

Antibacterial assessment of *Ziziphus mauritiana* Lam on inhibition of the growth and biofilm of *Streptococcus mutans*

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ABSTRACT

Background: *Streptococcus mutans* (*S. mutans*) causes dental caries. *S. mutans* biofilms are inhibited by the antibacterial properties of *Ziziphus mauritiana* Lam (*Z. mauritiana* Lam). **Purpose:** Evaluating the potential of *Z. mauritiana* Lam in inhibiting the growth and biofilm formation of *S. mutans* ATCC 25175 *in vitro*. **Methods:** This study used the ethanolic extract of *Z. mauritiana* Lam as the test material and *S. mutans* as the research subject. Spectrophotometry (620 nm) was used to assess the growth of *S. mutans*, the inhibition of *S. mutans* biofilm using a 1% crystal violet staining was measured by spectrophotometry (520 nm), and the visualization of the biofilm mass was conducted with an electric microscope (200x). **Results:** At all concentrations, *Z. mauritiana* Lam displayed excellent growth inhibition of *S. mutans* 0.04–0.09 (< 300 CFU/mL) and was able to inhibit the formation of *S. mutans* biofilm with a strong scale of optical density (OD) 0.4 at 24 hours incubation time. At the same time, the incubation time of 48 and 72 hours tended to have moderate-scale biofilm inhibition (OD 0.2–3.9). At a concentration of 25%, the biofilm mass decreased by a relatively small size, the same as the positive control group. At 50%, 12.5%, and 6.25%, it was seen that the *S. mutans* biofilm mass experienced a dominant loss. **Conclusion:** *Z. mauritiana* Lam can be bacteriostatic against the growth of *S. mutans* and can inhibit the formation of biofilms by degrading the structure and mass of *S. mutans* biofilms.

Keywords: biofilm; dental caries; growth; *Streptococcus mutans*; *Ziziphus mauritiana* Lam

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INTRODUCTION

Streptococcus mutans (*S. mutans*) has been reported as the primary agent in caries pathogenesis. These bacteria can form biofilms and produce acid from carbohydrate fermentation. A biofilm is a collection of microorganisms that can adhere to solid surfaces, such as tooth enamel, tooth roots, or dental implants.¹ Microorganisms in biofilms can produce an extracellular matrix and live surrounded by this matrix. The process of attachment of bacteria to the tooth surface occurs in the early stages of biofilm formation. Specific and nonspecific interactions control bacterial attachment to the tooth surface.² Glucans mediate particular interactions, while nonspecific interactions involve hydrophobic interactions. This interaction activity can increase the or biofilm formation.³

The increased biofilm formation by *S. mutans* on the dental pellicle may make it easier to enhance the stability of quorum-sensing formation with other oral bacteria.⁴ This property can interfere with the mucosal defense system or the activity of the tooth pellicle protein to stem the adhesion of *S. mutans*, thus making it easier for *S. mutans* to increase its growth rate.⁵ Biofilm eradication and inhibiting the growth of *S. mutans* is one strategy to reduce dental caries infection.⁶

Efforts to eliminate *S. mutans* as normal flora in the mouth with antibiotics are considered inappropriate because they can disrupt the ecological balance of the oral cavity.⁷ The extended use of antibiotics tends to increase the resistance of *S. mutans*, lead to an increase in biofilm formation and growth. It will be a severe problem in the treatment of dental caries.⁸ Karikalan and Mohankumar⁹

demonstrated that prolonged exposure to antibiotics leads to the development of resistance in *S. mutans* against ampicillin and Penicillin G. Throughout history, humans have used plants as anti-microbial agents to prevent tooth infections and dental caries.¹⁰ One plant whose properties may help prevent biofilm formation and reduce the growth of *S. mutans* by *Z. mauritiana* Lam.¹¹

Z. mauritiana Lam is reported to have antifungal and antibacterial properties. Its high antioxidant content allows this plant to neutralize bacterial virulence activities, including inhibiting the growth and formation of *S. mutans* biofilm as a prerequisite for dental caries.¹¹ In addition, *Z. mauritiana* Lam contains secondary metabolites such as flavonoids, saponins, and tannins that aid in reducing bacterial growth and removing dental plaque.¹² This study aims to evaluate the antibacterial properties of *Z. mauritiana* Lam in inhibiting the growth and formation of *S. mutans* biofilm in vitro.

MATERIALS AND METHODS

This study acquired ethical clearance under No. 324.KE/FKG/2021 from the Faculty of Dentistry, Universitas Syiah Kuala, Banda Aceh, Indonesia. The research was conducted in vitro, using *Z. mauritiana* Lam as a test material to evaluate its antibacterial potential for inhibiting the growth and formation of biofilm *S. mutans* ATCC 25175. The concentration and incubation time were used as a reference to create an overview of the optimum inhibition of each concentration and incubation time. *Z. mauritiana* Lam was obtained from Aceh Besar District, Province of Aceh, Indonesia (5.570704102637194, 95.35980633540842).

The extraction of assay materials and Gas chromatography/mass spectrometry (GCMS) analysis of chemical compounds were adopted from Soraya et al.² One kilogram of *Z. mauritiana* Lam leaves was chopped and macerated in five liters of 96% ethanol for 24 hours while stirring every four hours, after which it was decanted and filtered. The resulting residue was macerated for 48 hours with fresh 96% ethanol. The filtrate was then evaporated using a rotary vacuum evaporator to obtain a concentrated extract, which was then heated to 45 °C any remaining ethanol.

The spectrophotometric assessment of *S. mutans* showed its growth was initiated by preparing *Z. mauritiana* Lam extract at various concentrations. Each well of the 96-well plate received 50 µL of Tryptic Soy Broth (TSB) medium, was incubated for 15 minutes, and then washed twice with (Phosphate-buffered saline) BS (pH 7.0). The *S. mutans* was then placed in a 25 µL well in the medium and incubated for 15 minutes at room temperature (27°C). Each well of 100 µL contained a predetermined concentration of test material (1:4), which was then incubated in an anaerobic atmosphere for 24, 48, and 72 hours. Using spectrophotometry (Bio-Rad, USA) with an optical density (OD) of 620 nm, the quantity of *S. mutans* was measured based on turbidity.¹³

S. mutans biofilm was measured using the 1% violet crystal method, according to Gani et al.¹⁴ The *Z. mauritiana* Lam was extracted at 50%, 25%, 12.5%, 6.25%, and 3.125%. They were coated on a 96-well plate with 100 µL of TSB medium, allowed to incubate for 15 minutes, and rinsed with PBS (pH 7.0), after which 25 µL of *S. mutant* was added to each well, then allowed to incubate for 15 minutes at room temperature. *S. mutans* and assay material were added to each well containing a different test (triple serial), homogenized at 1,000 xg for five minutes, and incubated anaerobically for 24, 48, and 72 hours. The formation of *S. mutans* biofilms was evaluated by removing all solutions from the wells and washing them with PBS in a dishwasher at 1,000 xg for five minutes (repeated twice). Then, 150 µL of 1% violet crystal was injected into each well of the plate. The crystalline violet dye and biofilm protein were then homogenized for 10 minutes using a shaker at 100 xg. Each well was rinsed with 150 µL of PBS for five minutes, after which the solution was discarded and replaced with 150 µL of 70% ethanol for one minute. Following marking the 96-well plates containing biofilms based on the absorption of violet crystalline dyes, the plates were incubated at room temperature for 15 minutes. At 520 nm, the biofilm mass of *S. mutans* was measured using a spectrophotometer. Anti-biofilm evaluation was based on OD spectrophotometry: OD ≥ 0.4 (strong); OD = 0.2–0.39 (moderate); OD = 0.05–0.19 (low); OD < 0.05 (weak, no biofilm formation).¹³ The differences in the growth of *S. mutans* with biofilm formation and its correlation to incubation time were analyzed with the Kruskal-Wallis test with p < 0.05 as a significant reference.

Table 1. Growth inhibition of *S. mutans* after being influenced by *Z. mauritiana* Lam

<i>Z. mauritiana</i> Lam	N	Growth Inhibition of <i>S. mutans</i> (OD 620 nm)									*p-value
		24 h			48 h			72 h			
		Mean	S.Devt	Scale	Mean	S.Devt	Scale	Mean	S.Devt	Scale	
50%	3	0.099	0.005	10%	0.0890	0.002646	9%	0.0900	0.002	13%	0.004
25%	3	0.084	0.005	9%	0.0917	0.012423	9%	0.0870	0.06811	13%	
12.5%	3	0.058	0.060	6%	0.0943	0.112401	9%	0.0461	0.0461	7%	
6.25%	3	0.047	0.027	5%	0.0963	0.126255	10%	0.0941	0.0941	14%	
3.125%	3	0.055	0.017	6%	0.0853	0.078647	9%	0.0625	0.0625	9%	
CHX	3	0.081	0.005	8%	0.0837	0.007095	8%	0.0850	0.0850	13%	
<i>S. mutans</i>	3	0.539	0.114	56%	0.4627	0.019296	46%	0.2050	0.2050	31%	
*p-value			0.02			0.189			0.188		

*Kruskal-Wallis Test

RESULTS

This study evaluated the antibacterial properties of *Z. mauritiana* Lam in inhibiting the growth and formation of *S. mutans* biofilms. Table 1 summarizes the growth inhibition of *S. mutans* by various concentrations of *Z. mauritiana* Lam extract over 24, 48, and 72 hours. At a 50% concentration, the extract showed 10% inhibition at 24 hours, 9% at 48 hours, and 13% at 72 hours, with a significant p-value of 0.004 at 72 hours. The 25% concentration consistently inhibited growth by 9% at both 24 and 48 hours, increasing to 13% at 72 hours. The 12.5% concentration showed 6% inhibition at 24 hours, 9% at 48 hours, and 7% at 72 hours. At 6.25%, inhibition was 5% at 24 hours, 10% at 48 hours, and 14% at 72 hours. The 3.125% concentration consistently inhibited growth by 6% at 24 hours, 9% at 48 hours, and 9% at 72 hours. The control group using CHX showed consistent inhibition of 8% at both 24 and 48 hours, increasing to 13% at 72 hours. In contrast, the untreated *S. mutans* group showed significantly higher growth, with 56% at 24 hours, 46%

at 48 hours, and 31% at 72 hours. The p-values indicate significant inhibition at 24 hours (0.02), but not at 48 hours (0.189) and 72 hours (0.188).

The degradation of *S. mutans* biofilm mass due to the influence of various concentrations of *Z. mauritiana* Lam extract is depicted in Figure 1. At a concentration of 25% (B), the biofilm mass showed a slight reduction, similar to the positive control group (F). However, at concentrations of 50% (A), 12.5% (C), and 6.25% (D), there was a significant loss of biofilm mass, indicating that *Z. mauritiana* Lam extract is effective in inhibiting the formation of *S. mutans* biofilm in vitro. Table 2 shows the effect of *Z. mauritiana* Lam extract on inhibiting *S. mutans* biofilm formation over 24, 48, and 72 hours. At a 50% concentration, biofilm inhibition was 14% at 24 hours (0.53 ± 0.58), increasing to 18% at 48 hours (0.365 ± 0.481) and 17% at 72 hours (0.457 ± 0.397), with a p-value of 0.017 indicating statistical significance at 24 hours. At a 25% concentration, biofilm inhibition was 13% at 24 hours, 11% at 48 hours, and 13% at 72 hours. The 12.5% concentration showed the highest inhibition of 23% at 24

Table 2. The influence of *Z. mauritiana* Lam on the inhibition of *S. mutans* biofilm formation

<i>Z. mauritiana</i> Lam	N	Inhibition of <i>S. mutans</i> biofilm (520 nm)									*p-value
		24 h			48 h			72 h			
		Mean	S.Devt	Scale	Mean	S.Devt	Scale	Mean	S.Devt	Scale	
50%	3	0.53	0.58	14%	0.365	0.481	18%	0.457	0.397	17%	0.017
25%	3	0.48	0.69	13%	0.225	0.151	11%	0.352	0.216	13%	
12.5%	3	0.87	0.59	23%	0.271	0.052	13%	0.461	0.461	17%	
6.25%	3	0.67	0.11	18%	0.303	0.037	15%	0.376	0.376	14%	
3.125%	3	0.66	0.10	17%	0.352	0.026	17%	0.406	0.406	15%	
CHX	3	0.55	0.40	15%	0.545	0.399	26%	0.619	0.619	23%	
*p-value			0.02			0.061			0.172		

*Kruskal-Wallis Test

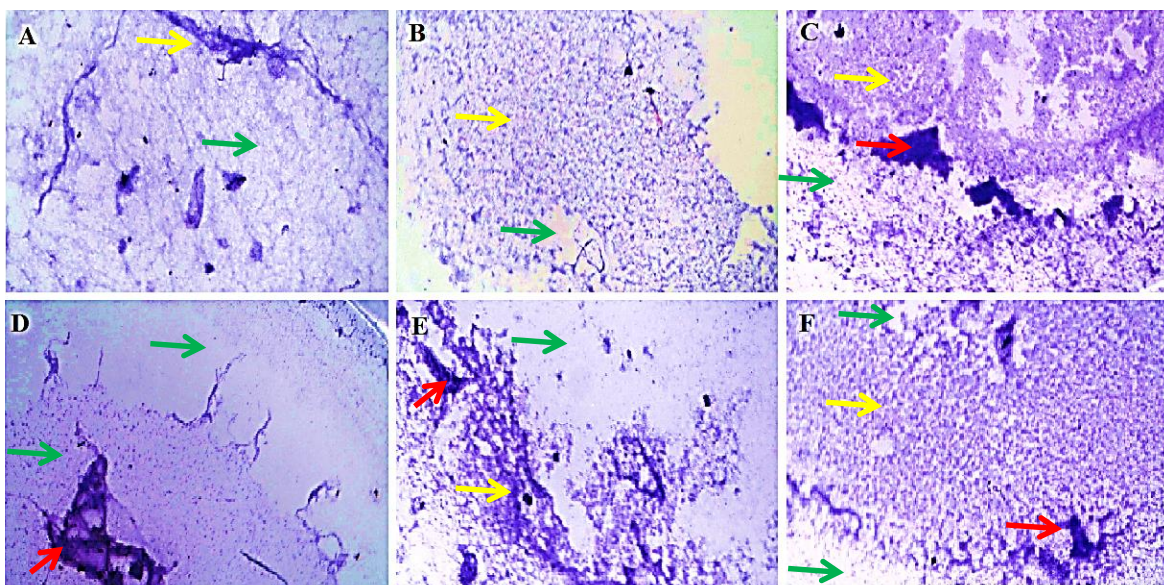


Figure 1. Mass of *S. mutans* biofilm. Mass degradation of *S. mutans* biofilm after being influenced by various concentrations of *Z. mauritiana* Lam extract. (A) 50%, (B) 25%, (C) 12.5%, (D) 6.25%, (E) 3.125%, (F) Positive control (CHX). Yellow arrow (biofilm mass), green arrow (missing biofilm mass), and red arrow (inactive biofilm mass). One percent crystal violet stain with 200x magnification.

hours, which decreased to 13% at 48 hours and 17% at 72 hours. At a 6.25% concentration, inhibition was 18% at 24 hours, 15% at 48 hours, and 14% at 72 hours. At a 3.125% concentration, biofilm inhibition was 17% at 24 and 48 hours and 15% at 72 hours. The control group using CHX showed consistent inhibition, with 15% at 24 hours, 26% at 48 hours, and 23% at 72 hours.

Overall, higher concentrations of *Z. mauritiana* Lam extract tended to show greater biofilm inhibition. The 50% concentration showed significant inhibition at 24 hours, while CHX, as the control, demonstrated consistent inhibition but was most effective at 48 hours. The p-values indicate statistical significance at 24 hours (0.02) but not at 48 hours (0.061) and 72 hours (0.172).

The extract of *Z. mauritiana* Lam demonstrated efficacy in reducing the growth frequency of *S. mutans*, leading to a consequent decrease in biofilm formation, as illustrated in Figures 1 and 2. The analysis revealed that, after 24 hours,

the 50% and 25% concentrations exhibited a notably close interaction compared to the other experimental groups. At the 48-hour mark, a marked similarity in the interaction was observed between the 25% and 12.5% concentrations. By the 72-hour mark, the interaction between the 25% and 6.25% concentrations was very close. This interaction was methodically analyzed based on the percentage of inhibition gain attributed to each concentration of the test substance.

DISCUSSIONS

This study evaluated the antibacterial properties of *Z. mauritiana* Lam on the growth and formation of *S. mutans* biofilms. The study focuses on these two virulence properties because they are critical in decreasing adhesion in dental caries' pathogenesis. *Z. mauritiana* Lam is reported to

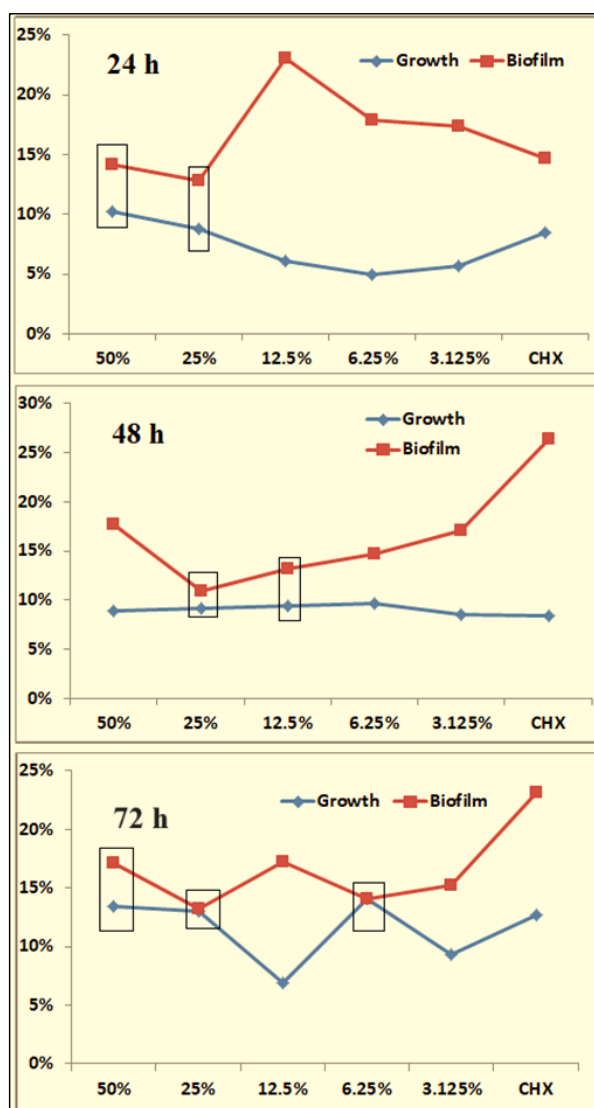


Figure 2. The relationship between growth inhibition and biofilm formation of *S. mutans* after being affected by *Z. mauritiana* Lam extract. The most substantial interaction occurred at 72 hours of incubation. The black box lines on the bar lines indicate the interaction between the growth and formation of *S. mutans* biofilms.

have several secondary metabolites that act as antioxidants, antifungals, and antibacterials. The antibacterial role tends to be facilitated by flavonoids, saponins, and tannins.¹⁵ Arma et al.¹⁶ reported that *Z. mauritiana* Lam contains antioxidant and antifungal compounds, such as Vitamin E, squalene, and phytol. Several of these compounds have been reported to reduce the virulence properties of *S. mutans* potentially.

Table 1 shows that *Z. mauritiana* Lam could inhibit the growth of *S. mutans*. All concentrations demonstrated excellent inhibitory ability against *S. mutans* 0.04–0.09 (< 300 CFU/mL). The power of *Z. mauritiana* Lam to inhibit the growth of *S. mutans* is related to the role of several active antibacterial compounds found in *Z. mauritiana* Lam. Jubair et al.¹⁷ reported that almost all plants are rich in secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found to have anti-microbial properties in vitro. Cellularly, antibacterial compounds increase the action potential on the surface of the cell membrane so that the cell surface exerts pressure, which causes changes in the membrane's permeability properties, which impacts membrane leakage.¹³ These impacts can cause the release of intracellular fluid and cells, causing lysis.¹⁸ According to Giovannoni et al.,¹⁹ releasing cellular contents from the cell wall causes the cell wall to degrade. This phenomenon suggests a possible antibacterial mechanism by which *Z. mauritiana* Lam inhibits the growth of *S. mutans* and the cellular response to dental caries treatment.

The release of intracellular fluid can cause a decrease in the cellular response to cell division activity so that cells do not form new daughters.²⁰ The fungistatic properties of *Z. mauritiana* Lam have increased the sensitivity of the cellular response to its growth, thus indirectly influencing the development and response of infection to the host. Miller and Zachary²¹ reported that DNA activity often influences mitotic failure, with intracellular fluid outside the cell membrane wall disrupting genetic communication and consequently affecting protein synthesis, which DNA facilitates.

Table 2 reports that *Z. mauritiana* Lam can inhibit the formation of *S. mutans* biofilms. The incubation time of 24 hours tends to result in strong biofilm inhibition, while the incubation time of 48 and 72 hours tends to yield moderate-scale biofilm inhibition. The biofilm inhibitory properties of *Z. mauritiana* Lam correlate with the intensity of bacterial growth (Figure 1). Based on the findings from this study, *Z. mauritiana* Lam can interfere with and inhibit the formation of *S. mutans* biofilms (Figure 1). This phenomenon indicates that *Z. mauritiana* Lam can interfere with communication between the bacterial cells involved in biofilm formation.¹⁶

The increase in conductivity caused by the chemical compounds in *Z. mauritiana* Lam could be involved in the interruption of communication between bacterial cells during quorum sensing formation,¹ which could interfere with the biofilm formation of *S. mutans*. Meanwhile, the

antibacterial compounds contained in *Z. mauritiana* Lam can inhibit biofilm formation by reducing the morphology or degrading the structure of the biofilm¹² so that the biofilm mass loses cohesion and cannot adhere to the pathogenesis of caries infection. This study reported that the extract of *Z. mauritiana* Lam degraded the biofilm's mass and decreased the biofilm's morphology.

From the perspective of biofilm inhibition modeling, Ghosh et al.²² reported that the inhibition of biofilms by antibacterial compounds could be initiated by inhibiting bacterial surface adhesion, disrupting the quorum-sensing system, disrupting the signaling of genetic information, interfering with biofilm maturation, and degrading biofilm mass. The small organic compounds present in several anti-infective plants can prevent bacterial infection by inhibiting the adhesion of the bacterial surface to the host cell and interfering with the quorum-sensing system in the biofilm formation process.²²

In conclusion, *Z. mauritiana* Lam can be bacteriostatic against the growth of *S. mutans* and can inhibit the formation of biofilms by degrading the structure and mass of *S. mutans* biofilms. The limitation of this study was that there was no attempt to examine how the antibacterial properties of *Z. mauritiana* Lam might interfere with the communication of *S. mutans* cells with other bacteria in the formation of quorum sensing and biofilms. This study gives hope that *Z. mauritiana* Lam may be used as an active preventative against dental caries.

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