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# Antibacterial and Antibiofilm Activity of Methanolic Extract of *Hopea ferrea* and It's Fractions

**Mohd Fithriee Zailani[1,2], Isna Athirah Othman[1,2], Fatin Nadiah Thaqifah Shahriman[1,2], Fatimah Salim[1,2], Juliana Jusoff[1] and Nurul 'Izzah Mohd Sarmin[1,3]***

[1]Atta-ur-Rahman Institute for Natural Product Discovery, Universiti Teknologi MARA, Puncak Alam Campus, 42300 Bandar Puncak Alam, Selangor, Malaysia

[2]Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia

[3]Centre of Preclinical Science Studies, Faculty of Dentistry, Universiti Teknologi MARA (UiTM) Sungai Buloh Campus, Jalan Hospital 47000 Sungai Buloh, Selangor, Malaysia

*Corresponding Author (e-mail: izzahsarmin@uitm.edu.my)

Biofilms in the oral cavity are a significant contributor to dental plaque, leading to conditions such as dental caries, gingivitis, and periodontitis. These microbial communities exhibit increased resistance to conventional antimicrobial agents, underscoring the need for novel antibiofilm compounds. *Hopea ferrea*, a Dipterocarpaceae tree species traditionally used in Southeast Asia for its medicinal properties, presents a potential source of such compounds. This study aims to isolate and identify bioactive compounds from *H. ferrea* and evaluate their antibacterial and antibiofilm activities against *Streptococcus mutans* (NCTC 10449), a cariogenic bacterium. Sample of plant *H. ferrea* was collected from Pulau Tuba, Langkawi, Kedah. The methanol extracts were screened using the agar diffusion method, and bioactive compounds were isolated from the bark of *H. ferrea* using Medium Pressure Liquid Chromatography (MPLC) and High-Performance Liquid Chromatography (HPLC). Methanol extracts of *H. ferrea* inhibited all tested strains, some extracts showed variable antibacterial activities. The minimum inhibitory concentration (MIC) of the potent extracts ranged from 1.56 mg/mL to 50.00 mg/mL while the minimum bactericidal concentration (MBC) was between 6.25 mg/mL to 25.00 mg/mL. This study contributes to the understanding of *H. ferrea*'s bioactive constituents and supports the development of natural source-derived antibiofilm therapies.

**Keywords:** *Hopea ferrea*; antibiofilm; bioactive compounds; antibacterial; MPLC; HPLC; NMR; IR; UV

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Dental caries, or tooth decay is a pervasive oral health problem significantly influenced by factors such as socioeconomic status, dietary habits, fluoride exposure, and systemic conditions like obesity and metabolic syndrome [3]. The prevalence of dental caries varies widely among different populations, often showing higher rates in specific demographic groups, with females and individuals with primary dentition being particularly affected [3, 4]. Research highlights a complex relationship between dental caries and systemic conditions; for instance, obesity in children and adolescents has been found to have a negative association with dental caries [5]. Additionally, low birth weight and preterm birth are significant risk factors for dental caries in primary teeth. Asthma medication further exacerbates this issue among children, necessitating a holistic approach to pediatric oral health [6]. The untreated dental caries detrimentally affects oral health-related quality of life, illustrating the crucial need for addressing this condition to enhance overall well-being [17, 18].

The root cause of dental caries is oral biofilm dysbiosis. Physiological dental biofilm is not cariogenic; however, when environmental conditions change, such as exposure to sugars in food, dental plaque biofilm will change and become pathological in composition and structure [2, 7]. *Streptococcus mutans* is recognized as the primary etiological agent of dental caries, contributing to the initiation and progression of biofilm formation on tooth surfaces [8, 9, 10]. The metabolic activities of *S. mutans* within biofilms, such as the production of organic acids from dietary carbohydrates, lead to the erosion of tooth enamel and the development of caries [14, 11, 12]. Furthermore, the interaction between different microorganisms within biofilms, such as *S. mutans* and *C. albicans*, can enhance the pathogenicity of plaque biofilms, further promoting the development of dental caries [10]. The acidic environment created by dental plaque biofilms is detrimental to tooth enamel, facilitating caries progression [15, 16]. The shift in microbial composition within biofilms towards aciduric and acidogenic bacteria underscores the significance of cariogenic biofilms in disease development [9], highlighting the importance of understanding and targeting biofilm formation in dental caries prevention and treatment.

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*Hopea ferrea* belongs to Dipterocarpaceae family which known to produce a range of bioactive compounds including stilbene and oligostilbenes. Members of this family such as Shorea and Dipterocarpaceae have shown significant antimicrobial, antioxidant and anti-inflammatory properties [20]. Stilbenoid especially resveratrol oligomers have demonstrated strong antibiofilm and antimicrobial activities, making it a promising natural agent for targeting biofilm related infection [21]. The objective of this study is to evaluate the antibacterial and antibiofilm activities of the methanolic extract of *H. ferrea* and its fractions against oral pathogenic bacteria, and to identify the specific fractions with potential therapeutic applications for combating biofilm-associated infections.

## EXPERIMENTAL

### Chemicals and Materials

Brain Heart Infusion broth and agar (BHI). *Streptococcus mutans* (NCTC 10449), chlorhexidine, petri dishes, 1 µL sterile loops, 96-well plates, blank disc, pipette, methanol, acetic acid, crystal violet, phospahate buffer saline tablet, acetonitrile. All chemicals used in the study were procured from Sigma Chemical Co. (St. Louis, Missouri) and it consists of analytical and HPLC grade. Glycerol stocks of *S. mutans* (NCTC 10449) were stored at -80°C for long-term preservation. The microbial strain was obtained from the Atta-ur-Rahman Institute for Natural Product Discovery (AuRIns).

### Preparation of Plant Extract

Stem bark from *H. ferrea* was collected in Pulau Langkawi, Malaysia, stembark was deposited in the Laboratory metabolite 2 of Atta-ur-Rahman Institute for Natural Product Discovery (AuRIns), Universiti Teknologi MARA Puncak Alam.

Bark of *H. ferrea* was dried a few days and pulverized into fine powder. Their methanol extract was prepared using the cold-extraction technique. In a 500 mL conical flask, 350 mL of methanol were added into 70.0 g of dried *H. ferrea* powder. The flasks were agitated at 150 rpm for 1 hour. Then, the methanol was extracted out and another 350 mL of fresh methanol was added. The flasks were treated as previous steps and repeated twice to pool a 900 mL of methanol extract. The extracted solvent was concentrated using a rotary evaporator with 1.45 Psi and 16°C and the amount of extract was noted. The crude extract then proceeded to MPLC, and 7 fractions were obtained and tested using bioassay guided methods.

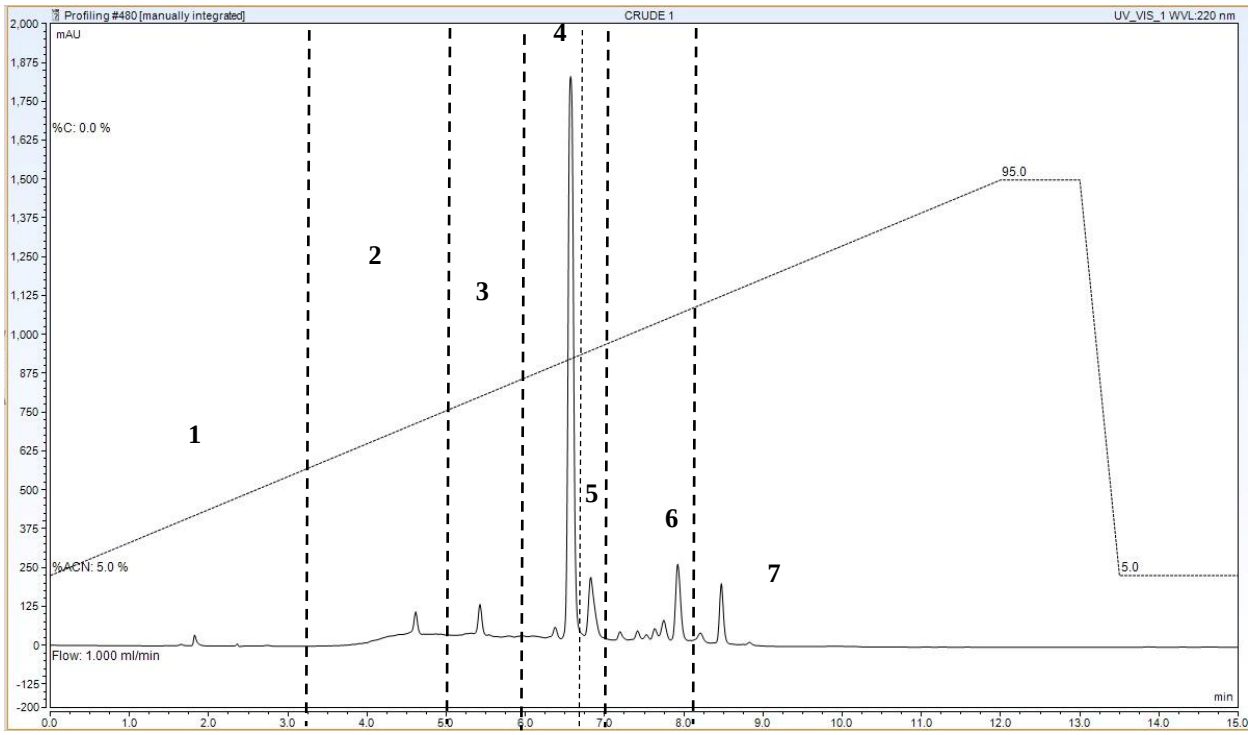
### Fractionation of *H. ferrea* Extract

Fractionation was done using Medium Pressure Liquid Chromatography (MPLC), where collectors can be configured to collect data based on three different parameters: peak gradient, peak threshold, or time. After a predetermined time, the system automatically switches tubes to collect time fractions. By doing this, all the compounds will be collected but in different fractions. If a certain fraction is active againts biofilm, study will focus more on the fraction, and it is easier to isolate the active compound.

Although the fractions collected may not be entirely pure, this technique helps narrow the focus on specific areas of the active compound mixture. Finer fractionation can be achieved with the use of current preparative, semi-preparative, or ultraviolet (UV) high-performance liquid chromatography (HPLC), which is frequently led by an online detection technique. Methanol Analytical grade (AR) was used to dissolve the sample after it was weighed for 4 g. After the sample has dissolved, it was combined with an L-sized "Inject-Column" and dried using a rotary evaporator. Along with the Hi-Flash 4 L column, the dried sample that has been combined with silica was poured back into the silica column at the MPLC instrument to be used and fixed. The disc-diffusion assay was studied further using the collected fractions.

The MPLC setup used a gradient elution system with two solvents, A (water) and B (acetonitrile), to separate compounds based on polarity. The gradient program, with stages labeled G1 to M3, gradually increases the acetonitrile concentration, aiding compound elution. The flow rate is set to 30 mL/min, and fraction collection is configured in a time-based mode, collecting approximately 27 mL per fraction across 89 tubes. The optical density detection range is set to 0.12, ensuring that only fractions with significant peaks are collected, corresponding to compounds in the sample. The chromatogram displays several peaks between 0 and 40 minutes, indicating the presence of different compounds separated by the gradient. This setup efficiently isolates bioactive compounds by exploiting differences in retention times and polarity.

The HPLC analysis utilizes a gradient elution method with acetonitrile as the primary solvent, gradually increasing its concentration to separate various compounds effectively. The UV detection wavelength is set at 220 nm, which is suitable for detecting aromatic compounds, such as those found in plant extracts. The flow rate is maintained at 1.0 mL/min, providing adequate resolution without excessively lengthening run times. The chromatogram in figure 1 shows multiple peaks with retention times ranging from early to later points in the run, reflecting the separation of compounds with different polarities. The HPLC system is optimized for profiling crude extracts, providing clear, distinct peaks that indicate the presence of individual bioactive components. This setup is suitable for further fractionation and isolation, as well as for preliminary identification of compounds within complex mixtures.




Note: Number 1-7 indicate fraction 1-7.

**Figure 1.** HPLC chromatogram of *H. ferrea*'s crude extract.

**Determination of Antibacterial Activity**

According to guidelines from the Clinical and Laboratory Standards Institute (CLSI), the extract fractions were tested in triplicate against the pathogens using the Kirby-Bauer disc diffusion method. Each disc contained a positive control 0.2% of chlorhexidine and a negative control of 5% DMSO (dimethyl sulfoxide).

To reach the 0.5 McFarland standard ($10^8$ cfu/mL), bacterium was cultivated overnight in BHI broth using a spectrophotometer to a standardised final Optical density (OD) of roughly 0.08-0.10. The bacterial suspension was next swabbed across the whole surface of the BHI agar. The 30 µL of unprocessed extracts were transferred to discs. After incubation at 37 °C for 24 hours, the mean diameter of the ZOI (zone of inhibition) will then be measured in triplicates.

**Biofilm Formation and Assessment**

Biofilm models were established in 96-well plates to simulate oral conditions. Broth cultures of *S. mutans* were inoculated into wells and allowed to form biofilms. Treatments were applied on day 1 by replacing a portion of the culture medium. Biofilm biomass was quantified using crystal violet (CV) staining after fixation, followed color development with acetic acid. Absorbance was measured at 620 nm, and data were analyzed to assess biofilm biomass and treatment efficacy (Figure 2).

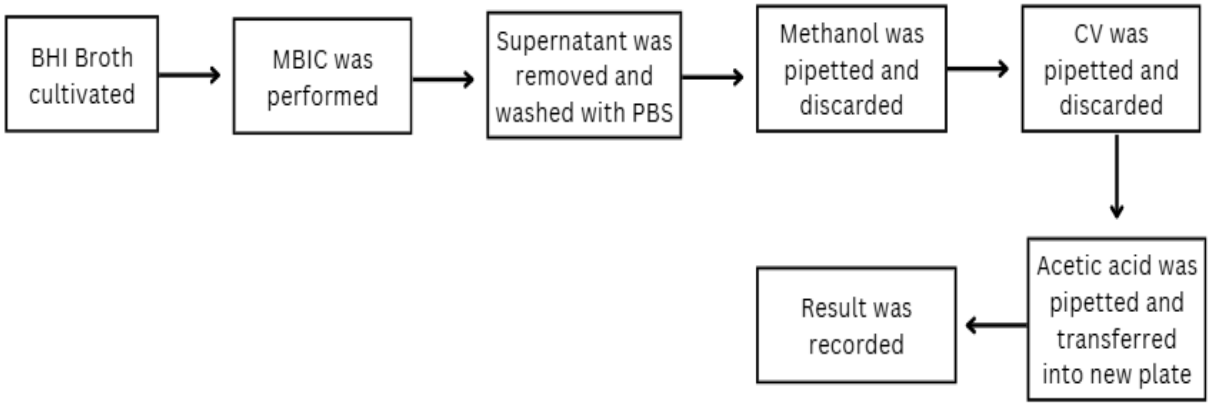



**Figure 2.** Flowchart of crystal violet (CV) staining.

A 5 mL sample of BHI broth was prepared in a 10 mL tube and inoculated with *S. mutans* bacteria. The inoculated broth was incubated for 3–4 days in a 5 % $CO_2$ environment. Following incubation, the bacterial suspension was standardized to an optical density (OD) of 0.5 McFarland at 620 nm using a spectrophotometer. The suspension was then mixed thoroughly using a vortex.

For the Minimum Biofilm Inhibitory Concentration (MBIC) assay, 2 % chlorhexidine was used as the positive control, and 1 % methanol served as the negative control. Each fraction was tested at a concentration of 10 mg/mL in 1 % methanol. The MBIC assay was performed in a 96-well plate, which was incubated for 24 hours at 37°C.

After incubation, the supernatant was carefully removed from each well, and each well was rinsed once with 200 µL of phosphate-buffered saline (PBS) to remove any non-adherent cells. To fix the biofilm to the well surface, 200 µL of methanol was added to each well and left for 15 minutes before being removed. The plate was then air-dried for 45 minutes.

Next, 1% CV was added to each well to stain the biofilm, left for 20 minutes, and then discarded. The plate was washed by immersing it in a container of tap water, then allowed to air-dry for a few minutes. Finally, acetic acid was added to each well to release the stain, and 100 µL from each well was transferred to a new 96-well plate. The optical density (OD) of each well in the new plate was measured at 620 nm using a microplate reader.

## RESULTS AND DISCUSSION

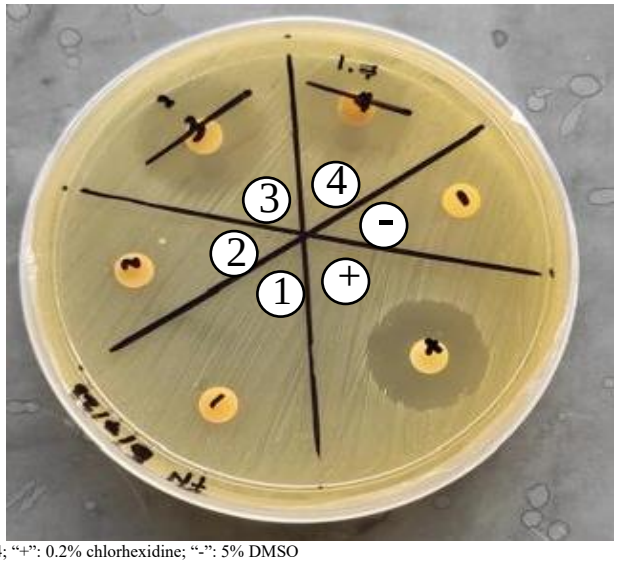
### Determination of MIC and MBC

The inhibitory effect of plant extract *H. ferrea* on the cariogenic bacterium *S. mutans* was investigated in vitro. The results revealed the antibacterial potential methanol extract of *H. ferrea*. The disc diffusion assay demonstrated varying degrees of antimicrobial activity from the fractions isolated from *H. ferrea*. Clear zones of inhibition were observed around certain discs, indicating that these fractions possess antibacterial properties. The size of the inhibition zones suggests the potential potency of the fractions against the tested bacterial strain [13].

Based on Figure 3, fraction 3 exhibited the largest inhibition zones at 23.67 ± 3.51 mm, followed by fraction 4 at 21.67 ± 4.04 mm. This could be attributed to a higher concentration of active phytochemicals or a more effective mechanism of action, which may disrupt bacterial cell walls or biofilm formation more efficiently [22].

In contrast, some fractions displayed either no zone of inhibition or very small zones, indicating weak or no antibacterial activity. This could be due to lower concentrations of the active compounds, the presence of resistant bacterial strains, or the inability of the compounds to diffuse effectively through the agar medium [23]. The active components in the plant extract usually interfere with the growth and metabolism of microorganisms in a detrimental way [23]. The activity is quantified by determining the MIC and MBC, which are used in the preliminary screening for antibiofilm activity.

The variability in inhibition zones across the samples highlights the importance of isolating and optimizing the most potent fractions of plant extracts for therapeutic use. These findings align with previous studies that demonstrate the selective antibacterial activity of plant-derived compounds. Further investigation into the chemical composition and the mechanism of action is needed to fully understand the potential of these bioactive agents in combating bacterial infections.




Note: Number 1 - 4 indicate Fraction 1 – 4; "+": 0.2% chlorhexidine; "-": 5% DMSO

**Figure 3.** Zone of inhibition (mm) for Fraction 1 – 4 against *S. mutans*.

**Table 1.** Average inhibition zone (mm) of Fractions against *S. mutans.*

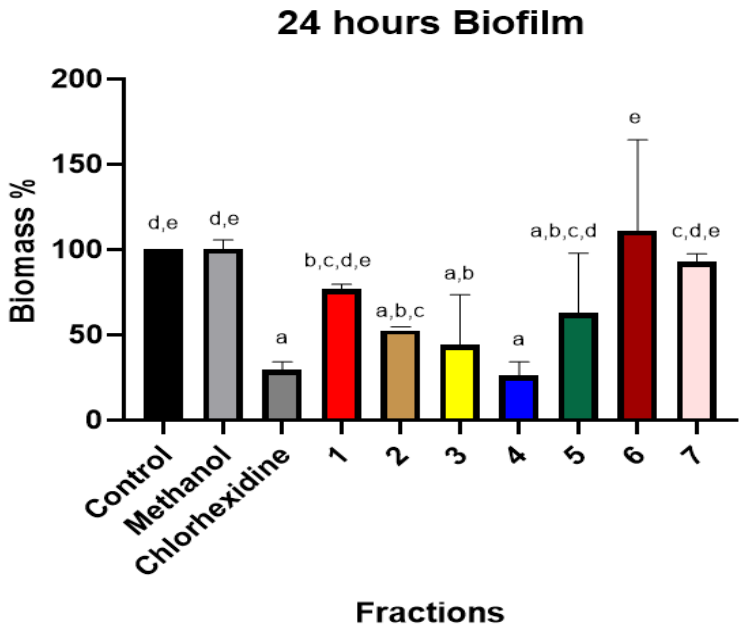
| Fractions | Inhibition zone (mm) |
|---|---|
| 1 | - |
| 2 | - |
| 3 | 23.67 ± 3.51 |
| 4 | 21.67 ± 4.04 |
| 5 | 9.06 ± 0.29 |
| 6 | 8.63 ± 0.21 |
| 7 | 6.89 ± 0.05 |
| 0.2% chlorhexidine | 20.00 ± 2.000 |
| Negative control (5% DMSO) | - |

Note: The results were showed as means ± standard deviation. Assay was done in triplicate.

**Biofilm Inhibition Activities**

Antimicrobial inhibition assays demonstrated a ZOI at concentrations of 10 mg/mL after 24 hours shown in figure 3. However, its effectiveness against mature biofilms was reduced, suggesting that the fraction is more effective at preventing the initial formation of biofilms rather than disrupting established ones. Antimicrobial assays underscored the potent activity of the *H. ferrea* extract against *S. mutans*, a primary causative agent of dental caries. MIC assay provided reliable indicators of bacterial viability, while the MBC assay reinforced the extract's bactericidal efficacy. Biofilm inhibition studies indicated a significant reduction in early-stage *S. mutans* biofilms, highlighting the extract's potential for preventive applications in dental care.

The biofilm biomass in control was set to 100%, serving as the baseline for comparison. The methanol shows a biofilm biomass slightly above the control, indicating minimal antibiofilm activity. This result is consistent with methanol being primarily a solvent rather than an active antibiofilm agent. As expected, chlorhexidine shows a significant reduction in biofilm biomass compared to the control, reaffirming its efficacy as a standard antibiofilm agent. The biofilm biomass is substantially lower than the control, indicating strong inhibition of biofilm formation.




Note: Number 1 - 7 indicate Fraction 1 – 7; "+": 2% chlorhexidine; "-": 1% methanol. The results were showed as means ± standard deviation. The different letters indicate significant differences at $p < 0.05$. Assay was done in triplicate.

**Figure 4. Biofilm** mass (%) after treatment with Fraction 1 – 7.

Figure 4 shows that fraction 4 demonstrated a significant reduction in biofilm formation, with biomass around 30% of the control, further suggesting it has strong antibiofilm effects with biomass reduced to about 20-30% of the control and making it a promising candidate for further investigation. It is believed that the bioactive compound of *H. ferrea* is at fraction 4, given the significant reduction of biofilm formation observed after 24 hours. Fractions 2 and 3 show moderate antibiofilm activities, reducing biofilm formation to around 40-70% of the control. These fractions have potential but are less effective than fraction 4. Fractions 6 and 7 either fail to inhibit biofilm formation or, in the case of Fraction 6, may even promote it.

The data supports the hypothesis that certain fractions derived from *H. ferrea* extracts possess significant antibiofilm properties against *S. mutans*. Fractions 4 stand out as particularly promising for further purification and characterization. This information will be vital in supporting research goals and advancing the understanding of natural antibiofilm compounds. The use of bark in this study is supported by previous finding that indicating the stem bark of medicinal plants often exhibit higher antimicrobial activity than leaves [19].

Additionally, a study conducted on various *Hopea* species, including *Hopea odorata*, explored the phytochemical composition and antimicrobial activity of the plant extracts. The results indicated that methanol extracts contained a variety of bioactive compounds such as polyphenols, flavonoids, and tannins, which contributed to their antimicrobial properties [1]. The study showed a significant activity against both Gram-positive and Gram-negative bacteria, suggesting that the genus *Hopea* holds potential for developing new antimicrobial agents. Although *H. ferrea* was not directly studied, the similarity in bioactive compound profiles across species suggests potential relevance.

## CONCLUSION

In this study, the bioactive compounds from *H. ferrea* demonstrated significant antibiofilm potential against *S. mutans* (NCTC 10449). The comprehensive analysis involved methanol extraction, MPLC and crystal violet assay to evaluate the efficacy of these fractions. The results showed some of these fractions exhibit not only antimicrobial properties but also potent antibiofilm activity, effectively inhibiting the formation of biofilms and disrupting pre-existing biofilms. This highlights their potential as alternative therapeutic agents to combat biofilm-associated infections, which are notoriously resistant to conventional antibiotics. The active fraction will proceed to isolation.

Further structural elucidation of the active compounds, combined with in-depth mechanistic studies, will be crucial to understand the precise molecular pathways through which these compounds exert their antibiofilm effects. Additionally, these findings open the door for future work exploring the synergistic effects of these fractions in combination with existing antibiotics, which could enhance treatment efficacy against resistant strains.

In conclusion, the active fractions derived from *H. ferrea* extracts show promising potential as sources of antibiofilm agents. Although pure bioactive compounds have yet to be fully isolated from these fractions, their efficacy against biofilms highlights their value for further research. The natural origin of these fractions supports the need for sustainable and alternative strategies in combating antimicrobial resistance. Future studies should focus on optimizing the fractionation and purification processes to isolate and identify the pure active compounds responsible for the antibiofilm activity, paving the way for new approaches in managing biofilm-associated infections. Additionally, quantifying and characterizing these bioactive components will provide a deeper understanding of their therapeutic potential and application.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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