et al., 2024).

Referring to Figure 1, Aq-SLE inhibited biofilm formation by both *S. mutans* and *S. sobrinus* in a dose-dependent manner. At the highest tested concentration (50.00 mg/mL), significant biofilm inhibition was observed in both species ($p < 0.0001$). *S. mutans* showed 91.32% inhibition while *S. sobrinus* showed 84.76% inhibition at this concentration.

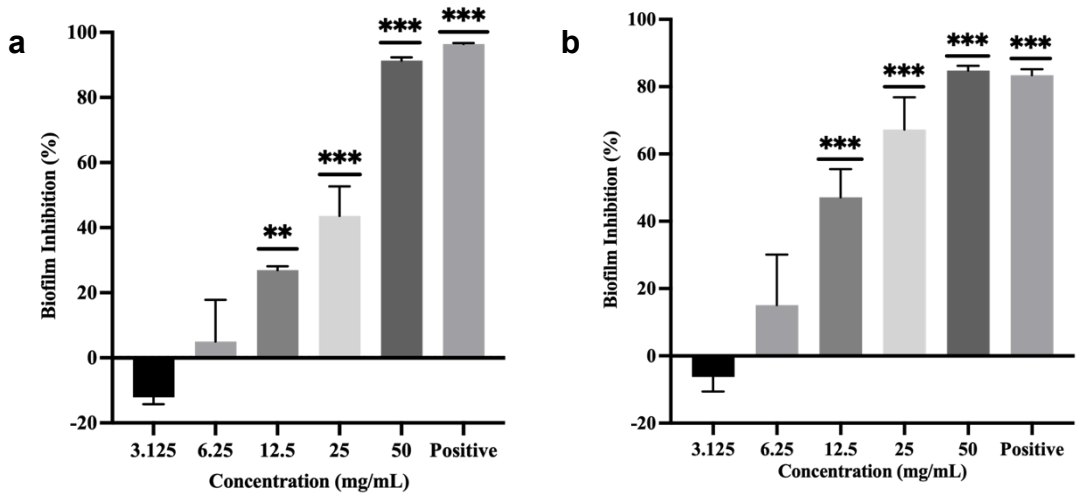

Figure 1 *S. mutans* (a) and *S. sobrinus* (b) Biofilm Inhibition (%) at Different Concentrations of Aq-SLE. * denote significance versus negative control (**p < 0.01, ***p < 0.0001). Positive control: 0.25% chlorhexidine.

Notably, at the lowest tested concentration (3.125 mg/mL), the result showed a negative inhibition value, which indicates that Aq-SLE exerts a potential sub-inhibitory effect. This concentration is referred to as sub-MIC levels. As reported in a previous study, Dong et al. (2012) found that sub-minimum inhibitory concentrations (sub-MICs) of chlorhexidine (CHX) and sodium fluoride (NaF) unexpectedly increased biofilm density in S. mutans. Their findings indicate that exposure to low levels of these agents would not inhibit, but might even increase, cariogenic biofilm formation and thus worsen dental caries. This result highlights the dangers of sub-MIC antimicrobial exposure, where stress-inducing responses could promote biofilm virulence instead of inhibiting it.

As shown in Figures 2 and 3, Aq-SLE reduced the adherence of *S. mutans* and *S. sobrinus* to resin surfaces in a dose-dependent manner. For *S. mutans*, the highest inhibition was observed at 200 mg/mL (2.3×10^5 CFU/mL), while S*. sobrinus* showed the lowest count at the same concentration (1.0×10^4 CFU/mL). TNTC results from untreated controls were normalized to 3.0×10^6 CFU/mL for comparative purposes. A clearer dose-response pattern was seen in *S. sobrinus*, whereas *S. mutans* showed an unexpected decrease in CFU at 12.5 mg/mL, possibly due to pipetting inconsistencies or uneven resuspension. Since this assay was conducted without biological replicates, the findings should be interpreted as preliminary. Single-run variability and the absence of a saliva-coated surface limit the generalizability of the data. Nonetheless, the results indicate that Aq-SLE possesses anti-adherence

activity at higher concentrations, requiring further investigation using standardized, replicated protocols and more physiologically relevant models.

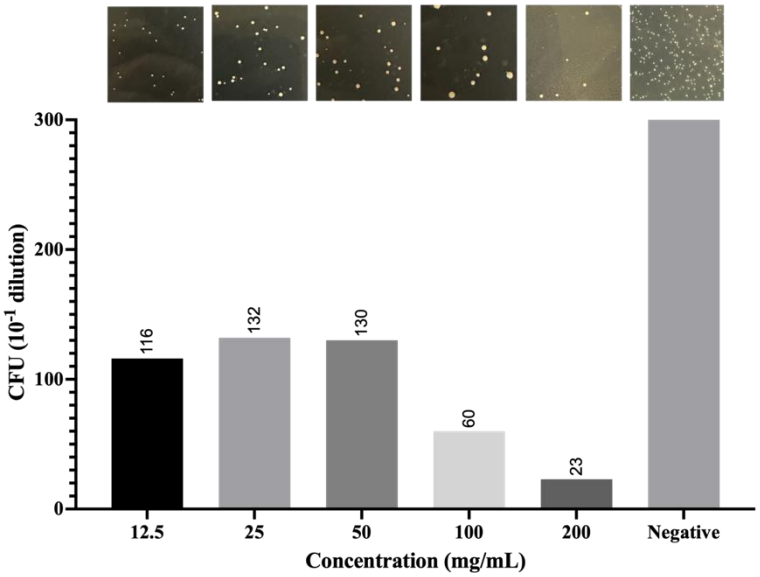

Figure 2 Anti-adherence activity against *S. mutans*. (Top) Visual plate comparison showing decreased colonization with increasing extract concentration. (Bottom) Quantitative CFU analysis.

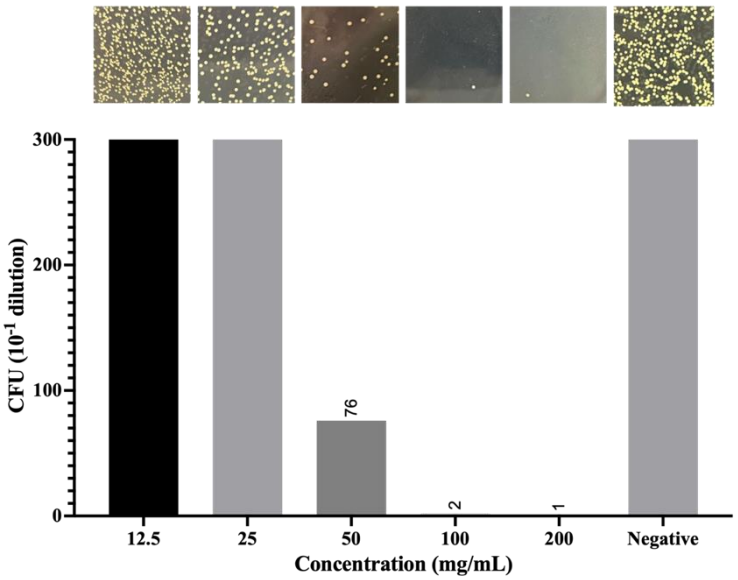

Figure 3 Anti-adherence activity against *S. sobrinus*. (Top) Visual plate comparison showing decreased colonization with increasing extract concentration. (Bottom) Quantitative CFU analysis.

Conclusion

Aqueous soursop (*Annona muricata*) leaf extract demonstrates significant in vitro anti-biofilm and anti-adherence activities against *S. mutans* and *S. sobrinus*. These results highlight its therapeutic potential as a natural oral care agent. Future work should include saliva-coated surface models, mechanistic validation, and formulation development for in vivo applications.

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