

Antimicrobial Activity of *Moringa Oleifera* L. Ethanolic Seeds Extract Against Orally Isolated *Prevotella Intermedia* from Periodontally Diseased Patients: An in Vitro Study

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ABSTRACT

Background: Periodontal disease is a chronic inflammatory condition with multiple contributing factors, marked by the progressive destruction of the supporting structures of the teeth. Synthetic antiseptics, including chlorhexidine, demonstrate efficacy; however, they are associated with risks such as resistance and adverse side effects. *Moringa oleifera* L. is a plant recognized for its extensive pharmacological properties, presenting a potential natural alternative owing to its anti-inflammatory, antioxidant, and antibacterial constituents. **Materials and Methods:** Ethanolic extracts were prepared via a maceration method and subsequently analyzed using gas chromatography–mass spectrometry (GC-MS) for the identification of bioactive compounds. *P. intermedia* was isolated clinically, confirmed through PCR and 16S rRNA sequencing, and evaluated using agar well diffusion, MIC, and MBC assays. **Results:** GC-MS analysis identified significant phytochemicals, including oleic acid (26.6%), octadecenoic acid (19%), vaccenic acid (14%), and hexadecanoic acid (11%), which are recognized for their antibacterial properties. The extract of *Moringa oleifera* L. seeds demonstrated a dose-dependent inhibitory effect on *P. intermedia*. The maximum inhibition zone measured 15 ± 0.6 mm at a concentration of 75 mg/ml, with both (MIC) and (MBC) established at 0.78 mg/ml. Chlorhexidine exhibited superior antibacterial activity; however, *Moringa* extract displayed notable effectiveness. **Conclusion:** *Moringa oleifera* L. seed extract exhibits significant antimicrobial properties against *P. intermedia*, serving as a promising natural complement to conventional periodontal treatments. Additional in vivo and biofilm investigations are suggested to assess its potential for clinical applications.

Keywords: Antibacterial Herbs, Biofilm, *Moringa Oleifera* L., Herbal Remedy, Periodontal Microbiology

INTRODUCTION

Periodontal disease is a multifactorial infectious condition characterized by progressive destruction of the tooth-supporting tissues. Initially presenting as reversible gingival inflammation, it can advance to irreversible damage if untreated. A limited group of microorganisms within the subgingival sulcus have been identified as central to its pathogenesis, with *Prevotella intermedia*—an anaerobic, gram-negative, black-pigmented bacterium belonging to the “orange complex”—frequently associated with conditions such as puberty-associated gingivitis, chronic periodontitis, and necrotizing ulcerative gingivitis [1,2]. Conventional periodontal management typically includes mechanical debridement combined with antiseptic rinses and systemic or local antibiotics. However, repeated use of these agents is linked to challenges such as antimicrobial resistance and undesirable side effects. This has intensified the search for effective, safe, and laboratory-validated plant-derived alternatives. *Moringa oleifera* L. (family Moringaceae) is widely studied for its pharmacological properties, including anti-inflammatory, antioxidant, and antimicrobial activities [3–5]. Its bioactivity is attributed to phytochemicals such as fatty acids, alkaloids, polyphenols, and tannins [6]. This activity, that stands against bacteria, is attributed to the existence of fatty acids, alkaloids, polyphenols, tannins, and other components that are considered bioactive in *Moringa oleifera* L. [6]. All of these broad-spectrum effects suggest the potential use of *Moringa oleifera* L. products as alternatives to chemical antiseptics, as well as possible adjuncts in the treatment of diseases that are periodontal. In spite of numerous studies that report the antibacterial effects of

Moringa oleifera L. against primary and secondary colonizers, its antimicrobial activity against anaerobic periodontal pathogens, specifically the ethanolic seed extract, is yet to be thoroughly explored. Consequently, this study measured the effects of *Moringa oleifera* L. ethanolic seed extract as antimicrobial against *Prevotella intermedia*.

MATERIALS AND METHODS

Plant Extraction

The ethanolic extract of *Moringa oleifera* L. seeds (Batch N. 8503004312, PT LTD, Coimbatore-641998, India) was prepared using the maceration process [7]. The dried seeds of *Moringa oleifera* L. were blended, and 100g of the seed material was then immersed in 1 liter of 96% ethanol, creating a 0.1g/ml mixture. Extract was filtered, and the rough material of the plant was removed through the use of the muslin cloth, by squeezing it in order to release any remaining solvent from the plant residue. The remaining extract ethanolic centrifuged at 9,000 rpm for around 10 min and later filtered. Finally, extract was dried using a spray dryer to produce a powdered form, which was stored in a sterile container in a refrigerator for use in following experiments.

Compounds Analysis in *Moringa oleifera* L. Seeds Extract

Each prepared extract solution was added to the GC-MS apparatus in a 1 μ L aliquot. A standard capillary column was used to achieve separation. After starting at 105°C and holding it there for six minutes,

the thermal program ramped up at a rate of 11°C per minute until it reached 240°C, where it remained steady for about eleven minutes. With the quadrupole and ion source temperatures set at 155°C and 245°C, mass spectrometric detection was carried out in electron impact mode at 1,550 V. Agilent ChemStation software was used for data collection and analysis. The obtained mass spectra were compared to reference spectra from the NIST database in order to identify the compound.

Plaque Sampling

Female participant who had undergone periodontal therapy, and used antibiotics during the last 4 months, and pregnant, smokers, or have systemic illnesses (like diabetes), were disqualified and not included in this study. In order to gather subgingival biofilms samples for the clinical isolation of *Prevotella intermedia*, ten male patients in good overall health who had stage 3 periodontal disease which is defined as having probing depths of at least 6 mm, clinical attachment loss of at least 5 mm, and the loss of radiographic bone that extends to the middle/apical or thirds of the root were chosen. Supra- and subgingival scaling using a sterile curette was done prior to subgingival plaque sampling so that any plaque and calculus were removed from the area in order to circumvent any contamination of the blood on the day of the sample. The patients were called back for subgingival plaque sample after a week. Samples were obtained from pockets in the manner of interproximal aspects of the upper left molar teeth that were actually deeper than 6 mm. The pockets at the interproximal aspect that is located at the upper left first molar was isolated with the use of cotton rollers. After that, a sterile paper point was carefully inserted into the periodontal pocket and held there for 60 seconds until tissue resistance was felt [8].

Identification, Isolation, and Confirmation *Prevotella Intermedia*

After cultivating suspected expanding colonies many times *P. intermedia* was first identified using a morphology of a colony, the production of black-pigment, staining of Gram, and anaerobic conditions. PCR and 16S rRNA gene sequencing were then used to validate the initial identification. To verify the existence of *P. intermedia*, certain pairs that are primer (MacroGen, Seoul) aimed at the 16s rRNA geneome at 575 base duos were employed, as stated below:

1. DNA isolation: A defined colony was obtained from each sample and placed in an Eppendorf test tube containing approximately 50 µL of sterile, hyper-deionized distilled water (DDW). The mixture was homogenized using a vortex mixer and subsequently incubated at 96°C for 10 minutes [8].

2. PCR analysis: Employing primers specific to *P. intermedia* targeting the 16s ribosomal RNA gene, conventional PCR was carried out as follows: Reverse primer 5'-TGA ACA TGT CTG TATCCT GGC T-3' and forward primer 5'-ATT GTT GGG GAG TAAACG GGC-3'. Three copies of the amplification were carried out utilizing a Veriti™ 96-well thermocycler PCR. A final volume of 20 µL was used to produce the reaction mixture, which included 10 µL of 2X Prime Taq Premix, 5 µL of template DNA, 1 µL of DDW, and 2 µL (10 pmol/L) of each *Prevotella intermedia* primer. The PCR cycle included of five minutes of initial denaturation at 96°C, amplification (which included 30 seconds of DNA-denaturation at 97°C, 30 seconds of hardening at 63°C, 30 seconds of extension at 73°C, an ultimate extension at 73°C for five minutes). Following a 35-minute, 80-volt 2% agarose gel electrophoresis of the PCR result, 4µl of ethidiumbromide had been

applied to make a stain over the gel. The molecular mass was determined using a 100 bp + DNA ladder. The bands on the gel were purified using Fermentas, UK's genomic JET™ Gel extraction kit [9]. At last, the PCR product sequencing was finished (MacroGen, Seoul).

Bacterial Suspension Preparation

Pure isolated colonies of *P. intermedia* from agar plates were inoculated with 3 ml of Mueller Hinton Broth, which contained 6µg/ml of hemin and 2µg/ml of menadione, using sterile loops. The bacterial suspension was calibrated to 1.5×10^8 CFU/ml using a 0.5-McFarland turbidity standard at an optical density of 650 nm [10].

Agar Diffusion Test

The antibacterial susceptibility of *P. intermedia* to *M. oleifera* L. seeds extract was assessed [11]. An L-shaped loop was used to inoculate 50 µL of *P. intermedia* from the bacterial suspension that is prepared onto Mueller-Hinton Agar (MHA) plate, which were allowed to dry. Six-millimeter holes were punched into each MHA plate using a sterile micropipette tip. Volumes of 100 µL of extract concentrations of *M. oleifera* L. (12.5, 25, 50, and 75 mg/ml) were added to the well holes. 100µl of DW and non-ethanolic 0.12% chlorhexidine (CHX; priokin) were used as positive and negative controls, respectively. Each plate was incubated anaerobically for 48 hours at 36°C. Using a millimeter ruler, the inhibition zone measured as the mean width of inhibition zone surrounding each well that held the extracts' solution test. Inhibition zones presence greater than 8 mm suggests the tested extract was effective against the microorganisms [12].

MIC and MBC Determination of the Extract

In order to measure the minutest inhibitory concentrations (MICs) of *M. oleifera* L.'s ethanolic seed extract, a 2-fold serial of macro dilution procedure was to be used. Mueller Hinton Broth (MHB) was divided into ten test tubes, each holding 900 µL. Next, there was an addition of 900 µL of 50 mg/ml seed extract aimed at the first tube. The extract was successively diluted, from the first toward the eighth tubes, into the resulting concentrations: 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19, and 0.09 mg/ml. In tube 9, there was a use of MHB as the negative control, and 900 µL of a mixture of 0.12% CHX and, in tube 10, as a positive control, MHB was used. Each dilution received 100 µL of *P. intermedia* suspension, making a whole volume of 1,000 µL for each tube, all the test tubes were made to be incubated for 49 hours and at 36.5°C in a jar that is anaerobic (Oxoid™ AnaeroJar). The MIC was calculated, in a tube with no turbidity, as being the lowest concentration of the extract. After incubation, bacteria (turbidity) growth was assessed in a visual manner, by the eyes, and later verified via a spectrophotometer. In order to evaluate the precision and repeatability of the outcomes, every antimicrobial evaluation was carried out three times in duplicate. Following MIC analysis, 50 µL from tubes that showed no signs of bacterial growth were cultivated on MHA plates for 49 hours at 36°C with a gas pack that was anaerobic within an anaerobic jar. The lowest dosages of ethanolic extract of *M. oleifera* L. seed that resulted in zero growth or fewer than three colonies indicated a killing activity of 99–99.5%. These values were utilized to calculate the minimum bactericidal concentration (MBC) [13].

Statistical Analysis

The data analysis was done using the SPSS version 26.0 software. For the antimicrobial sensitivity test, the results were initially evaluated at a 5% level, then performed to see whether there existed a difference amid the groups at a significance threshold of 0.5%.

Results

The Analysis of ethanolic Seed Extracts of the *Moringa oleifera* L. by GC-MS

As seen in figure 1, the *M. oleifera* L. seed' GC-MS analysis yielded 6, 8, 9, and 11 peaks, respectively.

There was a noteworthy rise in oleic acid (26.6%), octadecenoic-acid (19%), vaccenic-acid (14%), and hexadecenoic-acid (11%) among the main constituents of the extracts. Table 1 displays every component of the extract. Through spectral data comparison between the component components and the NIST, the given percentage and the type of phytochemical elements that were represented by means of the peaks were identified and also classified.

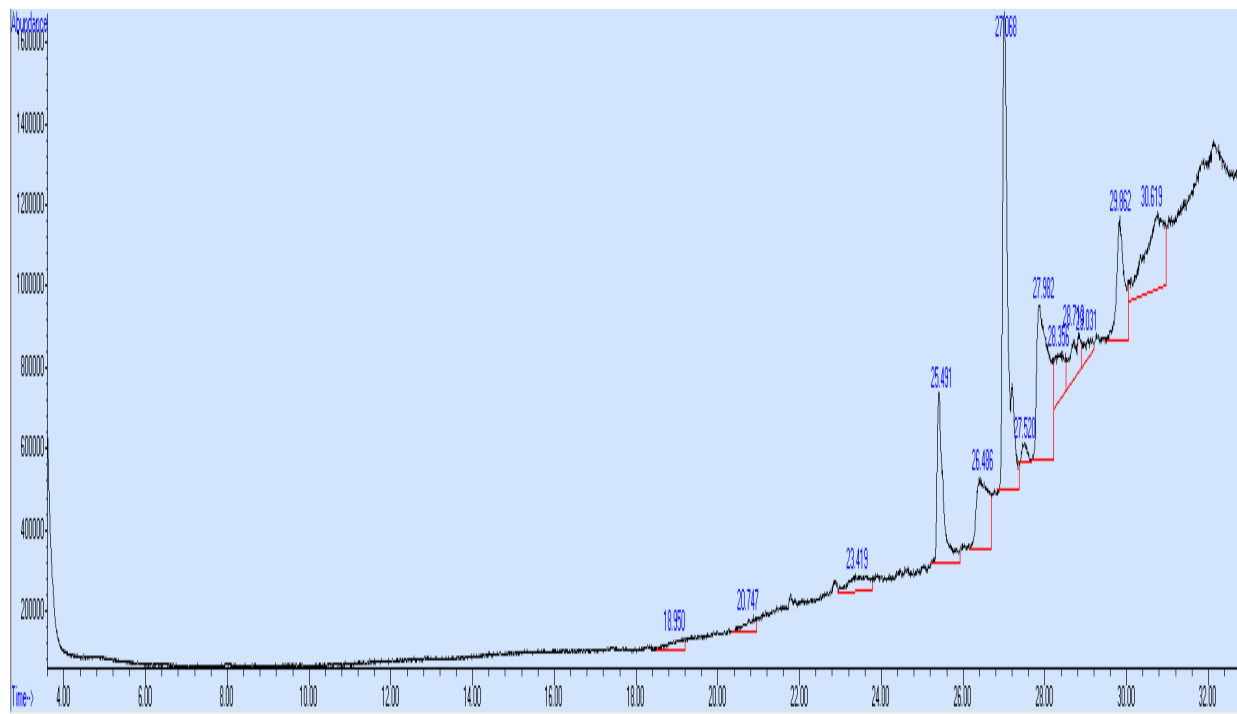


FIGURE 1: GC-MAS Analysis of *M. Oleifera* Ethanolic Seed Extract

Identification of *P. Intermedia*

Anaerobic bacteria with black pigmentation were found in 70% ($n=7$) of the specimens examined. The colonies were circular, tiny, had convex development, and were opaque after 48 hours. Figure 2 illustrates the appearance of black colored colonies on lysed blood after 7–10 days. Figure 3 illustrates how 16s rRNA sequencing demonstrated that every pure colony shared the same genetic makeup as the ATCC 25611 strain.

Assessment of the Ethanolic Extract for Antimicrobial Properties.

M. oleifera L. seeds exhibited dose-dependent antibacterial activity on all of the tested doses, according to the results of the agar well

diffusion test. At a dosage of 12.5 mg/ml, the lowest mean-inhibition zone was measured at 5.5 ± 0.5 mm, while the maximum inhibition zone was found at 75 mg/ml, measuring 15 ± 0.6 mm. Conversely, when compared to *M. oleifera* L. seeds, CHX 0.12% still had the largest noteworthy inhibitory zone, measuring 20.3 ± 0.4 mm, as shown in table 2.

By employing serial macro dilutions and OD, the MIC value of the seeds of *M. oleifera* L. against *P. intermedia* were determined. At 0.78 mg/ml ($OD=0.021$), the lowest concentration that exhibited antibacterial activity against *P. intermedia* was found. MBC values were calculated in Table 3 and were 0.78 mg/ml.

Table 1: GC-MAS Component *M. Oleifera* Ethanolic Seed Extract

Peck	Retention Time	Area%	Name
1	18.946	1.29	Alloxan 3-Aziridinopropionaldehyde
2	20.747	1.43	2-Carbamy1-9-[beta-d-ribofuranosyl] hypoxanthine
3	23.415	2.91	1N, N-Dimethylethanesulfonamide
4	25.488	9.47	Pentadecanoic acid, 14-methyl-, methyl ester
5	26.490	8.17	1,6-Penta-O-galloyl-β-D-glucose
6	27.068	26.6	oleic acid
7	27.518	1.02	2-Methyl-Z, Z-3,13-octadecadi enol
8	27.985	19	Octadecenoic acid
9	28.359	14	vaccenic acid
10	28.716	4.04	1H,3HQuinoline-2,5-dione, 1-4-fluorophenyl
11	29.030	10.5	hexadecanoic acid
12	29.863	8.98	Cyclopropane carboxamide, 2-cyclop 1,5,9- Undecatriene, 2,6,10-trimeth yl-, (Z)-
13	30.619	14.01	cis-11-Hexadecenal

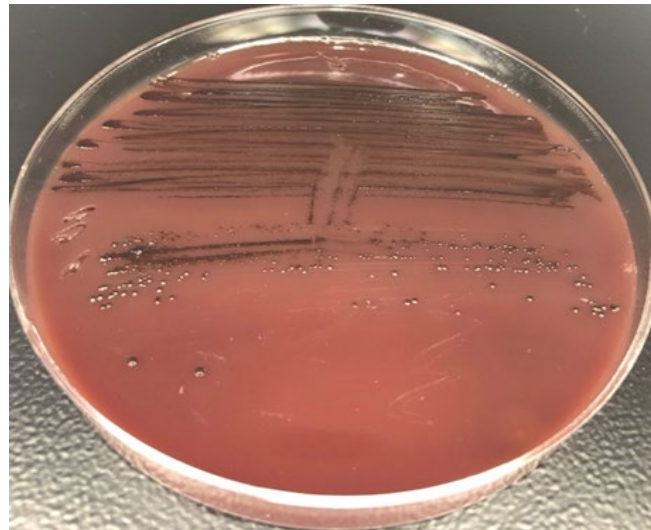


FIGURE 2: Pure Black Pigmented *P. intermedia* 7 Days after Subculturing.

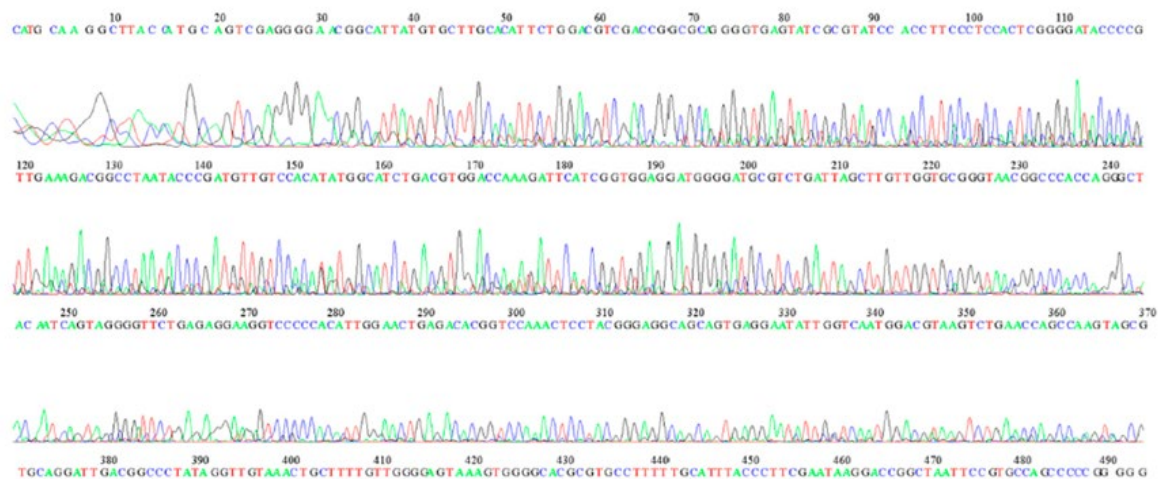


FIGURE 3: Genomic Sequencing Graph of *P. intermedia* at 16s RNA

Discussion

One notion that is centuries-old and has been broadly employed in various impoverished nations as a common healthcare therapy is medicine that is plant-derived. Furthermore, it has been established that these medicinal herbs can be used to treat oral conditions, particularly periodontal disorders [14]. While chemical antiseptics, like CHX, have many benefits when used as a therapy adjunct for periodontal illnesses, there are also drawbacks. For example, teeth discoloration and altered taste perception. These factors make it difficult to determine whether chemical antiseptics would continue to be the gold standard in the absence of a viable substitute. *P. intermedia* is thought to be crucial to the development of periodontal disorders. Several research works have focused on the antimicrobial properties of natural herbs in relation to *P. intermedia*. While a number of both in vivo and in vitro studies have documented the application of *M. oleifera* L. extracts against primary and secondary colonizers, there was a dearth of information on these extracts' antibacterial efficacy against *P. intermedia*, which also required investigation. Therefore, the purpose of this research was to assess the antimicrobial capabilities of an ethanolic seed extract of the periodontal disease-causing microbe, *Mycoplasma oleifera* L. Therefore, the purpose of this

research was to assess the antimicrobial capabilities of an ethanolic seed extract of the periodontal disease-causing microbe, *Mycoplasma oleifera* L [15].

Table 2 demonstrates the dose-dependent inhibitory effects of *M. oleifera* L. seeds on *P. intermedia* across all administered doses. The seeds of *M. oleifera* L. demonstrated antibacterial properties in the present study, with minimum inhibitory concentrations (MICs) recorded at 0.78 mg/ml, as illustrated in table 3. Table 1 presents the notable increases in oleic acid (26.6%), octadecenoic acid (19%), vaccenic acid (14%), and hexadecanoic acid (10.5%) observed in *M. oleifera* L. seeds. The antibacterial effects of each of these elements on *P. intermedia* have been demonstrated in vitro, thereby corroborating the findings of the current investigation. By changing the shape of phospholipid composition and affecting cell lysis and the integrity of the cell membrane, the presence of phenolic chemicals and fatty acids, especially oleic acid, contributes to this antibacterial impact [16]. Also, research has shown that oleic acid inhibits hemagglutinin production, which is important for *P. intermedia* adhesion and virulence [17]. In addition, the *P. intermedia* cell membrane is disrupted and messenger ribonucleic acid synthesis is impaired due to the high content of 10-trans-11-octadecenoic acid in *M. oleifera* L. seeds, which reduces the viability and proliferation rate of *P. intermedia* [18].

Table 2: Sensitivity of Ethanolic Extracts

Extract	Inhibition Zone (mm)	Standard Deviation	P-value
CHX (0.12%)	20.3000	0.484*^	0.000
Seeds 75 mg	15.2667	0.639*^	
Seeds 50 mg	11.0000	0.258*^	
Seeds 25 mg	9.2112	0.326*^	
Seeds 25 mg	5.5667	0.529*^	

CHX: Chlorhexidine; * Comparison to CHX by Kruskal, Significance at $P < 0.05$.
^Comparison

Table 3: MIC, MBC, OD values of Moringa oleifera L. seed and chlorhexidine 0.12% and broth against *P. intermedia*

Extract	MBC (mg/ml)	MIC (mg/ml)	OD (SD)	Versus
Mo seeds	0.78	0.78	0.021 (0.00122)	CHX
CHX	0.06	0.06	0.014 (0.00040)	Broth

MIC: Minimum Inhibition Concentration; CHX: Chlorhexidine 0.06%; OD: Optical Density *Comparison by Mann-Whitney Test, Significance Level at < 0.05

Existing in a biofilm is preferable to achieve a free planktonic state when there is a polymicrobial interaction [19]. In the current work, maceration procedures were used to extract seeds from crude plants. To prevent harm to potentially thermolabile components in plant extracts from alternative heat-dependent extraction methods, the solid plant mass was soaked in 96% ethanol away from any sources of heat,

and at room temperature, in order to extract the contents. This study's potential inability to identify certain physiologically active components in *M. oleifera* L. seeds by GC-MS analysis was one of its drawbacks. This could be because the components were either not extracted by the solvent used, or they were extracted previously but were not picked up by the GC-MS analysis. However, it is advised that in order to identify components that cannot evaporate or break down at high temperatures, alternative techniques for phytochemical screening be used in further research, such as high-performance liquid chromatography [20]. More research is advised in order to confirm and verify the study's findings, especially with regard to the combination's anti-biofilm action, which may offer useful information for the creation of oral health products.

Conclusions

The antibacterial activities of Moringa oleifera L. against clinically isolated *P. intermedia* were clearly demonstrated by this current in vitro study. More research needs to be done to determine and separate these oil extracts' physiologically active components. Furthermore, it is strongly advised to carry out in vivo investigations to verify their efficacy and employ them in conjunction with mechanical periodontal therapy.

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