ORIGINAL ARTICLE

Antibacterial and antibiofilm effect of *Moringa oleifera* leaves on bacteria associated with endodontic-periodontal lesions: an experimental study

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ABSTRACT

Introduction: Sodium hypochlorite (NaOCl) is the gold standard irrigation solution in root canal treatment; however, its clinical use is limited by its potential toxicity to periodontal tissue. *Moringa oleifera* is a natural plant known for its antibacterial properties. The study aims to analyze the potential antibacterial and antibiofilm effects of *M. oleifera* leaves on bacteria associated with endodontic-periodontal lesions. **Methods:** An experimental study was conducted using samples of *P. gingivalis* ATCC 33277 and *S. sanguinis* ATCC 10556 cultured on BHI agar before exposure to *M. oleifera* leaf extract at concentrations of 80, 60, 40, and 20%. A 5.25% NaOCl solution served as the positive control, while distilled water was used as the negative control. The minimum inhibitory concentration (MIC) was determined using the tube dilution method with BHI medium and defined as the lowest concentration that inhibited bacterial growth. The minimum bacterial concentration (MBC) was determined by subculturing inoculum from tubes that showed no visible growth in the MIC test. A standard plate count method was used to measure the growth density of *P. gingivalis* and *S. sanguinis*. The antibiofilm assay evaluated biofilm attachment using crystal violet staining. Biofil density was quantified using spectrophotometry at 570nmto measure the amount of transmitted light. A One-way ANOVA Test was applied to the normally distributed data, while the Kruskal-Wallis Test was used for non-normally distributed data. Post-hoc tests were performed to determine significant differences between groups, with a significance level set at $p < 0.05$. **Result:** The antibacterial test showed a significant effect of *M. oleifera* leaf extracts on the elimination of *P. gingivalis* ($p = 0.002$) and *S. sanguinis* ($p = 0.001$). The antibiofilm tests also showed a significant difference between among *M. oleifera* extract groups. **Conclusion:** *M. oleifera* leaf extract at concentrations of of 60 and 80 % exhibits antibacterial and antibiofil effects against *P. gingivalis* and *S. sanguinis*.

KEYWORDS

Moringa oleifera, *Porphyromonas gingivalis*, *Streptococcus sanguinis*, Antibacterial, Antibiofilm

INTRODUCTION

The pulp and periodontal diseases are caused by polymicrobial infection, in which the diversity of the microbiome and its interactions with the host contribute to complications in the treatment procedure.[1] Research by Altaf et al. reported that among 685 patients with dental caries, 102 patients (14.89%) presented with

endodontic-periodontal lesions).[2] Microorganisms are one of the main causes of dental caries which, if left untreated, can progress to pulp and periodontal tissue disease.[3] Interactions among microorganisms usually occur in the manifestation of pulp and periodontal disease due to the presence of various bacterial colonizations with their distinct characteristics.[4]

Endodontic treatment is a procedure aimed at cleaning the root canal system, which consists of mechanical preparation and disinfection using irrigation solutions.[5] Mechanical preparation is carried out using endodontic needles to create an obstacle-free path from the pulp chamber to the apical end, allowing the solution material to reach the entire main pathway of the root canal system and its branches.[6] The use of irrigation solutions as a disinfection agent with antibacterial and antimicrobial properties during root canal treatment procedures plays essential role in significantly reducing microorganism colonies. Commonly used root canal irrigants include sodium hypochlorite, chlorhexidine, and EDTA (*ethylenediaminetetraacetic acid*). *Sodium hypochlorite* (NaOCl) remains the gold-standard irrigation solution due to its high effectiveness in eliminating pathogenic microorganisms within the root canal system.[5, 6]

The NaOCl concentration commonly used for endodontic treatment is 5.25%. *Ghivari et al.* stated that this concentration was proven to be the most effective in eliminating *Enterococcus faecalis*, *Porphyromonas gingivalis*, *Streptococcus mutans* , and *Staphylococcus aureus,* achieving nearly 100% effectiveness with a contact time of 10 seconds.[7] *Martin et al.* stated that NaOCl exhibits increasing toxicity as the concentration increases.[8] The use of NaOCl solution can cause degeneration of healthy tissue, resulting in tissue death. Extrusion of NaOCl solution from the tooth apex into the periapical tissue area can cause complications.[9] Limitation of NaOCl is its toxicity to tissue, especially when it comes into contact with the periradicular area.

Research into the use of natural ingredients in dentistry has been widely carried out, with the expectation that they may serve as substitutes for chemical agents, and one of the plants that has an antibacterial effect is *M. oleifera* leaves.[10] *M. oleifera* is the most widely cultivated species of the *Moringa genus* and belongs to the *Moringaceae* family. *M. oleifera* leaves are widely used as a medicinal herbal ingredient because they have high nutritional content and have been applied in the management of bacterial-fungal infections, inflammation, and malnutrition.[11,12] Research conducted by *Shailemo et al.* showed that *M. oleifera* leaf extract was effective as an antibacterial agent against *E. faecalis,* which plays a role in endodontic treatment failure.[13] The phytochemical content of *M. oleifera*, specifically tannin (9.36%), is known to act as a potent antibacterial agent.[12,14] Furthermore, research by *Saad et al*. indicate that *M. oleifera* exhibits no toxic effects on Human Dental Pulp Stem Cells (DPSC), and may possess the potential to induce osteogenic differentiation.

In cases of periodontitis with periapical lesions, opportunistic infection involving *P. gingivalis* antigens has been reported with a prevalence reaching 78.7%.[15] This bacterium is often found in cases of pulp tissue disease due to endo-perio interactions in which bacteria enter through the apical foramen as a connecting route between the tooth and periodontal tissue. *S. sanguinis* is a Gram-positive commensal bacterium that plays a role as an initiator and provides attachment for the accumulation of microorganisms on the tooth surface. Although not the most dominant pathogen, *S. sanguinis* has a prevalence of 6.6% in contributing to periapical abscesses and endo-perio lesion interactions.[16]

The fimbriae of these bacteria are involved in the process of attachment to the tooth surface by mediating the adhesion process of *S. sanguinis* to the pellicle layer of hydroxyapatite, which is lubricated by saliva and contains various types of proteins, as an initial step in biofilm formation.[16] Research by Kurniawan et al. showed that a mixture of 20% *M. oleifera* extract with 10% propolis was able to inhibit biofilm formation by *P. gingivalis*.[17] The study also stated that *M. oleifera* concentrations of 40 and 80% mixed with 10% propolis had an antibacterial effect

comparable to that of 0.7% tetracycline. In addition, research by Rochyani analyzed the inhibitory effect of *M. oleifera* leaf extract on the biofilm formation of *E. faecalis.*[18] The research used extract concentrations of 20%, 40%, 60%, and 80%.

Therefore, based on these previous studies, the concentrations of *M. oleifera* leaf extract used were 80%, 60%, 40%, and 20% tested against *P. gingivalis* and *S. sanguinis* as a potential root canal irrigant compared to 5.25% sodium hypochlorite solution as a positive control and distilled water as a negative control. *P. gingivalis* and *S. sanguinis* were chosen as the bacteria in this study because they are associated with the process of biofilm formation, which plays a role in endodontic-periodontic lesion interactions.

The novelty of this study lies in the evaluation of *M. oleifera* leaf extract as a natural ingredient tested against two bacteria that cause endodontic-periodontal lesions: *P. gingivalis* and *S. sanguinis.* This evaluation was performed on both mono-species and dual-species biofilms, thus better representing the actual infection conditions in the oral cavity. In addition, this study not only assessed the antibacterial activity but also the antibiofilm ability of *M. oleifera* leaf extract and compared it with a 5.25% NaOCl solution as the gold standard for root canal irrigant.

This provides an overview of the potential of *M. oleifera* as an alternative root canal irrigation material that is biocompatible and friendly to periodontal tissue. Therefore, the purpose of this study was to analyze the antibacterial and antibiofilm effect of *Moringa oleifera* leaves on bacteria associated with endodontic periodontal lesions.

METHODS

M. oleifera leaves were first cleaned with running water and then dried at room temperature in a ventilated room. *The dried* leaves were subjected to maceration by mixing with methanol for 3 days. The mixture was placed in a rotary shaker for 2 hours, and the extract was subsequently filtered using Whatman Filter Paper No. 1. The resulting macerate was concentrated using a rotary evaporator at 35°C until a thick *M. oleifera* extract was obtained. The thick fraction was evaporated to ensure complete removal of methanol solvent. The final fraction was then dissolved using 1% Tween 80 in 5% Dimethyl Sulfoxide (DMSO) to obtain the *M. Oleifera* stock solution, The desired concentrations of 20%, 40%, 60%, and 80% were prepared using the dilution formula M1 x V1 = M2 x V2.[19]

Brain Heart Infusion-Broth (BHI-B) medium was prepared by mixing 3.7 grams of BHI-B powder with 100 ml of sterile distilled water in an Erlenmeyer flask, stirring with a glass spatula, and heating on an electric stove until homogeneous, then covering the flask with sterile cotton. The BHI-B medium was sterilized in an autoclave at 121°C for 15 minutes. Sterility testing was performed by incubating the BHI-B medium at 37°C for 24 hours. Sterile BHI-B medium remains clear after incubation.

Brain Heart Infusion-Agar (BHI-A) medium was prepared by mixing 5.2 grams of BHI-A with 100 ml of sterile distilled water in an Erlenmeyer flask, stirring with a glass spatula, and heating on an electric stove until homogeneous, then covering the flask with sterile cotton. The BHI-A medium was sterilized in an autoclave at 121°C for 15 minutes. The sterilized BHI-A medium was poured into Petri dishes to a thickness of 2 mm and allowed to cool and solidify. Sterility testing was performed by incubating the BHI-A medium at 37°C for 24 hours. Sterile BHI-A media remains clear after incubation.[20]

BHI-B medium was dispensed into test tube (2 ml each) using a micropipette fitted with a blue tip. One loopful of *P. gingivalis* (ATCC 33277) was then inoculated into each test tube containing BHI-B medium, then tubes were closed. The test tube were placed into a desiccator to create anaerobic conditions and

incubated at 37°C for 24 hours. After incubation, the bacterial suspension was removed and diluted.

The bacterial suspension was diluted with sterile distilled water and homogenized using a vortex mixer. The turbidity was measured with a spectrophotometer until the absorbance level reached the 0.5 Mc Farland standard ($1.5x10^8$ CFU/ml). *S. sanguinis* (ATCC 10556) were inoculated into a sterile tube containing 2 mL of BHI-B medium. The reaction tube was placed in a desiccator and incubated at 37°C for 24 hours. After incubation, the *S. sanguinis* culture was homogenized using a vortex mixer for 30 seconds, and the turbidity was adjusted to the 0.5 Mc Farland standard (1.5×10^8CFU/ml).[21]

The dilution method was used to determine the minimum concentration of the *M. oleifera* extract required to inhibit the growth of *P. gingivalis* and *S. sanguinis*. Bacteria were taken from each culture using a loop needle to make a suspension with a standard McFarland turbidity level of 0.5 (1.5×10^8 CFU/ml). Then, the *M. oleifera* leaf fraction at specific concentrations (20, 40, 60, and 80%) was added (1 ml to each 0.1-ml-bacterial suspension. This mixture was then combined with 15ml of liquid BHI-B (45-50°C), and poured into a Petri dish.

The cultures were then incubated at 37°C for 24-48 hours. Observation of bacterial growth was carried out by assessing the level of turbidity in the medium. Bacterial growth was recorded using the notation (+) for turbid growth, and (-) for a clear medium. This clear medium indicates Minimum Inhibitory Level (MIC). Determination of MIC and Minimum Bactericidal Concentration (MBC) values was performed using the streak plate method from the results of the solid dilution on BHI-A medium, which was incubated for 18-24 hours at 37°C. MBC is indicated by the complete absence of growth along the streaks on the medium. A positive control (5.25% NaOCl) and a negative control (distilled water) were used .[22, 23]

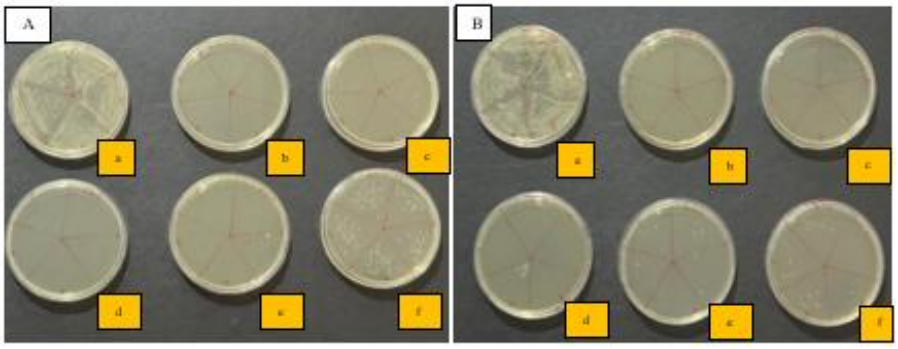

Figure 1. Antibacterial dilution test A. (*S. sanguinis*) and B. (*P. gingivalis*): a. negative control, b. positive control, c. 80%, d. 60%, e. 40%, f. 20%

P. gingivalis (200 µl) and *S. sanguinis* (200 µl) suspensions were added to the wells of a 96-well plate. Then, 200 µl of the *M. oleifera* leaf fraction was added to each bacterial suspension according to the concentration groups of 20%, 40%, 60%, and 80%. A positive control using 5.25% NaOCl and a negative control using 200 µl distilled water were prepared. After incubation for 24 hours, the contents were discarded, and the wells were rinsed with 300 µL of phosphate-buffered saline (PBS) three times. Biofilm staining was performed using 150 µl of crystal violet indicator for 15 minutes at room temperature (20-25°C). The stain was added to the microtiter wells, and the wells then rinsed again with PBS.

The microtiter plate was dried after PBS aspiration from each well. Then, 200 µL of 96% methanol solution was added and left for 15 minutes. A spectrophotometry set at a wavelength of 570 nm was used to measure the Optical Density (OD) of the microtiter plate as part of part of the inhibition test.

Meanwhile, in the biofilm attachment destruction test, after the biofilm was formed after 24 hours of incubation, turbidity appeared at the bottom of the wells. The plate was rinsed with PBS, and after exposure to the *M. oleifera* extracts, it was incubated for an additional 24 hours. Subsequently, 200 µL of 96% methanol solution was added and left for 15 minutes. A spectrophotometry at 570 nm was again used to measure the OD values as part of the destruction test. The same procedure was carried out for the dual-species biofilm of *P. gingivalis* + *S. sanguinis.*[24, 25]

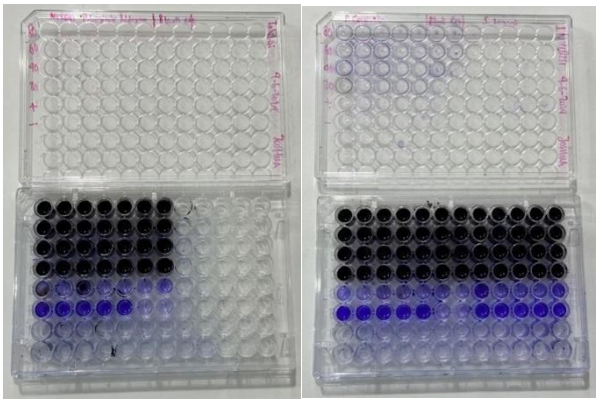

Figure 2. Antibiofilm assay test on *P. gingivalis* and *S. sanguinis*

As illustrated in Figure 2, differences in the color intensity indicate the antibiofilm effects of each group. Darker staining represents greater biofilm formation on the plate.

RESULTS

The results of the antibacterial test of *M. oleifera* extract against *P. gingivalis* bacteria are presented in Table 1. The Kruskal Wallis Test revealed a statistically significant difference between the treatment groups ($p < 0.05$).

Table 1. Kruskal-Wallis Test of Antibacterial Effect of *M. oleifera* Extract Against *P. gingivalis*

Moringa extract	Antibacterial *P. gingivalis* (cfu/ml) Median (Min-Max)	p
80 %	1.00 (0.00 – 1.00)	0.002*
60 %	1.50 (0.00 – 4.00)	
40 %	2.50 (1.00 – 4.00)	
20 %	0.00 (0.00 – 1.00)	
Positive control	0.00 (0.00 – 1.00)	
Negative control	671.00 (621 – 825)	

*significant at $p<0.05$

The Mann Whitney Test (Table 2) demonstrated no significant difference between the 80% and 60% *M. oleifera* extract concentrations in inhibiting *P. gingivalis* (p=0.762). However, the 80% extract was showed significantly greater an tibacterial activity compared with the 40% and 20% concentrations (p=0.046 and p=0.017, respectively). Additionally, no significant difference was observed between the 80% extract and the 5.25% NaOCl (p=0.186).

Table 2. *Mann-Whitney* Test of Antibacterial *M. oleifera* against *P. gingivalis*

Moringa extract		80 %	60 %	40 %	20 %	(+)	(-)
80 %	P value		0.762	0.046*	0.017*	0.186	0.018*
60 %	P value			0.557	0.019*	0.321	0.020*
40 %	P value				0.020*	0.026*	0.021*
20 %	P value					0.017*	0.020*
(+) control	P value						0.018*
(-) control	P value						

*significant at p<0.05

Further analysis of the Mann-Whitney significance test showed no significant difference in antibacterial activity between the 60% and 40% *M. oleifera* extracts (p = 0.557). However, a significant difference was observed between the 60% and 20% concentrations (p = 0.019). The antibacterial effect of the 60% was not significantly different from that of the 5.25% NaOCl positive control (p = 0.321). A significant difference was also observed between the 40% and 20% extract groups (p=0.020). In addition, both the 40% and 20% extracts showed significantly lower antibacterial activity when compared with the 5.25% NaOCl positive control (p=0.026 and p=0.017, respectively).

Table 3. Kruskal-Wallis Test of Antibacterial *M. oleifera* Extract against *S. sanguinis*

Moringa extract	Antibacterial *S. sanguinis* (cfu/ml) Median (Min-Max)	p
80 %	0.00 (0.00 – 1.00)	0.001*
60 %	1.00 (1.00 – 1.00)	
40 %	2.50 (1.00 – 4.00)	
20 %	5.00 (20.00 – 29.00)	
Positive control	0.00 (0.00 – 1.00)	
Negative control	960.00 (849 – 1125)	

*significant at p<0.05

Table 3 showed a significant difference among the groups in the antibacterial activity of *M. oleifera* extract against *S. sanguinis* (p<0.05). Post-hoc analysis in Table 4 indicated a significant difference between the 80% and 60% extract concentrations (p=0.040). The 80% extract also demonstrated significantly greater antibacterial activity compared with the 40% and 20% concentrations (p=0.026 and p=0.018, respectively). In contrast, there was no significant difference between the 80% extract and that of 5.25% NaOCl positive control (p = 1.000). The Mann-Whitney test further showed that the 60% extract exhibited significantly higher antibacterial activity than the 40% (p=0.047) and 20% (p= 0.014) concentrations.

Table 4. *Mann-Whitney* Test of Antibacterial *M. oleifera* against *S. sanguinis*

Moringa extract		80 %	60 %	40 %	20 %	(+)	(-)
80 %	P value		0.040*	0.026*	0.018*	1.000	0.018*
60 %	P value			0.047*	0.014*	0.040*	0.014*
40 %	P value				0.021*	0.026*	0.021*
20 %	P value					0.018*	0.021*
(+) control	P value						0.018*
(-) control	P value						

*significant at p<0.05

Table 4 also showed a significant difference between the 60% *M. oleifera* extract and the 5.25% NaOCl positive control in their antibacterial effects against *S. sanguinis* (p=0.040). The results of the Mann-Whitney significance test indicated that there was a significant difference between the 40% and 20% concentrations (p=0.021). In addition, both the 40% and 20% extracts exhibited significantly different antibacterial activity against *S. sanguinis* when compared with the positive control 5.25% NaOCl (p=0.026 and p=0.018, respectively).

Table 5. One Way Anova Test Inhibition Effect of Antibiofilm *M. oleifera* against *P. gingivalis*

	Sum of Squares	df	Mean Square	F	P
Intergroup	4.223	5	0.845	129.79	0.000*
Intragroup	0.117	18	0.007		
Total	4.340	23			

*significant at p<0.05

The antibiofilm inhibitory effect of the *M. oleifera* extract against *P. gingivalis* are described in Table 5. The statistical analysis revealed a significant difference between the tested groups (p<0.05).

Table 6. *Tamhane* Test Inhibition Effect of Antibiofilm *M. oleifera* against *P. gingivalis*

Moringa extract		80 %	60 %	40 %	20 %	(+)	(-)
80 %	Mean value		-0.0295	-0.08025	-0.3345	0.038225	-1.15900
	P value		0.233	0.005*	0.001*	0.136	0.018*
60 %	Mean value			-0.05075	-0.305*	0.067725	-1.12950
	P value			0.047*	0.001*	0.024*	0.019*
40 %	Mean value				-0.25425	0.118475	-1.07875
	P value				0.001*	0.010*	0.022*
20 %	Mean value					0.372725	-0.82450
	P value					0.003*	0.042*
(+) control	Mean value						-1.197225
	P value						0.017*
(-) control	Mean value						
	P value						

*significant at p<0.05

The test results showed that there was no significant difference between the 80% and 60% concentrations of *M. oleifera* extract in inhibiting *P. gingivalis* biofilm formation (p=0.233). However, the 80% concentration demonstrated a significant difference compared with the 40% and 20% concentrations (p=0.005 and p=0.001, respectively), and no significant difference when compared with the 5.25% NaOCl positive control (p=0.136).

The 60% concentration was observed to be significantly different from the 40% and 20% concentrations (p=0.047 and p=0.001, respectively), as well as from the 5.25% NaOCl positive control (p=0.024). In addition, there was a significant difference between the 40% and 20% concentrations (p=0.001), and both concentrations were significantly different from 5.25% NaOCl (p=0.010; p=0.003).

Table 7. One Way Anova test inhibition effect of Antibiofilm *M. oleifera* against *S. sanguinis*

	Sum of Squares	df	Mean Square	F	P
Intergroup	5.126	5	1.025	105.989	0.000*
intragroup	0.174	18	0.010		
Total	5.301	23			

*significant at p<0.05

Table 7 describes the inhibitory effect of *M. oleifera* extract on *S. sanguinis* and shows significant differences between the test groups (p=0<0.05). Table 8 illustrates that there was a significant difference between *the* 80% and 60% concentrations of *M. oleifera* extract in inhibiting *S. sanguinis* biofim formation (p=0.013). Significant differences in antibiofilm effectiveness were also observed for the 80% extract when compared with the 40% and 20% concentrations. (p=0.006; p=0.018). in addition, the 80% extract showed a significant difference when compared with the 5.25% NaOCl positive control (p = 0.011). The Tamhane post-hoc test revealed that the 60% extract differed significantly from both the 40% and 20% concentrations in inhibiting *S. sanguinis* biofim formation (p=0.006; p=0.024).

Furthermore, there was a significant difference between the 60% *M. oleifera* extract and the 5.25% NaOCl positive control in inhibiting *S. sanguinis* biofilm formation (p=0.010). The Tamhane post-hoc test showed that there was no significant difference between the 40% and 20% *M. oleifera* extracts in their inhibitory effect on the formation of *S. sanguinis* biofilm (p=0.081). Apart from that, there was a significant difference between *M. oleifera* extract 40 and 20% in providing an inhibitory effect on *S. sanguinis* biofilm formation when compared to the positive control NaOCl 5.25% (p = 0.005, p = 0.015).

Table 8. *Tamhane* Test Inhibition Effect of Antibiofilm *M. oleifera* against *S. sanguinis*

Moringa extract		80 %	60 %	40 %	20 %	(+)	(-)
80 %	Mean value		-0.068	-0.21025	-0.416	0.026825	-1.30725
	P value		0.013*	0.006*	0.018*	0.011*	0.021*
60 %	Mean value			-0.14225	-0.348	0.0948250	-1.23925
	P value			0.006*	0.024*	0.010*	0.024*
40 %	Mean value				-0.20575	0.237075	0.1097
	P value				0.081	0.005*	0.033*
20 %	Mean value					0.442825	-0.89125
	P value					0.015*	0.041*
(+) control	Mean value						-1.334075
	P value						0.020*
(-) control	Mean value						
	P value						

*significant at p<0.05

Table 9. One Way Anova Test Inhibition Effect of Antibiofilm *M. oleifera* against *Dualspecies*

	Sum of Squares	df	Mean Square	F	P
Intergroup	3.540	5	0.708	348.348	0.000*
Intragroup	0.037	18	0.002		
Total	3.577	23			

*significant at p<0.05

Table 9 describes the antibiofilm inhibitory effect of *M. oleifera* extract against *P. gingivalis* & *S. sanguinis.* The statistical analysis showed a significant difference between the varios treatment groups (p<0.05).

Table 10. *Tamhane* Test Inhibition Effect of Antibiofilm *M. oleifera* against *Dualspecies*

Moringa extract		80 %	60 %	40 %	20 %	(+)	(-)
80 %	Mean value		-0.05575	-0.089	-0.13625	0.050725	-1.064
	P value		0.001*	0.003*	0.000*	0.013*	0.004*
60 %	Mean value			-0.03325	-0.0805	0.106475	-1.00825
	P value			0.181	0.004*	0.001*	0.005*
40 %	Mean value				-0.04725	0.139725	-0.975
	P value				0.056	0.004*	0.005*
20 %	Mean value					0.186975	-0.92775
	P value					0.002*	0.005*
(+) control	Mean value						-1.114725
	P value						0.004*
(-) control	Mean value						
	P value						

*significant at p<0.05

Table 10 showed that there was a significant difference between the 80% and 60% concentrations in providing an inhibitory effect on the formation of dual species biofilm (p=0.001). The difference in antibiofilm effectiveness seen in the 80% *M. oleifera* extract was significant when compared to concentrations of 40% (p=0.003) and 20% (p<0.001) concentrations. Furthermore, the 80% *M. oleifera* extract exhibited a significant difference in inhibitory effect on dual-species biofilm formation when compared to the 5.25% NaOCl positive control (p=0.0013). This

indicates that even the highest concentration of the extract was significantly inferior to the gold standard against the dual species biofilm.

The results of the Tamhane significance test showed that there was no significant difference between the 60% the 40% *M. oleifera* extracts (p=0.181) in providing an inhibitory effect on dual species biofilm formation. However, a significant difference was observed when the 60% extract was compared to the 20% extract (p=0.004).

Similarly, there was no statistically significant difference between the 40% and the 20% extract concentrations (p=0.056). Crucially, the 60% (p=0.001), 40% (p=0.004), and 20% (p=0.002) *M. oleifera* extracts all exhibited a significant difference in inhibitory effect on dual-species biofilm formation when compared to the 5.25% NaOCl positive control. This collectively demonstrates that all tested *M. oleifera* concentrations were significantly inferior to the gold standard when chanllenged by the dual species biofilm.

Table 11. One Way Anova Test Destruction Effect of Antibiofilm *M. oleifera* against *P. gingivalis*

	Sum of Squares	df	Mean Square	F	P
Intergroup	5.750	5	1.150	204.498	0.000*
intragroup	0.101	18	0.006		
Total	5.851	23			

*significant at p<0.05

Table 11 describes the antibiofilm destruction effects of *M. oleifera* against *P. gingivalis.* The One-Way ANOVA Test revealed significant differences between the treatment groups (p<0.05).

Table 12. *Tamhane* Test Destruction Effect of Antibiofilm *M. oleifera* against *P. gingivalis*

Moringa extract		80 %	60 %	40 %	20 %	(+)	(-)
80 %	Mean value		-0.068	-0.1065	-0.189	0.0365	-1.36425
	P value		0.024*	0.000*	0.001*	0.034*	0.009*
60 %	Mean value			-0.0385	-0.121	0.1045	-1.29625
	P value			0.201	0.002*	0.017*	0.010*
40 %	Mean value				-0.0825	0.143	-1.25775
	P value				0.033*	0.000*	0.012*
20 %	Mean value					0.2255	-1.17525
	P value					0.003*	0.009*
(+) control	Mean value						-1.40075
	P value						0.009*
(-) control	Mean value						
	P value						

*significant at p<0.05

Table 12 illustrates the results of the antibiofilm destruction assay against *Porphyromonas gingivalis.* The 80% and 60% concentrations of *M. oleifera* extract showed a statistically difference between them (p=0.024, with the 80% concentration being superior. The 80% extract also demonstrated significantly higher destruction effects when compared to the 40% (p < 0.001) and 20(p = 0.001) concentrations. However, when compared to the 5.25% NaOCl positive control, the 80% *M. oleifera* extract exhibiting a significant difference (p = 0.034), indicating it was significantly less effective than the gold standard in biofil destruction.

The 60% *and* 40% concentrations showed no significant difference (p=0.201), although the 60% extract was significantly superior when compared to the 20% extract (p=0.002). Similar to the 80% concentration, the 60% extract was also significantly less effective than the 5.25% NaOCl positive control (p = 0.017).

Finally, the 40% extract concentration was significantly superior when compared to the 20% concentration (p=0.033). Both the 40% (p < 0.001) and 20% (p = 0.003) *M. oleifera* extracts demonstrated significant difference in destruction effect when compared to the NaOCl 5.25% positive control. Collectively, all tested *Moringa oleifera* concentrations were significantly inferior to the gold standard in destroying established *P. gingivalis* biofilms.

Table 13. One Way Anova Test Destruction Effect of Antibiofilm *M. oleifera* against *S. sanguinis*

	Sum of Squares	df	Mean Square	F	P
Intergroup	10.412	5	2.082	683.582	0.000*
intragroup	0.055	18	0.003		
Total	10.467	23			

*significant at p<0.05

Table 13 describes the antibiofilm destruction effect of *M. oleifera* against *S. sanguinis* biofilms. The statistical analysis showed that there were significant differences between the test groups (p<0.05).

Table 14. *Tamhane* Test Destruction Effect of Antibiofilm *M. oleifera* against *S. sanguinis*

Moringa extract		80 %	60 %	40 %	20 %	(+)	(-)
80 %	Mean value		-0.0725	-0.15975	-0.2585	0.0801	-1.8255
	P value		0.312	0.001*	0.000*	0.054	0.001*
60 %	Mean value			-0.08725	-0.186	0.1526	-1.753
	P value			0.142	0.006*	0.010*	0.001*
40 %	Mean value				-0.09875	0.23985	-1.66575
	P value				0.008*	0.003*	0.001*
20 %	Mean value					0.3386	-1.567
	P value					0.001*	0.002*
(+) control	Mean value						-1.9056
	P value						0.001*
(-) control	Mean value						
	P value						

*significant at p<0.05

Table 14 describes the results of the Tamhane significance test for the antibiofilm destruction assay against *Streptococcus sanguinis* (S. *sanguinis)*.

The 80% and 60% concentrations of *Moringa oleifera* extract showed no statistically significant difference between them (p = 0.312) in their destruction effect. The 80% extract was significantly superior to the 40% (p=0.001) and 20% (p<0.001) concentrations. Notably, no significant difference was found between the 80% *M. oleifera* extract and the 5.25% NaOCl positive control (p=0.054) in destroying *S. sanguinis* biofilms.

The 60% and 40% extracts also showed no significant difference (p=0.142) but the 60% extract was significantly superior to the 20% extract (p=0.006). However, there the 60% was significantly less effective than the NaOCl 5.25% positive control.

Finally, the 40% concentration was significantly superior to the 20% concentration (p=0.008). Both 40% (p=0.003) and 20% (p=0.001) *M. oleifera* extracts were significantly less effective than the 5.25% NaOCl positive control.

The antibiofilm destruction effect of the *M. oleifera* extract against dual species biofilm of *P. gingivalis* and *S. sanguinis* are summarized in Table 15. The Kruskal Wallis Test revealed a statistically significant difference between the treatment groups, indicating that at least one group differed from the others (p < 0.05).

Table 15. ***Kruskal-Wallis*** **Test Destruction Effect of Antibiofilm** ***M. oleifera*** **against** ***Dualspecies***

Moringa extract	Antibiofilm dualspecies (cfu/ml) Median (Min-Max)	p
80 %	0.0890 (0.079 – 0.108)	0.001*
60 %	0.1570 (0.155 – 0.203)	
40 %	0.2150 (0.193 – 0.232)	
20 %	0.4105 (0.393 – 0.430)	
(+) control	0.0025 (0.0022 – 0.0031)	
(-) control	2.3065 (2.059 – 2.651)	

*significant at p<0.05

Table 16. ***Mann-Whitney*** **Test Destruction Effect of Antibiofilm** ***M. oleifera*** **against** ***Dual-species***

Moringa extract		80 %	60 %	40 %	20 %	(+)	(-)
80 %	P value		0.021*	0.021*	0.021*	0.021*	0.021*
60 %	P value			0.043*	0.021*	0.021*	0.021*
40 %	P value				0.021*	0.021*	0.021*
20 %	P value					0.021*	0.021*
(+) control	P value						0.021*
(-) control	P value						

*significant at p<0.05

The Mann Whitney Test (Table 16) showed that there was a significant difference between the 80% and 60% concentrations of *M. oleifera* in providing a destruction effect on dual species biofilm formation (p=0.021). A significant difference in antibiofilm effectiveness was also observed in the 80% extract when compared with the 40% and 20% concentrations (p=0.021; p=0.021). In addition, the 80% *M. oleifera* extract exhibited a significant difference in providing a destruction effect on dual-species biofilm formation when compared to the positive control 5.25% NaOCl (p=0.021).

The Mann-Whitney test further showed a significant difference between the 60% and the 40% concentration (p=0.043) in providing a destruction effect on the formation of dual species biofilm. A significant difference was also observed when the 60% extract was compared with the 20% concentration (p=0.021).

Moreover, the 60% extract demonstrated a significantly different destruction effect than the NaOCl 5.25% positive control (p=0.021). Significance differences were also observed between the 40% *and* 20% extracts (p=0.021). Furthermore, both 40% and 20% extracts showed significant different effects when compared to the NaOCl 5.25% positive control (p=0.004, p=0.002).

DISCUSSION

Endodontic infections are caused by multispecies interactions of microorganisms, which may exhibit complementary effects to each other. *Alfawzan et al.* stated that colonization of *E. faecalis* and *P. gingivalis* was found to be the dominant among anaerobic bacteria in cases of necrotic teeth with periradicular lesions and periodontal pockets, potentially contributing to endodontic-periodontal lesion interactions.[26] Peters et al. also reported that several other bacterial species, including *Porphyromonas, Fusobacterium, and Streptococcus,* are commonly detected in endodontic infections.[27]

Table 1-4 show antibacterial properties of *M. oleifera* leaf extracts against *P. gingivalis* and *S. sanguinis*. The results indicate that the 20% concentration demonstrates growth inhibition for both bacterial species, establishing it as the Minimum Inhibitory Concentration (MIC). It This finding is consistent with Figure 1, which shows the absence of bacterial colonies on BHI-A medium, further indicating the ability of *M. oleifera* leaf extracts to exert antibacterial activities against both bacterial species.

Figure 1 also indicated that the colony growth observed in the 80% concentration of *M. oleifera* was similar to that of the 5.25% NaOCl positive control. Based on this observation, the 80% concentration of the *M. oleifera* extract can be considered the Minimum Bactericidal Concentration (MBC) for both bacterial colonies. This finding is consistent with *Hansen et al.,* whose research showed that *M. oleifera* simplicia powder extract has potential antibacterial ability against *P. gingivalis* at concentrations of 40% and 80%, while the 20% concentration shows lower antibacterial effectiveness.[18]

This research also examined the antibiofilm properties of *M. oleifera* against both mono-species and dual-species biofilms. The findings presented in Tables 5-16 indicate that the 80% concentration of *M. oleifera* leaf extracts to inhibit and degrade biofilm formation by *P. gingivalis* and *S. sanguinis*. Tables 5-14 indicate that the 80% concentration of *M. oleifera* leaf extract shows no significant difference compared to 5.25%. This is in accordance with research conducted by Septiyani et al*.,* which reported that *M. oleifera* extract at concentrations of 40% and 80% effectively inhibited the formation of *P. gingivalis* bacterial biofilm.[5, 13]

However, Tables 15-16 show that the inhibitory and destruction effects of each extract concentration were weaker against the dual-species biofilm of *P. gingivalis* and *S. sanguinis*. This may be attributed to the ability of *P. gingivalis* fimbriae to bind to several *Streptococcus* species, including *S. sanguinis*, *S. oralis, S. gordonii and S. parasanguinis.*[17] Western blot analysis have demonstrated that purified recombinant *P. gingivalis* fimbrillin binds to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Streptococcus* species, suggesting that *P. gingivalis* can utilize its fimbriae to attach to these bacteria and promote cooperative interactions that enhance biofilm formation.[27, 28]

The results of this study clearly demonstrate a dose dependent relationship between the concentration of *M. oleifera* leaf extract and its efficacy. Increasing the extract concentration consistently enhanced both the antibacterial and antibiofilm effects against *P. gingivalis* and *S. sanguinis*. This finding aligns with previous study by Sopandani et al. which showed that higher concentration of *M. oleifera* extract were more effective in eliminating *E. Faecalis*.[8].

The potent antibacterial and antibiofilm abilities of the *M. oleifera* extract are influenced by the various bioactive compounds it contains, including flavonoids, saponins, tannins, terpenoids, and alkaloids.[29] Flavonoids interfere with communication signals between bacterial cells, thereby inhibiting the formation and attachment of bacteria to host cell.[30] Saponins act by disrupting bacterial adhesion and altering the permeability of bacterial cell walls.[31] Tannins also disrupt the permeability of bacterial cell walls, leading to cell wall shrinkage and impaired bacterial protein synthesis , ultimately resulting in bacterial cell lysis.[15] Terpenoids exert their action by forming polymeric reaction with porins in the outer membrane cell walls. Alkaloids interact with bacterial DNA and amino acid components, resulting in nuclear damage and cell lysis.[32, 33]

In this study, *M. oleifera* leaf extract concentrations of 60% and 80% showed antibacterial and antibiofilm effects that were comparable to those of 5.25% NaOCl, which is considered the golden standard for root canal irrigation solutions.[34] This finding aligns with the research by Alharbi et al*.,* which reported that a 100% concentration of *M. oleifera* extract exhibited bacteriostatic activity similar to that of 5.25% NaOCl.[35] These results suggest that *M. oleifera* extract has the potential to be considered as an alternative irrigation solution with strong antibacterial properties and low toxicity to periodontal tissue.

The major limitation identified in this study is that the high viscosity of concentrated *M. oleifera* leaf fractionsmay hinder their clinical use as root canal irrigation solutions. Therefore, further research is needed to determine extract concentrations that retain strong antibacterial and antibiofilm activity while

allowing appropriate dilution and flow characteristics suitable for endodontic irrigation.

CONCLUSION

There is antibacterial and antibiofilm activity from *Moringa oleifera* leaf extract on endodontic-periodontal lesion bacteria (*Porphyromonas gingivalis* and *Streptococcus sanguinis)*. The concentration 60% and 80 % *M. oleifera* leaf extract show the most significant bacteriostatic effect, comparable to 5.25 % NaOCl. This suggests that *M. oleifera* extracts has potential as a biocompatible root canal irrigant. However, further research is required to confirm its safety., mechanism, and clinical effectiveness as an endodontic irrigation solution.

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