

Article

# Inhibitory Effect of Plantago Major Extract on Biofilm Formation by Enterococcus faecalis from Teeth Infections

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**Citation:** Modher, M. N., Yaseen, S. M., Alshanon, A. F., Hussein, S. M., Raheem, Y. S. Inhibitory Effect of Plantago Major Extract on Biofilm Formation by Enterococcus faecalis from Teeth Infections. American Journal of Biology and Natural Sciences 2025, 2(7), 122-131.

Received: 14<sup>th</sup> Jun 2025  
 Revised: 21<sup>st</sup> Jun 2025  
 Accepted: 07<sup>th</sup> Jul 2025  
 Published: 15<sup>th</sup> Jul 2025



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**Abstract:** Due to the pathogen's resistance to conventional antibiotics, the potential of *Plantago major*, a medicinal herb with known antibacterial and anti-inflammatory properties, was evaluated as an alternative treatment. The current study aimed to assess the antibacterial efficacy of an extract of *P. major* leaves against *Enterococcus faecalis* isolated from teeth infections. *E. faecalis* isolates from infected root canals were tested for biofilm-forming ability using the 96-well microtiter plate assay. Antibiotic susceptibility was assessed using standard disc diffusion testing. The antibacterial and anti-biofilm activities of *P. major* extract were evaluated through disc diffusion and microdilution assays to determine inhibition zones and minimum inhibitory concentrations (MIC). Among the isolates, 29.5% were strong biofilm producers, 40% moderate, and 30.5% weak. The *P. major* extract exhibited inhibition zones ranging from 9 to 22 mm. MIC values 128 µg/mL. Most isolates were resistant to erythromycin, clindamycin, trimethoprim, and tetracycline, while imipenem remained effective. Biofilm formation was effectively reduced at all tested concentrations, with the greatest inhibitory effect observed at a concentration of 500 mg/mL. *Plantago major* extract demonstrated antibacterial and anti-biofilm activity against multidrug-resistant *E. faecalis*. These findings suggest its potential as a complementary therapeutic agent in managing persistent root canal infections.

**Keywords:** Antibacterial Agents, Medicinal Plants, Antibiotic Resistance, *Plantago*, *Enterococcus faecalis*

## Introduction

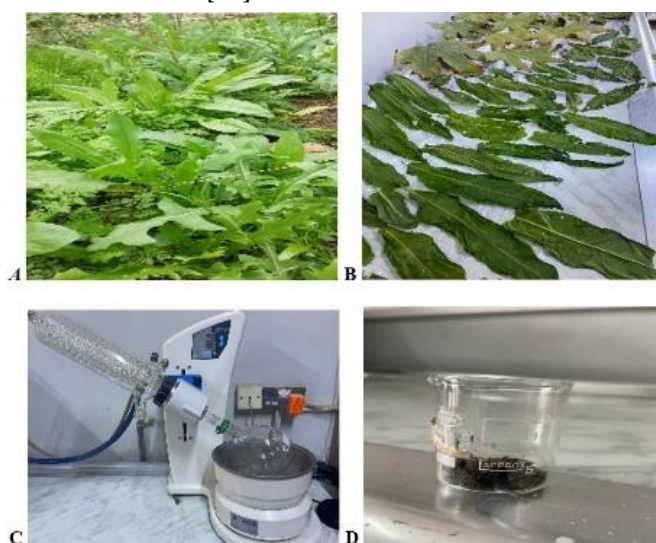
Enterococci are prevalent human commensals that have adapted to thrive in complex, oxygen-deficient environments such as the gastrointestinal tract, oral cavity, and female genital tract [1]. Although once considered harmless, Enterococci have been recognised since the early 20th century as potential human pathogens [2]. Currently, they are the third most common cause of hospital-acquired infections. Drug-resistant strains of *Enterococcus* pose significant challenges in clinical therapy. Among them, *E. faecalis* accounts for up to 90% of human enterococcal infections [3]. *E. faecalis* has been strongly associated with endodontic infections, particularly in cases of treatment failure. While it may be present

in early stages, it is more frequently isolated after unsuccessful endodontic therapy, as demonstrated by culture-based studies. The rapid emergence of antibiotic resistance makes persistent infections difficult to manage. *E. faecalis* is a Gram-positive, facultatively anaerobic, non-spore-forming bacterium. The cells are ovoid, measuring between 0.5 and 1  $\mu\text{m}$  in diameter, and appear singly, in pairs, or short chains. Most strains are non-motile and lack haemolytic activity. Colonies on blood agar are typically round, matte, and well-developed [4]. In certain conditions, the ability of Enterococci to form biofilms offers an ecological advantage. A strong correlation has been observed between biofilm-forming *E. faecalis* strains and cases of infective endocarditis. This may be partially explained by the presence of specific virulence factors, including adhesins and secreted pathogenic molecules that facilitate biofilm formation and disease progression [5]. Effective periodontal therapy requires the elimination of bacterial biofilms. Currently, chlorhexidine is considered the gold standard adjunct in periodontal treatment. However, reports of its adverse effects have driven the search for safer alternatives, particularly among natural agents with fewer side effects [6][7]. *P. major*, commonly known as “greater plantain,” “broadleaf plantain,” or “common plantain,” is a perennial herbaceous plant with unbranched underground stems. Owing to its antibacterial, anti-inflammatory, astringent, and wound-healing properties, it has been widely used in traditional medicine [8]. Native to Europe and Asia, *P. major* is now widespread across North America, Western Asia, much of Europe, North Africa, and Latin America. It grows easily in temperate and cold climates, is uncultivated, and often considered a weed. With a life cycle of 6–7 months, *P. major* reaches 15–30 cm in height and flowers from May to October. Its bioactive compounds include mucin, pectin, flavonoids, tannins, bactericidal agents, plantamajoside, baicalein, ursolic acid, catalpol, salicylic acid, zinc, potassium salts, and oleanolic acid. Despite its documented pharmacological properties, limited research has specifically addressed the antibacterial effect of *P. major* against *E. faecalis*. Therefore, this study aimed to evaluate the antibacterial activity of various concentrations of *P. major* methanol extract against *E. faecalis* [9].

## Materials and Methods

### Protocol for acquiring the plant specimen and its extract

A total of 3.2 kg of *P. major* leaves were collected from Khanaqin, northern Iraq, and transported to Al-Nahrain University illustrated in Figures A1 and B1. After rinsing with purified water, the leaves were oven-dried at 40°C, ground into powder, and sieved for uniformity. For extraction, 100 g of the powder were mixed with 200 mL methanol in a Soxhlet apparatus for 24 hours. The solvent was then evaporated Figure C1, yielding 2–3 mL of concentrated dark green extract Figure D1, which was stored in sterile vials under controlled conditions [10].



**Figure 1.** Extraction methods: A. *P. major*/ B. Using a system of forced air circulation to dry /C. Soft extraction by rotavapor / D. Samples of the extracted material.

### Analysis using Gas Chromatography-Mass Spectrometry (GC-MS)

Gas Chromatography–Mass Spectrometry (GC-MS) was used to identify the main phytochemicals in the hydroalcoholic extract of *P. major*. Analysis was performed on a Perkin-Elmer Clarus 680 system with an Elite-5MS capillary column and helium as the carrier gas (1 mL/min). Ionisation occurred at 70 eV with a scan range of 40–600 m/z. A 1 µL sample was injected at 250°C. The oven programme started at 52°C for 3.5 minutes, increased by 10°C/min to 280°C, and held for 10 minutes. Compounds were identified by retention times and peak areas compared to chromatogram references.

### Obtaining the Strains and Examination

One hundred root canal samples were collected under aseptic conditions using sterile paper points and files, then placed in Eppendorf tubes with 1 mL brain heart infusion (BHI) broth. Samples were stored cold and transported to the microbiology lab for analysis. Upon arrival, they were cultured on Pfizer-specific *Enterococcus* medium and incubated anaerobically at 37°C for 24 hours. Colonies were assessed macroscopically, and one colony per plate underwent biochemical and Gram-stain evaluation to confirm bacterial identity and morphology.

### Assay for antibiotic sensitivity by the disc diffusion method:

The antimicrobial susceptibility of isolates was assessed using the Kirby-Bauer disc diffusion method with 12 antibiotics shown in Figure 3. Mueller-Hinton agar plates (4 mm depth) were inoculated with a 0.5 McFarland-standardised suspension ( $1.5 \times 10^8$  CFU/mL) using sterile swabs. Antibiotic discs were placed aseptically, and plates were incubated anaerobically at 37°C for 24 hours. Inhibition zones were measured in millimetres and interpreted as sensitive, intermediate, or resistant per standard guidelines [11].

### Antibacterial Activity Assessment

#### agar well diffusion method conducted on an agar plate

The antibacterial activity of *P. major* was evaluated using the agar well diffusion method. Bacterial cultures were inoculated on Mueller-Hinton agar, and sterile discs impregnated with *P. major* extract (500, 250, and 125 mg/mL) were applied. Plates were incubated at 37°C for 24 hours, and inhibition zones were measured in millimetres to assess antibacterial efficacy [12].

### Minimal Inhibitory Concentration (MIC) Test

The minimum inhibitory concentration (MIC) of *P. major* extract was determined using resazurin microdilution methods. In the macrodilution assay, serial two-fold dilutions were prepared in Mueller-Hinton broth with added bacterial suspension. For microdilution, 96-well microtitre plates received 100 µL broth and 10 µL of a 1:20 diluted 0.5 McFarland bacterial suspension (final  $\sim 5 \times 10^5$  CFU/mL). Ten wells were used for extract dilutions, with two controls: broth only and broth with inoculum. Due to the extract's colour, resazurin was used to distinguish live from dead cells and improve turbidity assessment.

### Discovering biofilms of *E. faecalis*

The biofilm-forming ability of *E. faecalis* was assessed using a 96-well microtitre plate assay with crystal violet staining. Bacteria were grown in TSB with 1% glucose ( $1.5 \times 10^8$  CFU/mL), and 200 µL was added to each well, followed by 24 h incubation at 37°C. Wells were washed with PBS, dried, and stained with 0.1% crystal violet for 15 minutes. After rinsing and air drying, bound dye was solubilised with 96% ethanol. OD was measured at 630 nm to quantify biofilm biomass. TSB-only wells served as negative controls. Biofilm levels were categorised based on absorbance (Table 1).

**Table 1.** Bacterial adherence classification using the tissue culture plate technique [13], [14].

OD Values	Adherence	Biofilm formation
< OD C	none	none
OD < OD ≤ 2*ODC	Weakly	Weakly
2*ODC < ODT ≤ 2*ODC	Moderately	Moderately

40 DC	Strong	Strong
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C= control, t = test, OD = Optical density at 630 nm

### Inhibitory effect of *P. major* on *E. faecalis* biofilm formation

The previously described biofilm assay was used to assess both the biofilm-forming capacity of 60 isolates of *E. faecalis* and their response to *P. major* extract. Selected strains were treated with extract concentrations of 500, 250, and 125 mg/mL in 96-well microtitre plates and incubated at 37°C for 24 hours. Non-adherent cells were removed, and residual biofilms were stained with 0.1% crystal violet. After rinsing and drying, the dye was solubilised, and OD was measured at 630 nm. Biofilm inhibition was expressed as a percentage reduction compared to untreated controls, following the study's calculation formula [15].

$$\% \text{ of inhibition} = \frac{\text{OD in control} - \text{OD in treatment}}{\text{OD in control}} \times 100.$$

## Results

### Isolation and identification of bacteria:

Out of 100 samples tested, 60 *Enterococcus faecalis* isolates were obtained by culturing on Pfizer-selective agar plates, exhibiting robust growth. The colonies were round, grey, and approximately 2 mm in diameter. Notably, each colony displayed a centrally located black zone, surrounded by additional dark pigmentation extending into the surrounding medium, as shown in Figure 2.



**Figure 2.** *Enterococcus faecalis* grown on Pfizer Selective medium.

### GC-Mass spectrometry

The GC-MS analysis enabled the identification of key bioactive compounds present in the extract of *P. major*. The major detected constituents are summarised in Table 2.

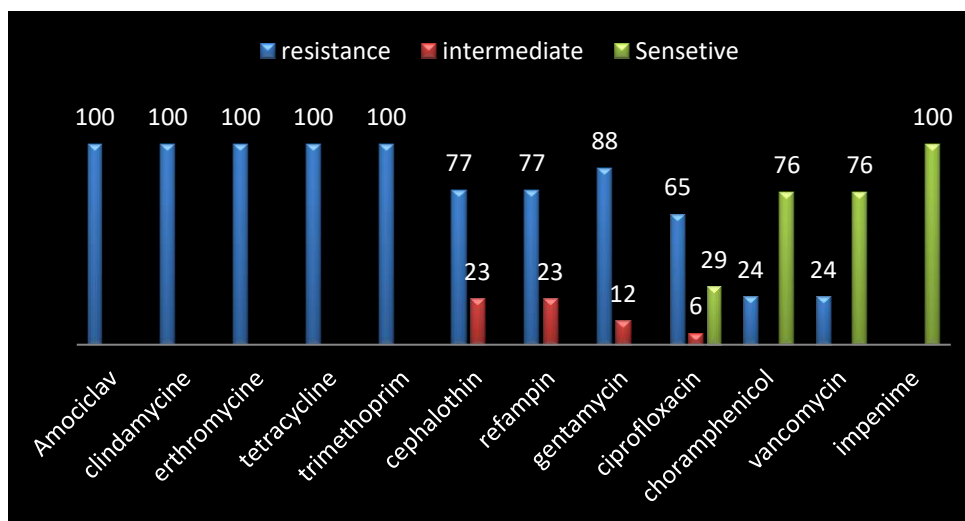
**Table 2.** Chemical constituents detected in the extract of *P. major* plants using GC-MS analysis.

Peak number	Compound	Retention time (min)RT
1	Hydrazinecarboxylic acid	4.203
2	Acetic acid	5.580
3	Carbamic acid	6.004
4	Benzoic acid	6.748
5	Hydrazinecarboxamide	7.155
6	1,2-Hydrazinedicarboxamide	7.899
7	1,4-Butanediol	8.574

8	Benzenemethanamine	9.457
9	Pyrimidinetrione	10.227
10	Dimethyldodecylsilyloxy	10.626

#### Assay for antibiotic sensitivity using the disc diffusion technique:

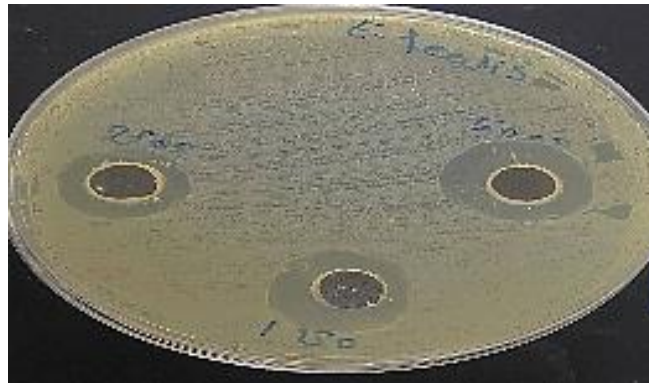
The antimicrobial susceptibility results revealed that all *E. faecalis* isolates were resistant to erythromycin, clindamycin, tetracycline, and trimethoprim. In contrast, complete sensitivity was observed to imipenem across all tested isolates. Sensitivity to the remaining antibiotics varied significantly ( $P < 0.05$ ), with some isolates exhibiting intermediate susceptibility. A high level of resistance was also recorded against gentamicin, rifampicin, and cephalothin. Regarding chloramphenicol, 76% of the isolates were classified as sensitive, 24% as intermediate, and the remainder as resistant. As for ciprofloxacin, 65% of the isolates demonstrated resistance, 29% were sensitive, and a small percentage exhibited intermediate susceptibility. These patterns are graphically represented in Figure 3. Antibiotic susceptibility testing revealed that all *E. faecalis* isolates exhibited complete resistance to the  $\beta$ -lactam antibiotic combination amoxiclav (amoxicillin–clavulanic acid).



**Figure 3.** Antibiotic susceptibility of *E. faecalis* isolates from root canals. Bar chart showing resistance, intermediate, and sensitivity rates across 12 antibiotics. Full resistance was observed for five drugs; imipenem showed 100% sensitivity.

#### Antibacterial activity of *P. major* on *E. faecalis*.

The antibacterial activity of *P. major* against *E. faecalis* was evaluated using the agar well diffusion method. The extract at a concentration of 500 mg/mL exhibited the highest inhibitory effect, producing a clear zone of inhibition measuring 19 mm. Lower concentrations of 250 mg/mL and 125 mg/mL resulted in inhibition zones of 17 mm and 15 mm, respectively. A clear dose-dependent relationship was observed, with the inhibition zone decreasing as the extract concentration decreased. Among all tested concentrations, 500 mg/mL demonstrated the most significant antibacterial effect (Figure 4). These findings are consistent with previous studies [16], which reported that pure *P. major* extracts produced an average inhibition zone diameter of 12.47 mm against a range of tested micro-organisms.



**Figure 4.** Inhibition zones of *P. major* extract against *E. faecalis*. Agar diffusion test shows clear zones around wells with different extract concentrations, indicating antibacterial activity.

### Minimal Inhibitory Concentration (MIC) Test

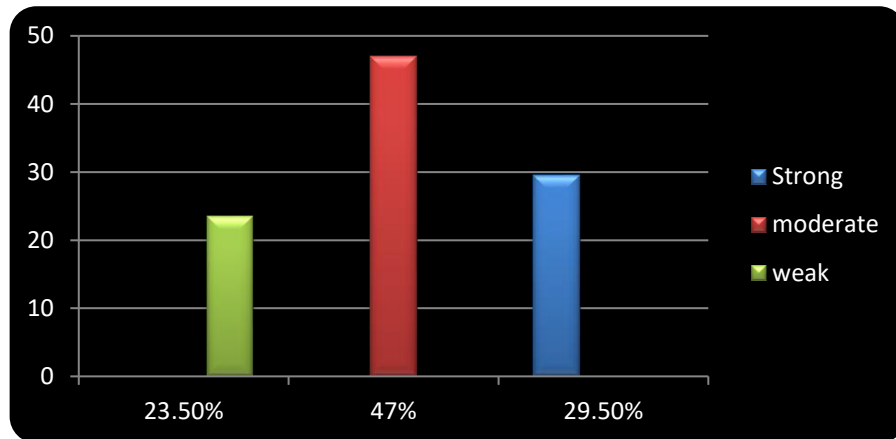
The most resistant *E. faecalis* isolate was subjected to MIC analysis using the 96-well microtitre plate method. Serial dilutions of *P. major* extract were tested at concentrations of 1024, 512, 256, 128, and 64  $\mu\text{g/mL}$ . The results demonstrated that the extract exhibited substantial antibacterial activity, with a minimum inhibitory concentration (MIC) of 128  $\mu\text{g/mL}$ . Additionally, sub-inhibitory concentrations (Sub-MIC) were observed at 64  $\mu\text{g/mL}$ , indicating potential for biofilm inhibition and modulation of bacterial behaviour at lower doses. These findings are visually presented in Figure 5.



**Figure 5.** A microtiter plate showing the MIC-tested concentration produced by adding 0.02% resazurin. Wells-colored blue indicates the absence of growth, while wells-colored pink provides evidence of bacterial growth.

### Biofilm detection of *Enterococcus faecalis*

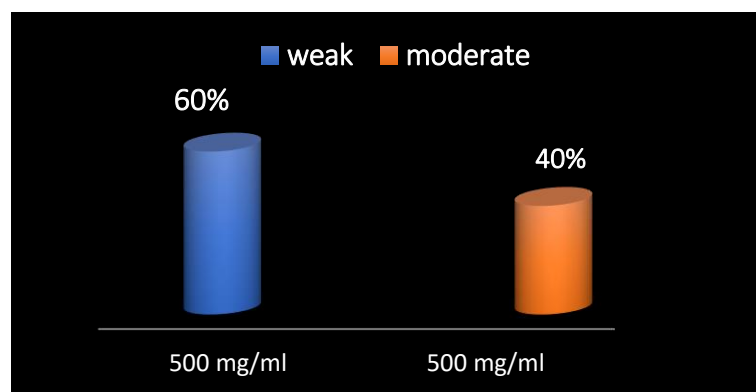
Pre-sterilised 96-well polystyrene microtitre plates were employed to assess the biofilm-forming ability of *E. faecalis* isolates. The extent of biofilm development was quantified by measuring the optical density at 630 nm. The results revealed variability in biofilm formation among the isolates under standardised experimental conditions. Out of 60 isolates obtained from root canal samples, 18 isolates (29.5%) exhibited strong biofilm production, 28 isolates (47%) demonstrated moderate production, and 14 isolates (23.5%) were classified as weak biofilm producers, as illustrated in Figure 6.



**Figure 6.** Biofilm formation levels among *E. faecalis* isolates. Bar graph illustrating distribution: 47% moderate, 29.5% strong, and 23.5% weak producers.

### Plant extract's impact on the production of biofilms

Biofilm inhibition assays were performed using *P. major* extract at concentrations of 500, 250, and 125 mg/mL against the 18 *E. faecalis* isolates (29.5%) previously identified as strong biofilm producers. The results showed that biofilm formation was effectively reduced at all tested concentrations, with the greatest inhibitory effect observed at 500 mg/mL. Following treatment, 11 of the 18 isolates (60%) demonstrated a shift to weak biofilm production, while the remaining 7 isolates (40%) exhibited moderate biofilm formation. These findings indicate that *P. major* extract exerts significant inhibitory activity against *E. faecalis* biofilms, particularly at higher concentrations (Figure 7).



**Figure 7.** Anti-biofilm effect of *P. major* extract. At 5000 mg/mL, the extract reduced biofilm strength to weak (60%) or moderate (40%) levels.

### Discussion

This study underscores the promising antimicrobial and anti-biofilm properties of *Plantago major* methanolic extracts against *Enterococcus faecalis*, a pathogen frequently implicated in endodontic treatment failures and known for its multidrug resistance and robust biofilm formation. The extract demonstrated maximum antibacterial activity at a concentration of 500 mg/mL. This potency is likely due to the synergistic action of phytochemicals such as phenols, flavonoids, terpenoids, and tannins, which interact with the peptidoglycan layer, disrupt membrane integrity, and induce cytoplasmic leakage, ultimately leading to bacterial cell death [17]. These findings are consistent with previous reports on other *Plantago* species including *P. asiatica* [18], *P. ovata* [19], and *P. lanceolata* [20], all of which exhibited comparable antibacterial effects. The complete resistance of all *E. faecalis* isolates to the  $\beta$ -lactam antibiotic combination amoxiclav (amoxicillin–clavulanic acid) may be attributed to the

compound's large molecular structure, which can limit its ability to permeate bacterial cell walls and membranes. This observation highlights the urgent need for alternative therapeutic agents, such as plant-based compounds, to combat antibiotic-resistant pathogens. When comparing the present study's findings with those of [21], some variation in biofilm-forming capability was observed. While their study reported 49% of *E. faecalis* isolates as strong biofilm producers, our data showed a lower proportion (29.5%). Despite these differences, both studies affirm the heterogeneous nature of biofilm production among clinical isolates, emphasising the importance of individualised therapeutic strategies. Moreover, targeting biofilm formation represents a key approach in mitigating chronic infections and resistance development. In this context, *P. major* extract demonstrated effective inhibition of *E. faecalis* biofilm at all tested concentrations, with notable suppression at 500 mg/mL. Complementing this, quercetin—a known flavonoid—showed biofilm inhibition rates of 95%, 85%, and 70% at concentrations of 256, 128, and 64 mg/L, respectively, surpassing previously reported inhibition rates of 31.4% for biofilm formation and 7.5% for established biofilms [22]. These data further reinforce the potential of plant-derived agents in managing biofilm-associated infections. In conclusion, the methanolic extract of *P. major* demonstrates substantial antibacterial and anti-biofilm activities against *E. faecalis*, supporting its potential as a natural therapeutic alternative. However, given the in vitro nature of this study, further in vivo investigations are necessary to confirm its clinical efficacy and safety. Additionally, future research should explore the synergistic effects between *P. major* extracts and conventional antibiotics to develop more effective treatment strategies against resistant microbial strains.

## Conclusion

This study aimed to identify potential therapeutic plants that could be utilised in treating tooth infections, particularly those caused by *E. faecalis*. The findings demonstrated that methanolic extracts of *P. major* possess significant antimicrobial properties, effectively inhibiting the growth of *E. faecalis*. Furthermore, the study revealed the remarkable ability of *P. major* extracts to suppress the formation and development of *E. faecalis* biofilms, which are critical contributors to antibiotic resistance and persistent infections. These results highlight the potential of *P. major* as a natural and effective alternative for managing endodontic infections and combating biofilm-associated resistance. Future investigations are recommended to explore the precise mechanisms of action and evaluate its efficacy in clinical settings.

## Acknowledgments

The authors extend their sincere gratitude to the interviewees for their invaluable assistance throughout this study. Special thanks are also directed to the traditional healers for their significant contributions, particularly for sharing crucial knowledge about medicinal plants and their traditional applications, which greatly enriched this research.

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