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Antibiofilm potential of *Zanthoxylum zanthoxyloides* bark-extract against biofilm-producing *Streptococcus mutans* in dental caries

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Keywords

Streptococcus mutans; antibiofilm; dental caries; therapy; *Zanthoxylum zanthoxyloide*

Abstract

Streptococcus mutans is the major cause of dental caries, which is costly to treat. The burden is worse in low- and middle-income countries, due to poor access to oral health services and diets high in free sugars. Conventional antibiofilm agents in oral health often have side effects, prompting the search for safer and affordable alternatives. This study investigates the antibiofilm activity of *Zanthoxylum zanthoxyloides* bark extract against biofilm-forming *S. mutans*. Dental plaque and tooth samples were collected from hospitals after ethical approval. *S. mutans* was identified using standard microbiological methods on Mitis Salivarius agar supplemented with Bacitracin and potassium tellurite. Biofilm formation was assessed with Congo Red agar, biofilm-related genes *gtfB* and *gtfC* were detected using polymerase chain reaction (PCR) and biofilm production quantified using a spectrophotometric assay. The chewing stick was authenticated and its ethanolic bark extract was obtained. The Minimum Biofilm Inhibitory Concentrations (MBIC) of the extract was determined at 0.2-100 mg/mL. Extract bioactive compounds were subsequently identified using Gas Chromatography-Mass Spectrometry (GC-MS) analysis. From 254 samples, 63 isolates were identified, with 61% being biofilm producers. *gtfB* and *gtfC* were detected in 56% and 50% of the isolates respectively. Biofilm production varied. The lowest concentration of the *Z. zanthoxyloides* that inhibited ≥ 80% of *S.mutans* biofilm was 0.20 mg/mL. The study confirms *Z. zanthoxyloides*, has significant biofilm inhibitory activity against *S. mutans*, supporting its potential as an alternative or complementary therapy.

Introduction

Dental caries, commonly known as tooth decay, is a prevalent condition affecting both children and adults worldwide. It is classified as a periodontal disease and originates with microbial colonization of microorganisms within the oral cavity. The early habitants of the mouth are predominantly facultative anaerobes, which include *Neisseria* spp., *Streptococci gordonii. Streptococcus mitis*, *Actinomycetes* Sp, *Streptococcus mutans* among others (Lemos et al. 2019). These early colonizers resist the cleansing effects of saliva and tongue movements, forming a microbial community that influences both oral health and disease progression (Liu et al. 2025).

The oral *Streptococci* include both commensal and pathogenic bacteria. Under certain conditions, commensal species can

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shift to become opportunistic pathogens, triggering disease and damaging the host. As these bacteria grow and metabolize, they alter the oral environment by changing factors like the redox potential (Eh), pH and colonize to form dental plaque. Acidic byproducts from bacterial fermentation of carbohydrates dissolve the calcium phosphate in the enamel, leading to tooth demineralization and the onset of dental caries (Machiulskiene et al. 2020). The process accelerates when the oral pH drops below 5.5, further dissolving tooth minerals. If remineralization does not occur, the carious lesion progresses, resulting in gradual tissue loss. A study into pH of plaque said that the critical pH for increased demineralization of dental hard tissues (enamel and dentine) is 5.5. The Stephan curve illustrates how quickly the plaque pH can fall below 5.5 after a snack or meal (Muhammad and Ahmed 2023).

S. mutans is widely recognized as the primary etiologic agent in dental caries, playing a critical role in dental plaque formation due to its ability to adhere to tooth surfaces and ferment carbohydrates, producing acids that de-mineralize tooth enamel (Rathee and Sapra, 2025). *S. mutans* is a highly virulent microorganism that produces biofilms, acids, and bacteriocins such as mutacins, which contribute to its pathogenicity (Lemos et al. 2019). The cariogenic *S. mutans* encodes the *gtf* operon, a glucosyltransferase enzyme-producing set of genes, namely, *gtfB*, *gtfC*, and *gtfD* (Baty et al. 2022). The enzymes utilize the glucose that results from sucrose fermentation to synthesize glucans, the building block of the biofilm-associated exopolysaccharide matrix (glucose polymer) (Bowen and Koo 2018).

The protein-bacterium interactions are sucrose dependent mechanism and sucrose independent mechanism. The sucrose dependent mechanism depends mainly on the production of glycosyltransferases *gtfB* and *gtfC* and *gtfD*, which are responsible for synthesis of glucan, while the sucrose independent adhesion is initiated by interaction of salivary agglutinins and *S. mutans* with the surface associated protein P1 (also known as I/II antigen, SpaP or Pac1. *gtfB* and *gtfC* enzymes synthesize water-insoluble glucans which are rich in -1,3-glucosidic linkages while *gtfD*, produces water-soluble glucans which are rich in -1,6-glucosidic linkages (often called dextran) (Lu et al. 2019). They are the major constituents of plaque or biofilm matrices. *gtfB* (formally known as *gtf*I) synthesizes primarily insoluble glucan rich in α-1,3-linkages, *gtfC* (*gtf*S produces a mixture of soluble (with mostly α-1,6-linkages) and insoluble glucans, while *gtfD* (*gtf*S) forms predominantly soluble glucans. These enzymes convert sucrose to fructose and glucose, and the enzymes further utilize the glucose that results from sucrose fermentation to synthesize glucans, the building block of the biofilm-associated exopolysaccharide matrix (glucose polymer) (Bowen and Koo 2018). Glucans are sticky and help in the formation of a robust extracellular polysaccharide EPS matrix in dental plaques, followed by biofilm formation which enhances the adhesion of *S. mutans* to tooth surfaces and to each other, promoting the formation of a stable biofilm (dental plaque). All *gtf*s have similar protein structure. *gtfB* and g*tfC* are homologous sharing ~75% of amino acid sequences while, *gtfD* possesses 50% sequence identity. The enzyme, *gtfB* (4.4 kb) and *gtfC* (4.3 kb) are in an operon arrangement separated by 198 bp (Van Hijum et al. 2006).

Research have shown that reducing *S. mutans* adhesion or inhibiting its acidogenic properties could mitigate its role in dental caries (Matsui and Cvitkovitch, 2010). Consequently, controlling factors such as biofilm formation and acid production is crucial for preventing disease progression. Scientific studies support the traditional use of *Zanthoxylum zanthoxyloides*, commonly known as the "toothache tree," in oral healthcare. The plant contains bioactive compounds such as alkaloids, flavonoids, saponins, and phenolic compounds, which have demonstrated antimicrobial efficacy (Ekunwe et al. 2022). The aim of this study was to isolate *S. mutans* strains from dental caries patients, assess their biofilm production, and evaluate the antibiofilm potential of *Z. zanthoxyloides* extract.

Furthermore, the study investigated the flavonoid-rich bioactive compounds in the extract that may contribute to its therapeutic properties.

Materials and Methods

The study was undertaken at Uniosun Teaching hospital and State Specialist Hospital, Asubiaro, Osogbo, Osun state, South west Nigeria. Osogbo lies on coordinates 7.7833°N latitude and 4.5667°E.

Ethical approval and sample collection

Ethical approval for this study was obtained from Uniosun Teaching Hospital under protocol number UTH/REC/2023/04/756. A total of 254 samples were collected based on the sample size formula for prevalence studies: $n = z^2p(1-p)/d^2$, with an additional 10% attrition rate, as described by Zayed et al. (2021). The collected samples included 119 decayed teeth and 135 plaque samples, which were placed in sterile plain bottles containing approximately 3 mL of prepared normal saline. The samples were then transported to the laboratory immediately.

Colonial, phenotypic and biochemical characteristics of the isolates

The samples were inoculated by streaking on Mitis Salivarius Agar (MSA) plates (HiMedia), supplemented with 1% potassium tellurite and modified with 0.5 IU/mL bacitracin (Sigma, USA). The inoculated plates were incubated at 37°C for 48 hours following the methodology outlined by Al-Mudallal et al. (2008). Gram's staining, sucrose fermentation and catalase test were performed on each isolate.

Qualitative determination of biofilm formers using Congo Red agar Method

Biofilm detection was performed according to Mohamed et al. (2013). Black coloration with dry consistency on Congo Red agar indicated biofilm-producing isolates.

Confirmation of biofilm producing genes by Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was utilized to amplify biofilm-associated genes using species-specific primers targeting the glucosyltransferase (*gtfB* and *gtfC*) enzyme of *S. mutans*. The forward primer for GTFB was designated as: Forward: 5' AGCAATGCAGCCAATCTACAAAT 3'Reverse: 5' ACGAACTTTGCCGTTATTGTCA 3' (Shemesh et al., 2007), while that for *gtfC* was Forward: 5' GGTTTAACGTCAAAATTAGCTGTATTA GC 3' Reverse: 5' CTCAACCAACCGCCACTGTT 3' (Ueda et al. 1988; Van Hijum, 2006). The primers were synthesized by Inquaba Biotechnical Sequencing Service, South Africa. The reaction was carried out by adding 6µl of purified template DNA into PCR tubes, followed by the addition of 1.0 µL each of forward and reverse primers and 7.5µl green master mix containing Tag polymerase (promega, USA), dNTPs (deoxyribonucleic triphosphate) mix, MgCl2 (Magnesium Chloride), Buffer and dye, the volume was adjusted with 4.5 µL nuclease-free de-ionized water to make up 20µL. Genomic DNA was extracted using a commercial extraction kit, followed by PCR amplification. Polymerase chain reaction was carried out in a Gene Amp 9700 PCR System Thermal cycler (Applied Bio system Inc., USA). It was conducted using the following thermocycling conditions; an initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing of primer at 55 °C for 30 seconds, with extension at 72 °C for 45 seconds, with a final extension at 72°C for 5 minutes. The amplified gene fragments were visualized under a UV transilluminator, and positive results were documented. The molecular weights were compared with the mobility of 100 DNA ladder base pair (bp) that ran alongside experimental samples in the gel.

Quantification of Biofilm produced from each isolate

Each isolate was inoculated in 3 mL of Brain Heart Infusion (BHI) broth supplemented with 2% sucrose and incubated at 37°C for 48 hours. The culture was then adjusted to a 0.5 McFarland standard using sterile normal saline. Subsequently, 2 µL of the dilution was transferred into another 2 µL

of BHI broth containing 2% sucrose to achieve a 100-fold dilution. The diluted culture for each isolate was thoroughly mixed and distributed into wells of a 96-well microtiter plate at 200 µL per well in triplicates. The plate was incubated at 37°C for 48 hours. Following incubation, the culture was discarded by inverting the plate, and each well was washed three times with sterile saline (0.9%). To fix the biofilm, 200 µL of methanol was added to each well and left for 20 minutes before being discarded. The plate was then allowed to dry. Following this, 200 µL of 0.1% crystal violet was added to each well and left for 15 minutes. After staining, the wells were washed three times with distilled water and left to dry completely. To dissolve the stained biofilm for measurement, 200 µL of 33% glacial acetic acid was added to each well. The Optical Density (OD) at 600 nm was then measured using a microplate reader. The average OD of each test isolate was calculated, and the mean OD of the control wells (without bacteria) was subtracted to obtain the final biofilm measurement. Each isolate was inoculated in 3 mL of Brain Heart Infusion (BHI) broth supplemented with 2% sucrose and incubated at 37°C for 48 hours. The culture was then adjusted to a 0.5 McFarland standard using sterile normal saline. Subsequently, 2 µL of the dilution was transferred into another 2 µL of BHI broth containing 2% sucrose to achieve a 100-fold dilution. The diluted culture for each isolate was thoroughly mixed and distributed into wells of a 96-well microtiter plate at 200 µL per well in triplicates. The plate was incubated at 37°C for 48 hours. Following incubation, the culture was discarded by inverting the plate, and each well was washed three times with sterile saline (0.9%). To fix the biofilm, 200 µL of methanol was added to each well and left for 20 minutes before being discarded. The plate was then allowed to dry. Following this, 200 µL of 0.1% crystal violet was added to each well and left for 15 minutes. After staining, the wells were washed three times with distilled water and left to dry completely. To dissolve the stained biofilm for measurement, 200 µL of 33% glacial acetic acid was added to each well. The OD at 600 nm was then measured using a microplate reader. The average OD of each test isolate was calculated, and the mean OD of the control wells (without bacteria) was subtracted to obtain the final biofilm measurement. The final measurement was classified into strong, moderate and weak biofilm formers following this equation; $ODc<OD \leq 2 \times ODc$ (Weak Biofilm producer), $4XODc \leq OD$ (Strong Biofilm producer), $2xODc \leq OD \leq 4xODc$ (Moderate biofilm producer), $OD \leq ODc$ (No biofilm production) following the procedure previously described in Mohammed et al. (2013) and Zayed et al. (2021), where, ODc is the Optical Density of the control well (well containing sterile hearth infusion broth), which served as a baseline measurement

Extract preparation

Z. zanthoxyloides was confirmed at the herbarium unit of the Department of Botany, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria, with herbarium accession number IFE 18310. Approximately 50 grams of *Z. zanthoxyloides* bark was soaked in 50 mL of ethanol in a 750 mL flask. The mixture was agitated intermittently and kept for two weeks. Thereafter, it was filtrated through Whatman No 1 filter paper and air-dried in a laminar airflow**.**

Determination of antibiofilm activity using microtiter plate assay

Each isolate was sub-cultured on Nutrient agar plates before being transferred to Brain Heart Infusion (BHI) broth and incubated at 37°C for 48 hours. The bacterial culture was then adjusted to a 0.5 McFarland standard using normal saline (1.5×10^8 CFU/mL). Subsequently, 2 mL of each isolate was transferred into 2 mL of fresh BHI broth to prepare the inoculum suspension. A 96-well microtiter plate was prepared for the biofilm inhibition assay. In the first column, 100 µL of double-strength BHI broth was added, while 100 µL of single-strength BHI broth was distributed into the remaining wells. The extracts were reconstituted by weighing 100mg of the extract and dissolved in100 µL of DMSO (Dimethyl sulfoxide) shaken for

complete dissolution. After complete dissolution, it was top up to 1 mL total volume, that is 100 mg extract plus 0.1 ml DMSO plus 0.9 ml sterile water to make 100 mg/ml in 1% DMSO to make stock solution. The first well of each row received 100 µL of the extract (100 mg/mL), which was mixed thoroughly. Serial dilutions were performed by transferring 100 µL from the first well to the next across ten columns, discarding 100 µL from the final column. Columns 11 and 12 served as controls: Column 11 represented biofilm formation by untreated isolates, while Column 12 assessed biofilm inhibition by 0.2% chlorohexidine, the plates were incubated at 37°C for 48 hours. After incubation, the plates were decanted by inversion and washed three times with sterile saline (0.9%). Methanol was added to each well and left at room temperature for 20 minutes before being discarded, allowing the plates to air dry. The biofilms were stained by adding 0.1% crystal violet to each well and left for 15 minutes, followed by three washes with distilled water. The plates were then inverted and left to dry at room temperature. To quantify biofilm formation, 200 µL of 33% glacial acetic acid was added to each well to dissolve the stained biofilm OD at 600 nm was measured using a microplate reader. The percentage inhibition of biofilm formation by the extract was calculated using the following formula:

$$1-\left(\frac{\text{average OD 600 of treated isolate}}{\text{average OD 600 of untreated isolate}}\right) \text{X } 100$$

For each extract, the minimum biofilm inhibitory concentration (MBIC) was determined as the lowest concentration that inhibited biofilm formation by ≥80% according to the published procedure (Kirmusaoglu 2019; Kwasny and Opperman, 2010).

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of Flavonoid Compound

The flavonoid content was analyzed using Gas Chromatography-Mass Spectrometry (GC-MS) with a Shimadzu QP2010 Plus model equipped with an AOC-20i auto-sampler and a gas chromatograph interfaced with a mass spectrometer. The mass spectrum data were interpreted using the National Institute of Standards and Technology (NIST) database, which contains over 62,000 reference patterns. The fragmentation spectra of unknown components were compared to those of known compounds stored in the NIST 11 library. The relative percentage of each compound was determined by calculating its average peak area relative to the total chromatogram area. The name, molecular weight, and structure of the active compounds in the *Z. zanthoxyloides* extract were identified according to previously established methodologies (Owokotomo et al. 2015; Oladele et al. 2021).

Statistical Analysis

The datasets obtained in this study were subjected to frequency distribution to determine the occurrence of MBIC percentage inhibition ranges. Graphical illustrations and interpretations were done using Microsoft Excel LTSC, 2019 and Flourish Studio software (https://flourish.studio/visualisations/heatmaps/).

Results

Phenotypic and biochemical identification of S. mutans in the Samples

At 72 hours of incubation, *S. mutans* isolates exhibited characteristic hard, raised, convex, opaque colonies with a rough, pale blue appearance and glossy texture (Plate 1). No observable growth was noted on some plates. On fermentation of sucrose, using phenol red as indicator, the colour changed from red to yellow and the isolates were catalase negative. Microscopically, they appeared as Gram-positive cocci arranged in chains (Figure 1). A total of sixty-three (24.80%) *S. mutans* were identified from the 254 samples. Out of these, fifty-one (80.95%) and twelve (19.04%) were recovered from

tooth samples and plaque samples respectively.

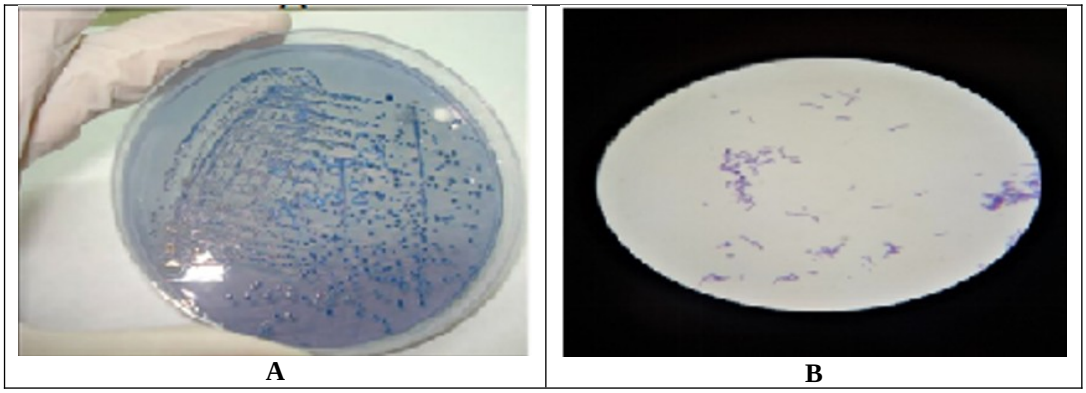


Figure 1: **A:** Appearance of *S. mutans* on *Mitis salivarius* agar plate.
B: Microscopic view of s. Mutans

Qualitative Determination and Molecular Confirmation of Biofilm-Producing Genes in S. mutans

Among the 63 *S. mutans* isolates, 59 exhibited black coloration with a dry consistency, confirming biofilm formation. The presence of glucosyltransferase (*gtfB*) and *gtf* genes, essential for glucan synthesis, was detected using species-specific primers at 517 bp and 319 bp, respectively. The *gtfB* gene was identified in 54 (91.5%) isolates, while 48 (81.35%) isolates carried the *gtfC* gene (Figures 2 and 3 respectively). Variations in gene presence were noted, emphasizing their crucial role in biofilm formation. The biofilm production categories of each isolate varied with three classified as weak biofilm-formers, seven, moderate biofilm formers and 44 were strong biofilm formers (figure 4).

Percentage Inhibition of Antibiofilm Activities of Z. zanthoxyloides extract on biofilm formers

All the isolates exhibited varying percentages of biofilm inhibition across *Z. zanthoxyloides* extract concentration, which ranged from 0.20-100mg/mL. Notably, some isolates demonstrated inhibition values ≥ 80% at different concentrations levels. These variations indicate the respective Minimum Biofilm Inhibitory Concentrations (MBIC) values for each isolate. The MBIC values differed among the isolates, with effective biofilm inhibition observed at relatively low concentrations of the extract (figure 5).

Figure 6 shows the comparison of minimum biofilm-inhibition concentration at ≥80% with other percentage inhibition ranges and their frequencies against *S. mutans*. The concentrations range from 0.20 mg/mL to 100 mg/mL, with each concentration further differentiated into 5 distinct MBIC percentage ranges (0-20%, 20.1-40%, 40.1-60%, 60.1-80%, and 80.1-100%). The frequency of the biofilm-producing *S. mutans* inhibition was represented within their specific concentrations and MBIC percentage ranges. At 100 mg/mL, no *S. mutans* was observed at the 0-20% MBIC range, while 16 *S. mutans* isolates were observed at the 60.1-80% and 80.1-100% MBIC. As the concentration decreases, there is a gradual reduction in the 80.1-100% MBIC percentage range. Despite the good MBIC at 50 mg/mL, a slight shift towards lower inhibition ranges compared to 100 µg/mL was observed, with 5 *S. mutans* isolates observed in the 0-20% MBIC percentage range. The 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, 3.13 mg/mL, 1.56 mg/mL, 0.78 mg/mL, and 0.39 mg/mL concentrations show a more varied distribution, with frequencies spread across different MBIC percentage ranges. It was also observed that the lowest concentration (0.20 mg/mL) still shows a decent spread of inhibition, with the highest frequencies (13 each) at the 40.1-60% and 60.1-80% MBIC

percentage ranges. Each isolate showed a high percentage of inhibition at different dilutions, with some isolates demonstrating more potent antibiofilm activity ≥80%. As the concentration decreases, the ≥80% inhibition potency reduces in frequency.

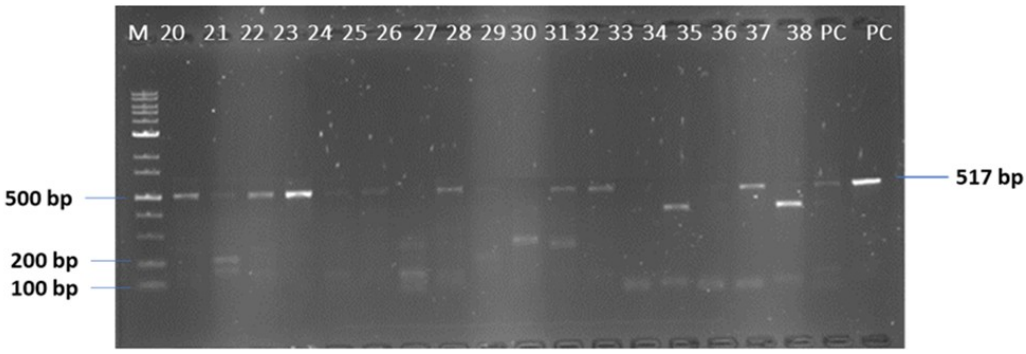


Figure 2: A representative gel electrophoresis image of PCR products from *S. mutans* with *gtfB* gene
Legend: Lane M: Molecular weight marker; Lanes 1-74, 75-96: Plaque samples.

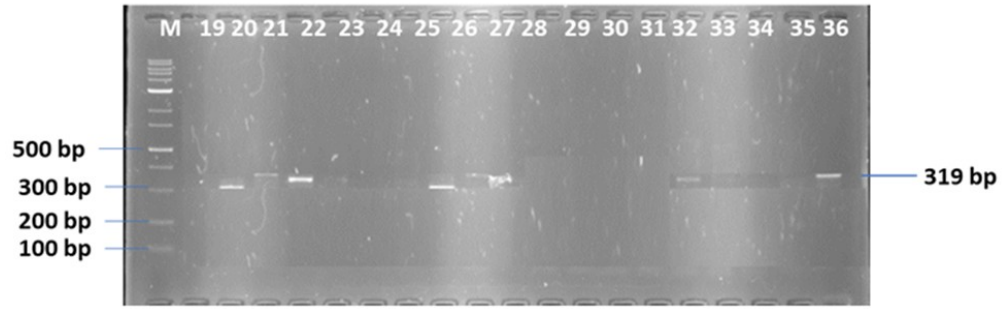


Figure 3: A representative gel electrophoresis image of PCR products from *S. mutans* with *gtfC* gene. Lane M: Molecular weight marker; Lanes 1-74, 75-96: Plaque samples.

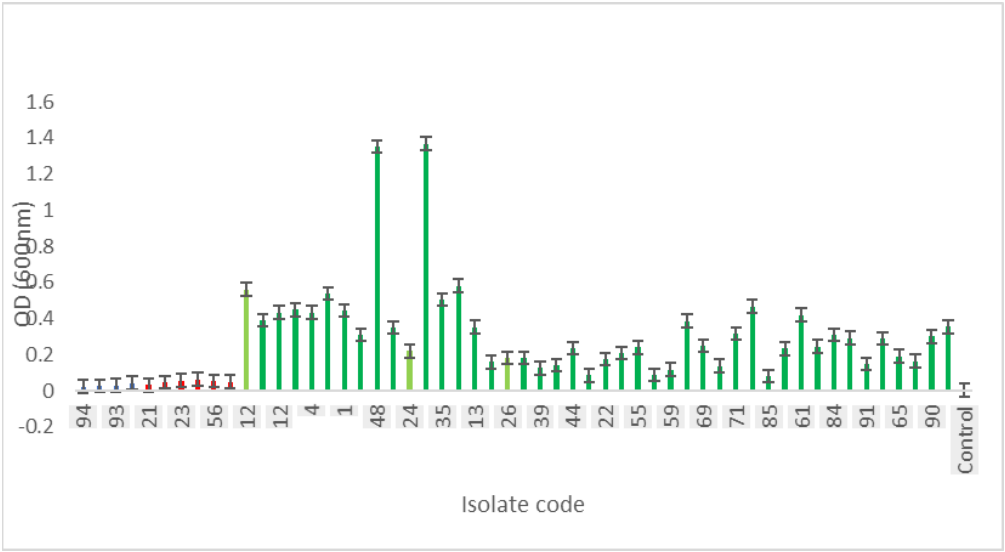


Figure 4: Biofilm production categories by the isolates

OD: Optical Density

Weak biofilm producers: 94, 79 and 93

Moderate biofilm producers: 3, 21, 53, 23, 57, 56, 74,

Strong biofilm producers:12, 10, 2, 4, 11, 1, 30, 48, 38, 24, 35, 29, 13, 34, 26, 37, 39, 40, 44, 47, 22, 50, 55, 60, 59, 73, 69, 92, 71, 62, 85, 66, 61, 72, 84, 75, 91, 80, 65, 89, 90, 95.

Flavonoid Constituents of Z. zanthoxyloides extract

Phytochemical screening of the extract revealed the presence of flavonoids. The GC-MS spectrum identified 16 flavonoid subclasses of phenolic compounds, including limonene luteolin and apigenin among others. The structures of these phytochemicals are shown in Figure 7 and Table 1, which provide detailed information for each compound.

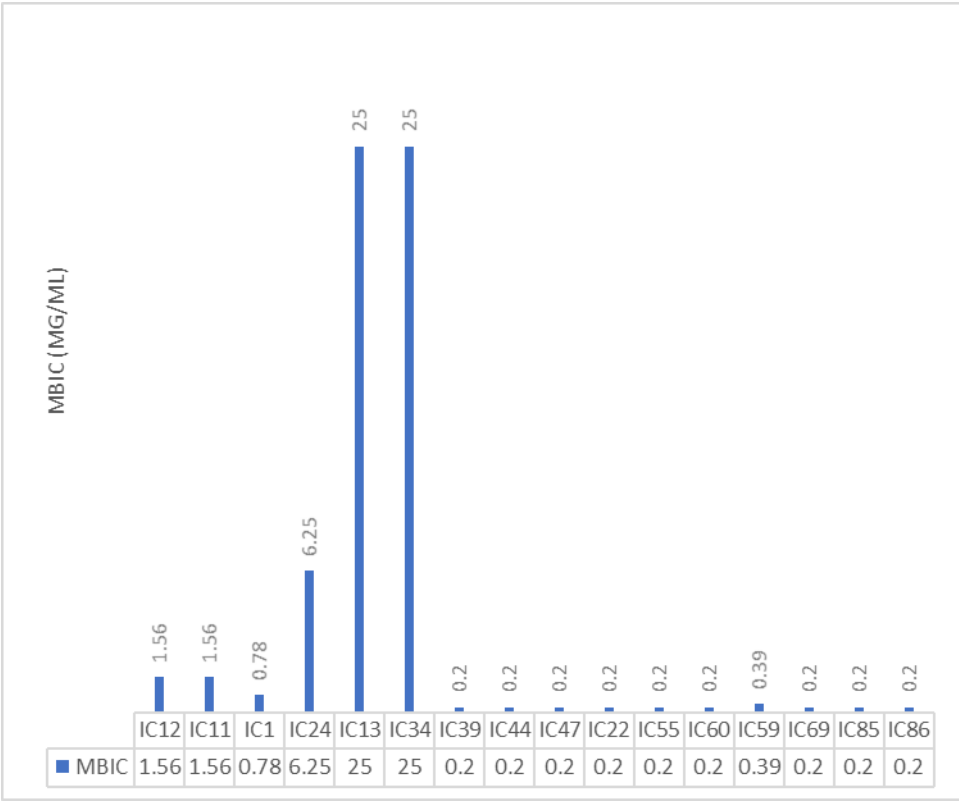


Figure 5: MBIC of the bark extract of *Z. zanthoxyloides* on *S. mutans* isolates
Legend
IC: Isolate code
MBIC: Minimum Biofilm Inhibitory Concentration

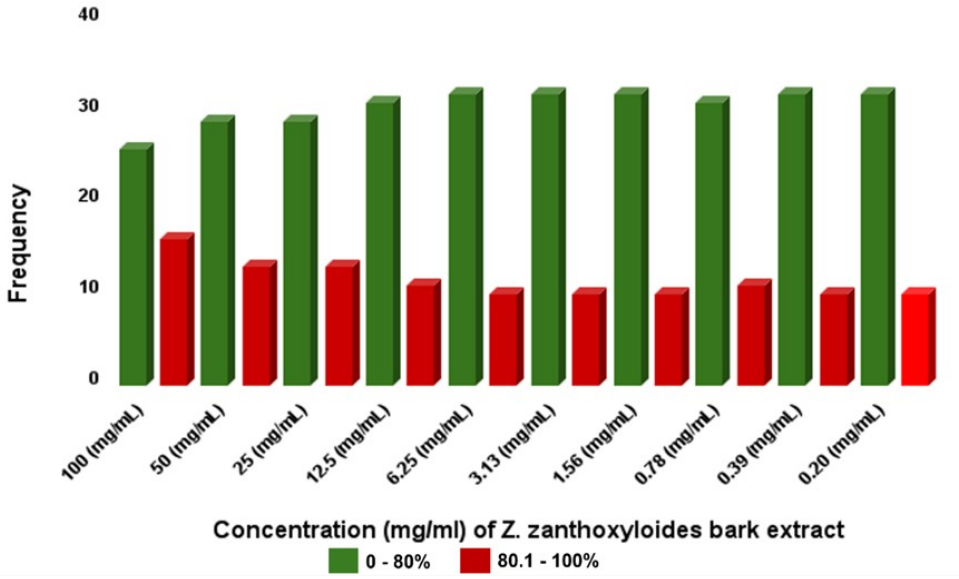


Figure 6: Comparison of minimum biofilm-inhibition concentration at ≥80% with other percentage inhibition ranges

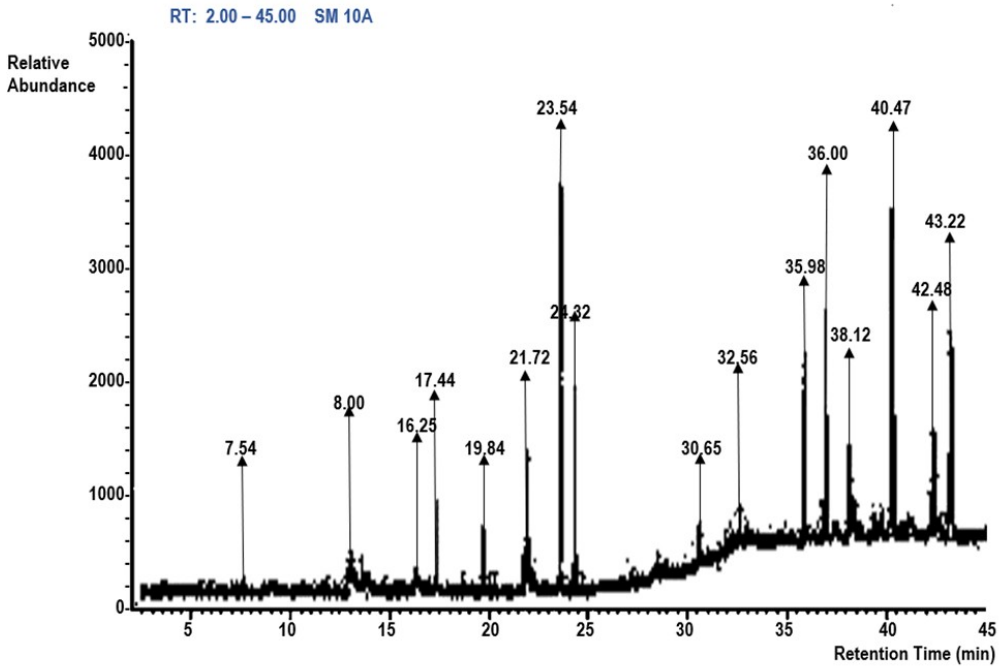
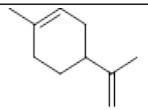
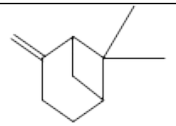
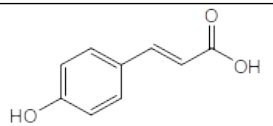
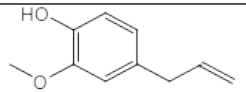
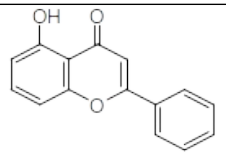
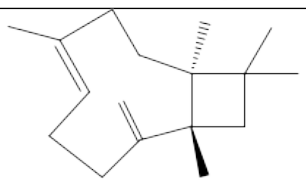
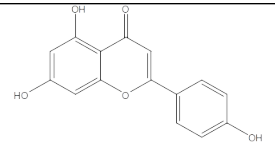
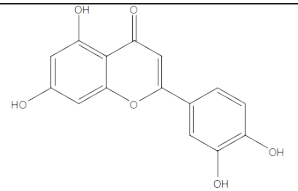
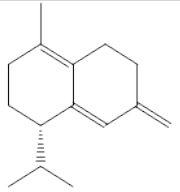
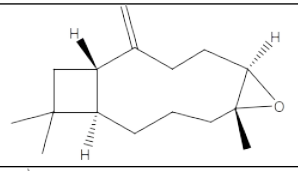
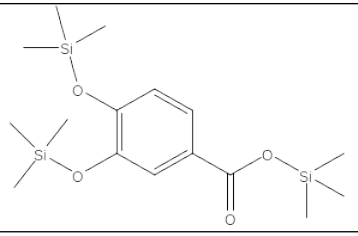
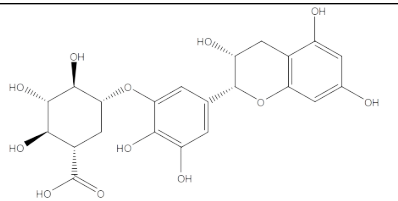
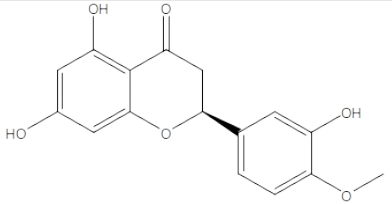
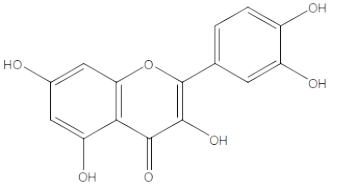
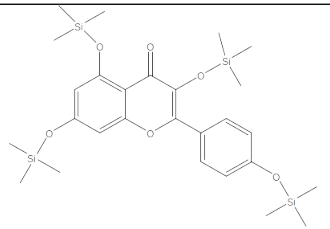
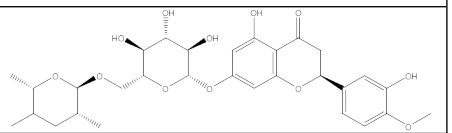


Figure 7: GC-MS spectrum of flavonoid rich bark extract of *Z. zanthhoxyloides*

Table 1: GC-MS Analysis and structures of compounds in Flavonoid-rich of *Z.zanthoxyloides* crude bark extract

Peak #	RT	Compound Detected	Mol. Formula	MW	Peak Area %	Comp %wt	m/z	Structures
1	7.54	Limonene	$C_{10}H_{16}$	136	0.80	1.85	43, 65, 136	
2	8.00	β-Pinene	$C_{10}H_{16}$	136	1.60	2.75	41, 93, 136	
3	16.25	p-Coumaric acid	$C_9H_8O_3$	164	1.20	2.84	91, 119, 164	
4	17.44	Eugenol	$C_{10}H_{12}O_2$	164	3.21	4.15	55, 77, 164	
5	19.84	5-Hydroxyflavone	$C_{15}O_3$	228	2.38	3.21	41, 57, 228	
6	21.72	Caryophyllene	$C_{15}H_{24}$	204	6.61	7.69	41, 93, 204	

7	23.54	Apigenin	$C_{15}H_{10}O_5$	270	14.22	17.20	41, 117, 270	
8	24.32	Luteolin	$C_{15}H_{10}O_6$	286	8.01	6.18	41, 153, 286	
9	30.65	Germacrene D	$C_{15}H_{24}$	204	3.28	4.13	41, 105, 204	
10	32.56	Caryophyllene oxide	$C_{15}H_{24}O$	220	3.92	4.75	41, 97, 128	
11	35.98	Protocatechoic acid, 3TMS derivative	$C_{16}H_{30}O_4Si_3$	370	8.77	5.83	73, 193, 370	

12	36.00	(-)-Epigallocatechin 3-glucuronide	$C_{21}H_{22}O_{13}$	482	10.40	6.29	41, 65, 482	
13	38.12	Hesperetin	$C_{16}H_{14}O_6$	302	5.68	6.27	51, 95, 302	
14	40.47	Quercetin	$C_{15}H_{10}O_7$	302	13.92	7.91	51, 77, 302	
15	42.48	Kaempferol, TMS	$C_{27}H_{44}O_6Si_4$	576	6.39	7.80	43, 157, 576	
16	43.22	Hesperidin	$C_{28}H_{34}O_{15}$	610	9.61	11.14	57, 137, 610	

Discussion

The inherent affinity to form biofilms by *S. mutans* has been pivotal in its ability to attach to tooth enamel. Among the 254 collected samples, 96 were identified as *S. mutans*, with 59 (61%) exhibiting biofilm formation capabilities. This suggests that certain oral microorganisms may produce peroxidase enzymes that counteract sucrose-dependent biofilm formation, thereby reducing the virulence of *S. mutans* (Lemos et al. 2019). Additionally, *Streptococcus sanguinis* may compete with *S. mutans* for colonization sites on tooth enamel, and its biochemical antagonism could explain the observed reduction in *S. mutans* populations during biofilm formation. This is in accordance with findings of Rita et al. (2012) who suggested that the ratio of *S. mutans* to *S. sanguinis* could serve as an indicator of caries risk, given that *S. sanguinis* has lower cariogenic potential than *S. mutans*. Furthermore, *S. salivarius* exhibits a much lower capacity to form biofilms compared to *S. mutans* (Bidossi et al., 2018).

S. mutans produces glucosyltransferase (*gtf*), an enzyme that converts sucrose to fructose and glucose, which are then incorporated into the growing glucan polymer, forming an exopolysaccharide matrix. This matrix enhances the adhesion of *S. mutans* to the salivary pellicle and other bacteria, thereby increasing the biofilm's resistance to chemotherapeutic agents and host immune responses (Banas 2004). Additionally, glucan binding proteins (glucosyltransferases) act as a specific receptor for glucan, facilitating microbial adhesion and biofilm formation (Krzyściak et al. 2014).

Therefore, the detection of these proteins is significant, as they contribute to the virulence of *S. mutans*. Variations in the presence of biofilm-related genes, specifically *gtfB* and *gtfC*, in 54 isolates (56%) and 48 (50%), respectively, suggest that not all *S. mutans* strains possess the protein antigens capable of causing harm to the host (Caufield et al. 2000; Renata et al. 2006).

Biofilm production by *S. mutans* isolates was further assessed using the microtiter plate method, providing a precise measurement of biofilm formation. Results indicated that decayed teeth samples produced significantly stronger biofilm than plaque sample. Among the decayed teeth, 44 samples were strong biofilm-producers, while others exhibited moderate to weak biofilm formation. *Z. zanthoxyloides* is known to contain bioactive compounds such as alkaloids, flavonoids, saponins, and phenolic compounds, which have shown efficacy against a wide range of microbial pathogens (Ekunwe et al. 2022).

GC-MS analysis revealed several active compounds, including apigenin, eugenol, quercetin, p-Coumaric acid and luteolin. Among these apigenin (14.22%), Quercetin (13.92%) and epigallocatechin 3-glucuronide (10.40%) exhibited the highest peak concentrations. Each of these compounds has documented antimicrobial, antioxidant and antibiofilm properties (Zayed et al. 2021). This is further evidenced by the reduction in biofilm quantity observed at various concentrations. The minimum biofilm inhibitory concentration (MBIC) varied for each concentration, further confirming the efficacy of *Z. zanthoxyloides* as a potential alternative or complementary treatment for oral microbial infection. These findings support the traditional use of *Z. zanthoxyloides* in ethnomedicine for wound healing and the treatment of dental problems, as also noted by Phuong et al. (2018).

Conclusion

Results from this study confirmed that *Z. zanthoxyloides* contains active phytochemical constituents responsible for the anti-biofilm activity against *S. mutans*. Biofilm formation by *S. mutans* makes it virulent and helps in resisting treatments, side effects of synthetic anti-biofilm agents are worrisome. Therefore, using (natural) plant- based material can serve as an antibiofilm or complimentary therapy. The presence of flavonoids in the plant's extracts, known for their antimicrobial and anti-biofilm properties, further supports its therapeutic application in oral health care.

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