Archives of Razi Institute, Vol. 77, No. 4 (2022) 1405-1419

DOI: 10.22092/ARI.2022.357513.2051

Original Article

Antimicrobial Effect of *Moringa Oleifera* L. and Red Pomegranate against Clinically Isolated *Porphyromonas gingivalis*: *in vitro* Study

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Received 17 January 2022; Accepted 1 April 2022
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Abstract

Moringa oleifera L. and red pomegranate extracts have been reported to inhibit gram-positive facultative anaerobe growth and inhibit the formation of biofilm on tooth surfaces. The current study aimed to assess the antibacterial effect of *M. oleifera* L. and red pomegranate extracts and their combinations against *Porphyromonas gingivalis.* The antimicrobial sensitivity, minimum inhibition concentrations (MIC), and minimum bactericidal concentrations after treatment with the aqueous extracts of *M. oleifera L.* and red pomegranate as well as their combination against clinically isolated *P. gingivalis* were determined using agar well diffusion and two-fold serial dilution. The anti-biofilm activity of the extracts and their combination was evaluated using the tube adhesion method. The phytochemical analysis was carried out using gas chromatography-mass spectrometry. It was found that *P. gingivalis* was sensitive to aqueous extract of *M. oleifera L.* seeds and red pomegranate albedo, however, not to *M. oleifera L.* leaves and red pomegranate seeds. The MIC value of *M. oleifera* L. seeds, red pomegranate albedo, and their combination were obtained at 12.5 mg/ml, 6.25 mg/ml, and 3.12 mg/ml against *P. gingivalis*, respectively. The extract combination had the highest anti-biofilm effect than *M. oleifera* L. seeds and red pomegranate albedo aqueous extracts at the minimum concentrations of 6.25 mg/ml, 25 mg/ml, and 12.5 mg/ml, respectively. The combination of red pomegranate albedo and *M. oleifera* L. seeds showed superior antibacterial and anti-biofilm effects against *P. gingivalis*, followed by red pomegranate albedo and *M. oleifera* L. seeds. This may highlight a promising alternative to the traditional chemicals that can be used as an adjunct in the treatment of periodontal diseases.

Keywords: Antibacterial herbs, Biofilm, Herbal remedy

1. Introduction

Periodontal disease is a multi-factorial, poly-microbial, diverse infection defined by damaging the tissues that support the teeth. It initiates as a reversible gingival inflammation, and non-treated disease can lead to irreversible destruction of the tooth-supporting tissues. Substantial data collected over the years have led only a tiny portion of microorganisms in the sub-gingival sulcus to contribute to the beginning and advancement of periodontal disease. *Porphyromonas gingivalis*, an anaerobic gram-negative secondary colonizer, represents one of the main keystone pathogens involved in the initiation and progression of chronic periodontitis following the transition of normally symbiotic microbiota to dysbiotic one (1). It is known that *P. gingivalis* have several virulence factors that enable this bacterium to infiltrate the gingiva, interact with other microorganisms, and trigger tissue damage with direct

and indirect pathways in adult periodontitis lesions, resulting in periodontal disease progression (2).

Professional scaling and root planing, antibiotic medications, and antiseptic mouthwashes have been adopted to treat periodontal disease. These medications and mouthwashes used frequently have adverse side effects, such as antibacterial resistance. The therapeutic effect of plants is a centuries-old concept that has received much interest in the past few years due to its biological effectiveness and safe use. In addition, oral infections used to be cured with these traditional plants in the past. Due to its wide range of antibacterial, antioxidant, and anti-inflammatory properties, plant-derived medicine can be used as an alternative stable, safe, and bioactive treatment for synthetic medications (3). One of these medicinal herbs is *Moringa oleifera* L. (Mo), belonging to the family of *Moringaceae*. Different parts of *M. oleifera* L. were used for their antihypertensive, anti-inflammatory, antioxidant, and antimicrobial properties (4). Early epidemiological data have reported that *M. oleifera* L. has an antimicrobial effect against primary colonizers of dental plaque, such as *Streptococcus salivarius*, *Streptococcus mutans*, *Streptococcus mitis*, and *Streptococcus anginosus* (5, 6). This antibacterial activity is due to the presence of polyphenols, alkaloids, tannins, fatty acids, and other bioactive components (4). Red pomegranate, one of the world's oldest fruits referred to as a symbol of fertility, prosperity, and abundance (7), has shown to exert diverse antibacterial effects against the growth of dental plaque-associated microbiota, such as *S. salivarius*, *S. mutans*, *Streptococcus sanguinis*, and *Aggregatibacter actinomycetemcomitans* (8). The antimicrobial activity may be attributed to the presence of several bioactive components, such as tannins, polyphenols, and flavonoids. All of these wide range influences favor the possible use of *M. oleifera* L. and red pomegranate products as substitutes to chemical antiseptics and probable adjunctive in the treatment of periodontal diseases (9).

Despite all the investigations reporting the antibacterial effects of *M. oleifera* L. and red pomegranate against primary and secondary colonizers, the antimicrobial effect of various extracts and/or combination of red pomegranate and *M. oleifera* L. against anaerobic periodontal pathogens has not been determined. Therefore, this study aimed to address the antimicrobial effects of different aqueous extracts of *M. oleifera* L. (seeds and leaves) and red pomegranate (albedo and seeds) and test their combinations against *P. gingivalis*.

2. Materials and Methods

2.1. Plant Extraction

Moringa oleifera L. (seeds and leaves) (B. NO. 850703004221, PVT LTD, coirnbatore-641108, India) and red pomegranate (albedo and seeds) aqueous extracts were prepared by the process of maceration (10). Similar maceration steps were conducted for all *M. oleifera* L. and red pomegranate components, except for red pomegranate albedo and seeds which were first removed and left to dry at room temperature for 1 month; afterward, the same maceration process continued. Briefly, each dried component was crushed and blended, and 100 g of each extract powder was immersed in 1 l of distilled water to have a 0.1 g/ml mix proportion. The extracts were filtered and rough plant matter was taken away using a muslin cloth, which was entangled to liberate extra solvent crude from its exhausting plant matter. The aqueous extract was then centrifuged at 8,000 rpm for 10 min (K Centrifuge, PLC series), and filtered with No. 1 filter paper (Whatman No 1, diameter 150 mm, Cat No 1001 150, England). The purified extracts were then frost-dried (CRIST ALPHA 2-4 LD plus Freeze dryer, Germany) to gain powdered extracts, which were stored in sterile containers at room temperature to be readily available for subsequent experiments.

2.2. Analysis of Compounds in *Moringa oleifera* L. and Red Pomegranate Extracts and their Combination

Gas chromatography-mass spectrometry (GC-MS) analysis (Agilent Technologies 7820A GC System, USA) was conducted to identify the components of the

extracts. The GC-MS system was loaded with 1 ul sample solution of each extract. A straightforward capillary column (30 mm×0.25 mm×0.25 µm film thickness) was used in the mass spectrometer. The temperature of the column was held at 100°C for 5 min, which then gradually raised to 250°C at a rate of 10°C/min with an isothermal hold at this temperature for 10 min. A continuous flow rate of 1 ml/min of helium was used as a carrier gas. The electron impact ionization mode of the mass spectrum (MS) was set at 1,500 V, with the ion source temperature at 240°C, and the MS quad temperature at 150°C. The GC-MSD Agilent Chem Station Software was used to collect mass spectral data. The extracted components were identified by comparing their MS values with those in the National Institute of Standards and Technology (NIST) collection.

2.3. Photochemical Screening of *Moringa oleifera* L. and Red Pomegranate Extracts and Their Combination

Phytochemical analysis was performed for the qualitative detection of alkaloids, flavonoids, steroids, tannins, saponins, and other phytochemicals for *M. oleifera* L. and red pomegranate extracts and their combination as follows. Alkaloids: 2 ml aqueous extract was mixed with 2 ml Mayer's reagent. The formation of a white precipitate indicates the presence of alkaloids. Flavonoids: 1 ml of aqueous extract was mixed with 1 ml of 10% sodium hydroxide. The formation of a yellow to orange color indicates the presence of flavonoids. Steroids: 2 ml sulphuric acid was added slowly on the tube's wall to 2 ml aqueous extract. The creation of 2 layers, including a lower yellowish-green layer and an upper red layer, indicates the presence of steroids. Tannins: 1 ml of aqueous extract was mixed with 1 ml of 10% aqueous ferric chloride. The formation of a dark blue or green color indicates the presence of tannins. Saponins: 1 ml of aqueous extract was mixed with 5 ml of distilled water; the tube was vortexed for 2 min. Lather formation indicates the presence of saponins (11).

2.4. Plaque Sampling

Participants who received periodontal treatment, used antibiotics in the last 3 months, were female, were pregnant, had systemic diseases (e.g., diabetes), and were smokers were excluded from the study. Seven systemically healthy male patients with stage 3 and 4 periodontitis (probing depth of ≥6 mm, clinical attachment loss of ≥5 mm, and radiographic bone loss extending to the middle/apical thirds of the root), were chosen to collect sub-gingival plaque samples for *P. gingivalis* clinical isolation. For sub-gingival plaque sampling, supra and sub-gingival scaling with sterile curette were first performed to remove any plaque and calculus within the area and avoid any blood contamination that occurred on the sampling day. After 1 week, the patients were recalled for sub-gingival plaque sampling. Cotton rolls were used to isolate the selected pocket at the buccal aspect of the upper right first moral, and the samples were taken from the pockets in the buccal aspects of upper right molar teeth that were deeper than 6 mm. A sterile paper point (F2 Dia- ProTTM) was then gently introduced into the periodontal pocket until tissue resistance was felt and kept in that position for 60 sec (12). Afterward, the paper point was removed carefully and streaked immediately over a Columbia agar plate supplemented with 1 µg/ml vitamin K1 (Himedia), 5 µg/ml hemin, and 5% horse blood (Oxoid, UK). Inoculated plates were cultivated for 5-7 days at 37ºC in an anaerobic jar (Oxoid™ AnaeroJar) with an anaerobic gas pack (thermo-scientific).

2.5. Isolation and Verification of *Porphyromonas gingivalis*

Following multiple culturing of suspected growing colonies of *P. gingivalis,* verification was justified depending on the pigment synthesis on the agar plates, gram-staining followed by microscopic examination for morphologic identification. Subsequently, DNA analysis was performed using polymerase chain reaction (PCR) assay as below:

1- Isolation of DNA: Well-defined colony was collected from each sample, mixed with 50 ul of disinfected hyper deionized-distilled water (DDW) in an Eppendorf test tube, loaded with a vortex mixer for proper homogenization, and incubated at 96°C for 10

min in a heat block (Thermo Fisher Scientific). The microtubes were centrifuged at 1,000 rpm for 5 min. Afterward, the supernatant was discarded, and the leftover precipitate was used as a DNA template (13).

2- PCR analysis: Conventional PCR was conducted using *P. gingivalis*-specific primers directed at the 16s ribosomal RNA gene (14) as follows: Forward primer 5´-AGGCAGCTTGCCATACTGCG-3´ and reverse primer 5´-ACTGTTAGCAACTACCGATGT-3´. The amplification was performed in triplicate using Veriti™ 96-well, Thermocycler PCR. The reaction mixture was prepared in a final volume of 20 µl containing 2 µl (10 pmol/l) of each *P. gingivalis* primers, 5 µl of template DNA, 1 µl of DDW, and 10 µl of 2X Prime Taq Premix (GeNet BioG-2000). The PCR cycle consisted of an initial denaturation at 96°C for 5 min, followed by amplification (consisting of DNA denaturation at 96°C for 30 sec, annealing at 63°C for 30 sec, extension at 73°C for 30 sec, and a final extension at 73°C for 5 min). The PCR product was then analyzed using 2% agarose gel electrophoresis for 35 min at 80 V, and 3 µl of ethidium bromide was added to stain the gel. As a molecular mass indicator, a 100bp + DNA ladder was used. The genomic JETTM Gel extraction package (Fermentas, UK) was used to purify the bands on the gel (15). Finally, the routine sequencing of the PCR product was completed (Macrogen, South Korea).

2.6. Preparation of Bacterial Suspension

Sterile loops were used to inoculate 3 ml Mueller Hinton Broth containing 5µg/ml of hemin and 1 µg/ml of menadione with pure isolated colonies of *P. gingivalis* from the agar plates. The bacterial suspension was adjusted to 5×10^5 CFU/ml at optical density (OD) 660 nm according to the 0.5 McFarland turbidity standard (16).

2.7. Antimicrobial evaluation of the extracts and their combination

All tests for antimicrobial assessment of the extracts were performed in triplicate on three separate times to ensure the accuracy and reproducibility of the results.

2.8. Agar well Diffusion Test

The antimicrobial sensitivity of *P. gingivalis* to *M. oleifera* L. and red pomegranate extracts was evaluated using the agar well diffusion method (17). Mueller Hinton Agar (MHA) plates were inoculated with 50 µl of *P. gingivalis* from the prepared bacterial suspension using an L-shape loop and left to dry. A sterile micropipette tip was used to punch holes of 6 mm in each MHA plate. One agar plate was used for each type of herbal extracts. Volumes of 100 µl of each concentration (25, 50, 75, and 100 mg/ml) of *M. oleifera* L. and red pomegranate aqueous extracts were applied to the well holes. As negative and positive controls, 100 µl of sterilized DW and non-ethanolic 0.12% chlorhexidine (CHX; periokin) were used, respectively. All the plates were incubated at 36°C for 48 h anaerobically. The inhibition zones were measured with a millimeter ruler and documented as the mean width of the zone of inhibition out around each well containing the solution test of the extracts (18). The inhibition zones of more than 8 mm indicated that microorganisms were susceptible to the extract being tested (19).

2.9. Determination of the MIC and MBC of *Moringa oleifera L.* and Red Pomegranate Extracts

The minimum inhibitory concentrations (MICs) of each aqueous extract of *M. oleifera* L. and red pomegranate were defined using a 2-fold serial macro dilution method. A volume of 900 µl of Mueller Hinton Broth (MHB) was distributed into every 10 test tubes, tagged as T1-T10. At the next steps, 900 µl of each extract (50 mg/ml) was added to the first tube and serially diluted from the 1st to the 8th tubes into the following concentrations: 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, and 0.39 mg/ml. The negative control was MHB in T9, while 900 µl of a mixture of 0.12% CHX with MHB was in T10 as the positive control. An amount of 100 µl of *P. gingivalis* suspension was added to each dilution, resulting in a total volume of 1,000 µl per tube. All test tubes were incubated in an anaerobic jar (Oxoid™ AnaeroJar) at 37°C for 48 h using an anaerobic gas pack (thermo-scientific). Following incubation, the growth of bacteria (turbidity) was measured visually by the eyes and confirmed by a spectrophotometer (EMC LAB, Germany, v-1100

DIGITAL SPECTROPHOTOMETER) at OD of 620 nm, and the MIC was determined as the lowest concentration of extract in a tube without turbidity. To assess the accuracy and reproducibility of the results, all antimicrobial assessments of the extracts were conducted in triplicate at three separate times. The same procedure was used to determine the MIC of *M. oleifera* L. (leaves and seeds) and red pomegranate (albedo and seeds) as described above.

After MIC determination, 50 ul from tubes with no visible growth of bacteria were cultured on MHA plates at 36°C for 48 h using an anaerobic gas pack in an anaerobic jar. The minimum bactericidal concentration (MBC) value was calculated as the lowest doses of aqueous extracts of *M. oleifera* L. and red pomegranate at which no growth or fewer than three colonies were obtained, indicating a killing activity of 99-99.5% (20).

2.10. Preparing a Combination of *Moringa oleifera* L. and Red Pomegranate Extracts

A test combination of *M. oleifera* L. and red pomegranate extracts was formulated at a concentration of 25 mg for *M. oleifera* L. extracts + 25 mg for red pomegranate extracts /1 ml MHB to be used as a reference concentration for determining the interactions of *M. oleifera* L. and red pomegranate extracts. A volume of 900 µl of MHB was distributed into every 10 test tubes, tagged as Cc1 to Cc8. An amount of 900 µl of extract combination was added to the 1st tube and serially diluted from the 1st to the 8th tubes into the following concentrations: 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, and 0.39 mg/ml. Mueller Hinton Broth alone was used as a negative broth in the Cc9 tube, while a mixture of non-alcoholic 0.12% CHX with MHB was used as the positive control in the Cc10 tube. Subsequently, 100 µl of *P. gingivalis* suspension was added to each dilution of the 10 test tubes, resulting in a total volume of 1,000 µl per tube, and incubated in anaerobic conditions in an anaerobic jar with an anaerobic gas pack at 37°C for 48 h. To assess the accuracy and reproducibility of the results, all antimicrobial assessments of the extracts were conducted in triplicate at three separate times.

The fractional inhibitory concentration (FIC) index of the created combination was calculated. The interaction of extracts in the combination was described as synergistic (FIC≤0.5), partial synergistic (0.5<FIC>1), additive (FIC=1), indifferent (1<FIC>4), or antagonistic (FIC≥4) based on the FIC value (21).

FIC index = FICMo + FICRp = (concentration of Mo, in combination/MICMo, in test alone) + (concentration of Rp, in combination/MICRp, in test alone).

2.11. Anti-Biofilm Assay of *Moringa oleifera* L. and Red Pomegranate Extracts and Their Combination

The detection of anti-biofilm activity of the extracts and their combination was investigated using the tube adhesion method (22). The solutions were poured from serial dilutions tubes, rinsed with phosphate-buffered solution (pH 7.3) for 1 min, and left to dry. The tubes were stained with 0.1% crystal violet for 1 min and rinsed by DW to remove any remaining stain. The test tubes were then left to dry upside down for 24 h. When a noticeable stain lined the tube's wall and bottom, it was viewed as a positive indication of biofilm formation. Biofilm formation was graded as none or weak, moderate, and strong. The stain was measured by visual eye with inter-examiner collaboration at 3 different times in triplicate.

2.12. Statistical Analysis

The SPSS version 26.0 for Windows package was used to analyze the data. The data were first assessed using the Kruskal-Wallis test at a 5% level for the antimicrobial sensitivity test. Afterward, at a significance level of 0.5 %, the Mann-Whitney U test was used to check if there was a difference between the groups.

3. Results

3.1. GC-MS Analysis of *Moringa oleifera* L. and Red Pomegranate Extracts and Their Combination

The GC-MS analysis of *M. oleifera* L. seeds, *M. oleifera* L. leaves, red pomegranate albedo, red

pomegranate seeds, and the combination of *M. oleifera* L. seeds and red pomegranate albedo revealed 13, 18, 17, 10, and 11 peaks, respectively, as shown in figure 1. The major components of the extracts showed a significant increase in oleic acid (24.6%), octadecenoic acid (18%), vaccenic acid (14%), and hexadecanoic acid (9.5%) within *M. oleifera* L. seeds, compared to other extracts. On the other hand, there was a significant increase in 3,4,5-trihydroxybenzoic acid (33.7%) followed by furfural (22.7%) in red pomegranate albedo, in comparison to other extracts. However, furfural showed the highest percentage (76.2%) in *M. oleifera* L. seeds and red pomegranate albedo combination, compared to other extracts, with a significant difference. There were no changes in other components either due to their presence in only one extract and absence in other extracts or because there was no significant difference. All of the extract components are presented in table 1. The percentage and nature of phytochemical constituents represented by the peaks were classified and recognized by comparing the spectral data of the components with the NIST.

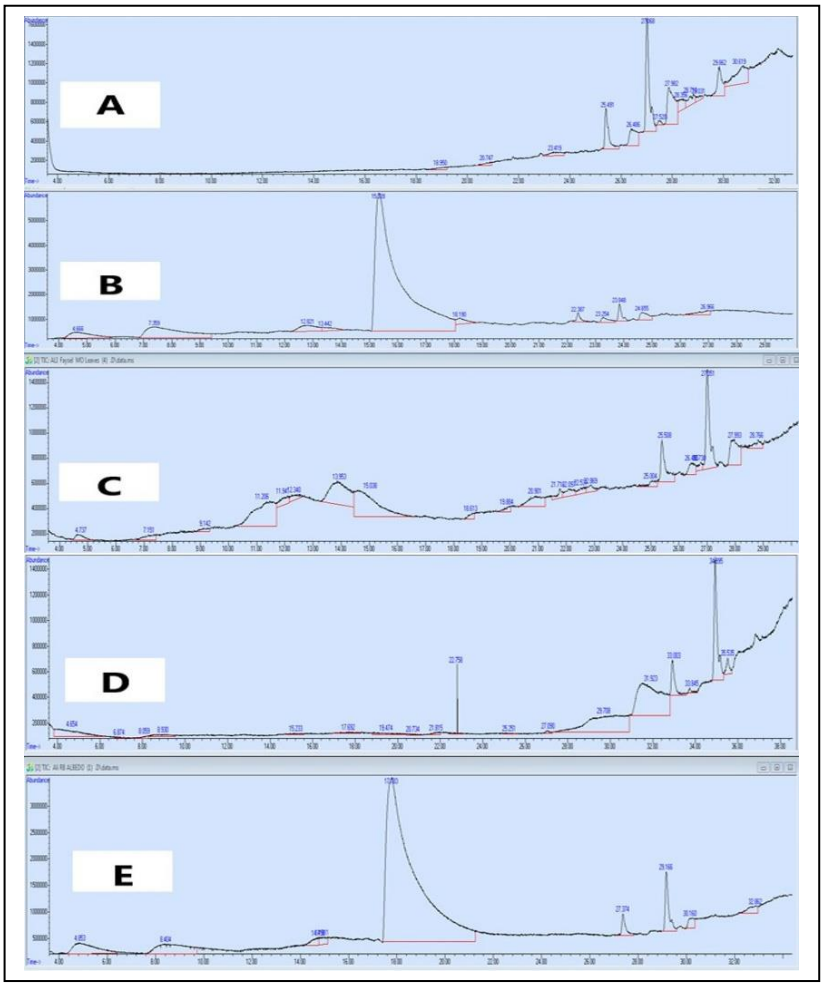

Figure 1. GC-MS chromatogram of (**A**) *Moringa oleifera* L. seeds, (**B**) *Moringa oleifera* L. seeds and red pomegranate albedo combination, (**C**) *Moringa oleifera* L. leaves, (**D**) red pomegranate seeds, and (**E**) red pomegranate albedo

Table 1. GC-MS analysis of the aqueous extracts

Peak No.	Identified compound	Peak area %				
		Mo seeds	Mo leaves	Rp albedo	Rp seeds	Mo seeds + red pomegranate albedo
1	3,5-Dimethyl-1H-pyrazole-1-carbothioamide	1.29	--	4.54*	--	3.33
2	2-Hexanol, 3-methyl	--	16.28	--	--	--
3	Carbonic acid, butyl 2-pentyl ester3-Hydroxy-N,N-	--	18.15	--	--	--
4	Furfural	--	--	22.7	--	76.21*
5	3,4,5-trihydroxybenzoic acid	--	--	33.77*	0.72	--
6	5-Hydroxymethylfurfural	--	--	--	--	76.21
7	Hexadecanoic acid, methyl ester	9.47*	--	1.43	--	0.86
8	Trans-11-Octadecenoic acid	18.09*	--	3.70	--	1.32
9	Oleic acid	24.64*	0.97	1.06	0.64	--
10	1H,3HQuinoline-2,5-dione, 1-4-fluorophenyl	4.04*	2.28	--	--	--
11	l-Gala-l-ido-octose	--	--	--	27.64	--
12	cis-Vaccenic acid	--	1.06	--	--	--
13	2-(2-Hydroxyethylthio) propionic acid	--	--	--	33.56	--

Mo: *Moringa oleifera* L.; Rp: Red pomegranate
* The highest peak area of the compound between all of the extracts.

3.2. Phytochemical Analysis

Phytochemical analysis was conducted for the qualitative detection of alkaloids, flavonoids, steroids, tannins, saponins, and other phytochemicals of *M. oleifera* L. and red pomegranate extracts and their combination (11). The results showed that the alkaloids were strongly present in *M. oleifera* L. seeds and the combination of *M. oleifera* L. seeds with red pomegranate albedo, while it was weakly present in both *M. oleifera* L. leaves and red pomegranate seeds. Flavonoids were only present in red pomegranate albedo, whereas they weakly existed in *M. oleifera* L. leaves. It was found that red pomegranate seeds were the only extract that contained steroids. Another finding was the presence of tannins in all plant extracts, except red pomegranate seeds. Finally, saponins were only present in red pomegranate seeds as shown in table 2.

Table 2. Phytochemical screening of *Moringa oleifera* L. and red pomegranate extracts and their combination

Phytochemical components	Test	Extracts				
		Mo seeds	Mo leaves	Rp albedo	Rp seeds	Mo seeds + red pomegranate albedo
Alkaloids	Wagner's and Hager's tests	+	±	-	±	+
Flavonoids	Alkaline reagent test and Ammonia test	-	±	+	-	-
Steroids	Salkowski test	-	-	-	+	-
Tannins	Lead acetate test and $FeCl_3$ test	+	+	+	-	+
Saponins	Frothing test	-	-	-	+	-

Mo: *Moringa oleifera* L.; Rp: Red pomegranate
(+) Presence, (±) Weak presence, and (–) Absence of phytochemicals

3.3. Identification of *Porphyromonas gingivalis*

Black-pigmented anaerobic bacteria were present in 60% (n=4) of the tested samples. After 48 h, the colonies were round, small, with convex growth, and opaque. Black pigmented colonies appeared after 7-10 days on lysed blood, as shown in figure 2A. Under a light microscope, the colonies appeared as gram-negative short coccobacilli following gram staining, as depicted in figure 2B. DNA sequencing revealed that all pure colonies were genetically identical to the ATCC 33277 strain, as seen in figure 3.

3.4. Antimicrobial Evaluation of *Moringa oleifera* L. and Red Pomegranate Extracts

The results of the agar well diffusion test revealed that red pomegranate albedo had antimicrobial activity at all tested concentrations in a dose-dependent manner. The lowest mean inhibition zone was 5.2±0.4 mm at 25 mg/ml concentration, whereas the highest inhibition zone was 14±0.4 mm identified at 100 mg/ml concentration. In a similar trend, *M. oleifera* L. seeds showed dose-dependent antimicrobial activity at all tested concentrations, with the lowest and highest mean values of inhibition zone estimated at 4.8±0.7 mm at 25 mg/ml concentration and 11.2±0.3 mm at 100 mg/ml concentration, respectively. On the other hand, CHX 0.12% still showed the highest significant inhibition zone of 19.4±0.3 mm compared to red pomegranate albedo and *M. oleifera* L. seeds. The intergroup comparisons between red pomegranate albedo and *M. oleifera* L. seeds at the same correspondent doses revealed significant differences at $p < 0.05$. However, no antibacterial activity was observed in none of the tested concentrations of *M. oleifera* L. leaves and red pomegranate seeds against *P. gingivalis*, as summarized in table 3.

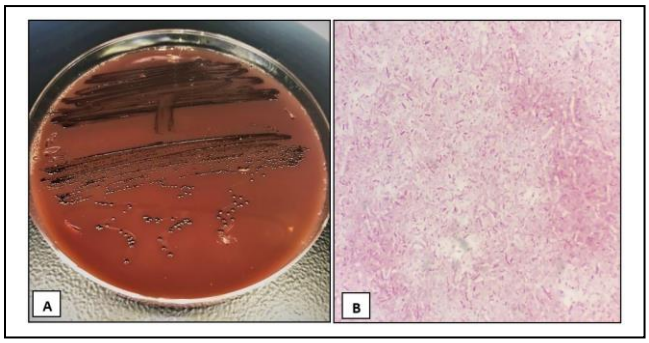

Figure 2. (**A**) After 7 days, *P. gingivalis* colonies appearing black-pigmented, spherical, smooth, and shiny, and (**B**) *P. gingivalis* appearing as gram-negative coccobacilli under a light microscope

Porphyromonas gingivalis ATCC 33277 chromosome, complete genome
Sequence ID: NZ_CP025930.1 Length: 2379874 Number of Matches: 4
Range 1: 230076 to 230429 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
654 bits(354)	0.0	354/354(100%)	0/354(0%)	Plus/Plus

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Query  1       AGGCGTGGGTATCAAACAGGATTAGATACCCTGGTAGTCCACGCAGTAAACGATGATTAC  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  230076  AGGCGTGGGTATCAAACAGGATTAGATACCCTGGTAGTCCACGCAGTAAACGATGATTAC  230135

Query  61      TAGGAGTTTGCGATATACCGTCAAGCTTCCACAGCGAAAGCGTTAAGTAATCCACCTGGG  120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  230136  TAGGAGTTTGCGATATACCGTCAAGCTTCCACAGCGAAAGCGTTAAGTAATCCACCTGGG  230195

Query  121     GAGTACGCCGGCAACGGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGAGGAA  180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  230196  GAGTACGCCGGCAACGGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGAGGAA  230255

Query  181     CATGTGGTTTAATTCGATGATACGCGAGGAACCTTACCCGGGATTGAAATGTAGATGACT  240
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  230256  CATGTGGTTTAATTCGATGATACGCGAGGAACCTTACCCGGGATTGAAATGTAGATGACT  230315

Query  241     GATGGTGAAAACCGTCTTCCCTTCGGGGCTTCTATGTAGGTGCTGCATGGTTGTCGTCAG  300
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  230316  GATGGTGAAAACCGTCTTCCCTTCGGGGCTTCTATGTAGGTGCTGCATGGTTGTCGTCAG  230375

Query  301     CTCGTGCCGTGAGGTGTCGGCTTAAGTGCCATAACGAGCGCAACCCACATCGGT  354
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Sbjct  230376  CTCGTGCCGTGAGGTGTCGGCTTAAGTGCCATAACGAGCGCAACCCACATCGGT  230429
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Figure 3. 16s RNA genome sequencing graph of isolated colonies of *P. gingivalis*

Table 3. Sensitivity (zone of inhibition) of *P. gingivalis* to different concentrations of *Moringa oleifera* L. and red pomegranate aqueous extracts

Extract type	Mean inhibition zone (mm)	Standard deviation	*P*-value
CHX (0.12%)	19.4000	0.38406	
Rp albedo 100 mg	14.0222	0.43237*****	
Rp albedo 75 mg	11.9667	0.18028*****	
Mo seeds 100 mg	11.2667	0.33912***** ^	
Mo seeds 75 mg	10.0000	0.15811***** ^	0.000
Rp albedo 50 mg	9.3444	0.58333*****	
Mo seeds 50 mg	8.1111	0.12693***** ^	
Rp albedo 25 mg	5.1556	0.40961*****	
Mo seeds 25 mg	4.8667	0.72973***** ^	
Mo leaves 100 mg		Resistant	
Mo leaves 75 mg		Resistant	
Mo leaves 50 mg		Resistant	
Mo leaves 25 mg		Resistant	
Rp seeds 100 mg		Resistant	
Rp seeds 75 mg		Resistant	
Rp seeds 50 mg		Resistant	
Rp seeds 25 mg		Resistant	

CHX: Chlorhexidine; Mo: *Moringa oleifera* L.; Rp: Red pomegranate
*****Comparison to CHX by Kruskal, significance at $P< 0.05$. ^Comparison to red pomegranate albedo at the same dose by Kruskal, significance at $P< 0.05$

3.5. MIC and MBC of Red Pomegranate Albedo and *Moringa oleifera* L. Seeds Extracts and Their Combinations

The MIC values of aqueous extracts of *M. oleifera* L. seeds and red pomegranate albedo as well as their combination against *P. gingivalis* were measured by means of OD using serial macro dilutions. The least concentration of red pomegranate albedo extracts that showed antibacterial activity against *P. gingivalis* was obtained at 6.25 mg/ml (OD=0.025), while for *M. oleifera* L. seed extract, the least concentration that exerted antibacterial activity was at 12.5 mg/ml (OD=0.024), suggesting that red pomegranate albedo had a higher antibacterial effect, compared to *M. oleifera* L. seeds. On the other hand, the highest antibacterial effect was observed using the combination of red pomegranate albedo and *M. oleifera* L. seeds extracts at concentrations of 1.56 mg red pomegranate albedo + 1.56 mg *M. oleifera* L. seeds. In addition, the FIC value of extract combinations was 0.37, indicating that this combination had a complete synergism effect. The aqueous extracts of red pomegranate albedo, *M. oleifera* L. seeds, and their combination showed bactericidal activity at a minimum concentration of 12.5 mg/ml, 25 mg/ml, and 6.25 mg/ml, respectively. The analysis of the OD values at MIC levels, MBC values, and combination values of both red pomegranate albedo and *M. oleifera* L. seeds are tabulated in table 4.

3.6. Anti-biofilm assay of *Moringa oleifera* L. and red pomegranate extracts and their combination

The results of Anti-biofilm activity tests revealed that the extract combination had a better anti-biofilm effect at a minimum concentration of 6.25 mg/ml, which was equivalent to 0.06% CHX, compared to the *M. oleifera* L. seeds and red pomegranate albedo aqueous extracts, which had a similar effect at a minimum concentration of 25 mg/ml and 12.5 mg/ml, respectively, as shown in figure 4.

Table 4. MIC, MBC, and OD values of aqueous extracts of red pomegranate albedo, *Moringa oleifera* L. seeds, and their combination and chlorhexidine 0.12% and broth against *P. gingivalis*

Extract	MBC (mg/ml)	MIC (mg/ml)	OD (SD)	Versus	*P*-value
Rp albedo	12.5	6.25	0.0257 (0.00132)	Mo Seeds	0.050
				Combination	0.014*
				CHX	0.000*
				Broth	0.000*
Mo seeds	25	12.5	0.0243 (0.00132)	Combination	0.010*
				CHX	0.000*
				Broth	0.000*
Combination	6.25	3.12 (1.56 mg red pomegranate albedo+1.56 mg *Moringa oleifera* L. seeds)	0.0240 (0.00087)	CHX	0.000*
				Broth	0.000*
CHX			0.0197 (0.00050)	Broth	0.000*

MIC: Minimum inhibition concentration; CHX: Chlorhexidine 0.06%; OD: Optical density
*Comparison by Mann-Whitney test, significance level at < 0.05

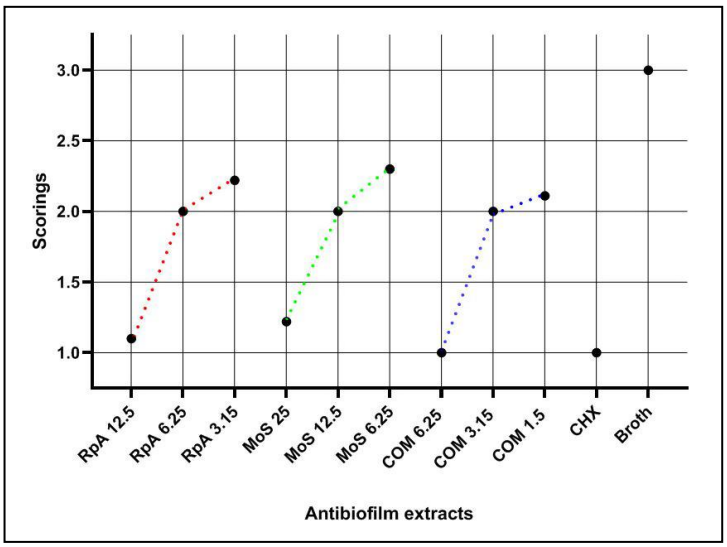

Figure 4. Anti-biofilm activity of the minimum concentrations of red pomegranate albedo and *Moringa oleifera* L. seeds extracts and their combination equivalent to 0.06% chlorhexidine

The scores showed that the combination of red pomegranate albedo and *M. oleifera* L. seeds had an anti-biofilm effect equivalent to 0.06% CHX at a minimum concentration of 6.25 mg/ml, followed by red pomegranate albedo and *M. oleifera* L. seeds at minimum concentrations of 12.5 mg/ml and 25 mg/ml, respectively. On the other hand, the anti-biofilm score of each extract/combination was increased with a decrease in extract concentrations. Score 1=absent or weak stain; 2=moderate stain; 3=strong. RpA: red pomegranate albedo, MoS: *Moringa oleifera* L. seeds, COM: red pomegranate albedo and *Moringa oleifera* L. seeds combination, CHX: chlorhexidine 0.06% (positive control). The experiment was conducted triplicate at three different times.

4. Discussion

Plant-derived medicine is a centuries-old concept and has been widely used in numerous developing countries as a common healthcare therapy. In addition, the application of these medicinal plants in the treatment of oral, in particular periodontal, diseases has been

documented (23). Despite the pros of using chemical antiseptics (e.g., CHX) as an adjunct in the treatment of periodontal diseases, several cons (e.g., teeth staining and change in the taste sensation) raise the question of whether it would remain a gold standard without a competitive alternative. The wide range of antibacterial, antioxidant, and anti-inflammatory properties attributed to herbal medicine is suggestive of their use as an alternative, stable, safe, and bioactive treatment for chemical medications.

Porphyromonas gingivalis, a keystone periodontal pathogen, is reported to play a pivotal role in the initiation and progression of periodontal diseases through a dysbiotic shift of the entire, and in particular, sub-gingival gram-positive and gram-negative periodontal microbiota (24). Targeting this pathogen may interfere with several pathways involved in the advancement of periodontal diseases, such as direct bacterial interaction and host immune modulation/evasion (25). Considering this, and in an attempt to use alternatives to antibiotics and antiseptics, numerous studies have concentrated on the antibacterial influences of natural herbs against *P. gingivalis*. Although several *in vitro* and *in vivo* investigations have reported the use of aqueous extracts of *M. oleifera* L. and red pomegranate against primary and secondary colonizers, very limited data were available regarding the antibacterial effects of these extracts against *P. gingivalis*, which also needed to be determined. Therefore, this research aimed to assess the antibacterial effects of *M. oleifera* L. (leaves and seeds), red pomegranate (albedo and seeds), and their combinations against this periodontal pathogen.

As shown in table 3, no sensitivity reactions of *M. oleifera* L. leaves and red pomegranate seeds were reported against *P. gingivalis*, while only red pomegranate albedo and *M. oleifera* L. seeds showed inhibitory effects against *P. gingivalis*. Therefore, the MIC values of red pomegranate albedo, *M. oleifera* L. seeds, and their combination were evaluated against *P. gingivalis* growing. The MIC values of the aqueous extracts of *M. oleifera* L. seeds and red pomegranate albedo as well as their combination against *P. gingivalis* were measured by means of OD using serial macro dilutions. In the present study, red pomegranate albedo, *M. oleifera* L. seeds, and their combination exerted an antibacterial effect at the MICs of 6.25 mg/ml, 12.5 mg/ml, and 3.12 mg/ml, respectively. Red pomegranate albedo had a significant increase in gallic acid content by 33% (also known as 3,4,5-trihydroxybenzoic acid) as analyzed by GC-MS analysis (Table 1). The gallic acid has shown to significantly inhibit the growth of *P. gingivalis* at MIC=1 mg/ml (26), which may partly support the inhibitory effect of red pomegranate albedo against *P. gingivalis* in our study. Gallic acid also damages the cell membrane of bacteria, causing cytoplasm leakage (27), resulting in the inhibition of DNA synthesis (28). In addition, 22.7% of furfural was observed in this study, which was shown to interfere with the DNA synthesis of *P. gingivalis*, affecting its survival. However, different MICs used in previous studies may be attributed to the differences in red pomegranate parts, extraction methods, environmental conditions, and *P. gingivalis* strains. On the other hand, *M. oleifera* L. seeds have been shown to have higher contents of oleic acid (24.6%), Trans-11-Octadecenoic acid (18%), and hexadecanoic acid (9.4%), compared to other extracts (Table 1). All of these components have been shown to exhibit antibacterial influences against *P. gingivalis in vitro* (29, 30), supporting our findings in the current study. Such antibacterial effect was due to the presence of phenolic components and fatty acids, especially oleic acid, that act by altering the morphology of phospholipid composition and destructing cell membrane and cell lysis (31). Moreover, oleic acid was found to inhibit the synthesis of hemagglutinin, which is an important virulence factor that functions as adhesins in *P. gingivalis* (32). In addition, the presence of 10-trans-11-Octadecenoic acid in high amounts in *M. oleifera* L. seeds inhibits the viability and proliferation rate of *P. gingivalis* by

damaging their cellular membrane and suppurating messenger ribonucleic acid synthesis (29).

However, the strongest antibacterial effect against *P. gingivalis* was observed for the combination between red pomegranate albedo and *M. oleifera* L. seeds with lesser MIC (3.12 mg/ml), compared to the effects of their extracts alone. This may be attributed to the highest content of furfural (76%), compared to other extracts, as analyzed by GC-MS analysis in this study (Table 1). It has been reported that furfural exerts antimicrobial action against both gram-positive and gram-negative actions by interaction with DNA Synthesis (33). The positive indication of tannins, flavonoids, and alkaloids found within the red pomegranate albedo, *M. oleifera* L. seeds, and their combination, as shown in table 2, may further explain the antibacterial effects against *P. gingivalis* growth (34). In addition, the FIC value of extract combinations was estimated at 0.37, indicating the complete synergism effect of this combination.

A polymicrobial interaction favors a mode of living in a biofilm rather than in a free planktonic state. With all pros related to biofilm formation, such as bacterial synergism, nutrient exchange, neutralizing harmful molecules, and antibiotic resistance (35), the bacteria need to stabilize this mode of living through a firm attachment on the available surfaces, such as teeth and soft tissues. Parallel to this, the investigation regarding the biological activity of the aqueous extracts of red pomegranate albedo and *M. oleifera* L. seed may further be enhanced by evaluating the anti-biofilm influences of these aqueous extracts. The tube adhesion method was used in the same tubes following MIC determination of the related extracts. The results showed that the highest anti-biofilm effect was observed with the combination extract (6.25 mg/ml, score=1) followed by red pomegranate albedo (12.5 mg/ml, score=1.1±0.3), while the minimum one was related to *M. oleifera* L. seeds (25 mg/ml, score=1.2±0.4). However, these anti-biofilm effects have been shown to reduce with a decrease in their concentrations in the target extract, which may be attributed to the attenuated effects of the bioactive compounds against bacterial adherence and survival.

The choice of solvent for plant extraction between water and ethanol was challenging. Although ethanolic extracts have a better antibacterial activity (36), it has been noted that they have limitations when used intraorally in certain circumstances, including alcoholic patients, nursing and pregnant women, children, and patients with xerostomia and diabetes. As a consequence, distilled water was chosen as a solvent for extracting plant components. However, using alcoholic solvents in other studies may account for comparable results regarding different MICs observed with red pomegranate and *M. oleifera* L. effects against *P. gingivalis.*

In the current study, the extraction of red pomegranate (seeds and albedo) and *M. oleifera* L. (seeds and leaves) crude plants was performed using maceration techniques. The constituents were extracted from a solid plant mass by soaking it in water at room temperature away from any heat sources to preserve possible thermolabile components in the extracts of the plants from damage when using other heat-dependent extraction techniques (10).

One of the limitations of this study was related to the possibility of not detecting some biologically active components of red pomegranate (seeds and albedo) and *M. oleifera* L. (seeds and leaves) by GC-MS analysis. The reason for this might be either that those ingredients were already extracted, however, not detected by GC-MS analysis or not extracted by the used solvent. Nevertheless, it is suggested to use other methods for phytochemical screening in future studies, such as high-performance liquid chromatography, to detect ingredients that cannot be decomposed at high temperatures or subjected to vaporization (37). In addition, more precise and reproducible methods, such as the tissue culture plate method (22), are needed to test the anti-biofilm activity of herbal extracts to overcome the difficulty in distinguishing weak and negative biofilm isolates while scoring.

To the best of our knowledge, this was the first study demonstrating the antibacterial effects of red

pomegranate albedo, *M. oleifera* L. seeds, and their combination extracts against the growth and biofilm formation of *P. gingivalis in vitro*. No sensitivity was observed using red pomegranate seeds and *M. oleifera* L. leaves against *P. gingivalis*. However, the highest antibacterial and anti-biofilm effects were obtained with the combination of red pomegranate albedo and *M. oleifera* L. seeds, followed by red pomegranate albedo and *M. oleifera* L. seeds. To validate and justify the results of this study, further investigations are recommended, in particular, regarding the anti-biofilm activity of the combination, which can be a promising insight for developing oral health products.

Authors' Contribution

Study concept and design: A. F. M.
Acquisition of data: F. B. H. A.
Analysis and interpretation of data: H. R. A.
Drafting of the manuscript: A. M. S.
Critical revision of the manuscript for important intellectual content: F. B. H. A.
Statistical analysis: F. B. H. A.
Administrative, technical, and material support: A. F. M.

Ethics

The study was conducted after obtaining approval from the Ethics Committee of Dentistry, University of Baghdad, Baghdad, Iraq (Ref. 256 at 20/03/2021). The research objectives and procedures were explained to all participants, and informed consent was obtained from all subjects.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

The authors would like to thank Assistant Professor Sarhang Sarwat Gul, the Dean of College of Dentistry, University of Sulaimani, Sulaymaniyah, Iraq, to facilitate the project work within the campus. Technical support for microbiology and plant extract was kindly provided by Smart Health Tower in Sulaymaniyah, and Hatim Hussein in Ibn Al-Baitar Research Center, Baghdad, Iraq.

References

1. Hajishengallis G, Lamont RJ. Breaking bad: Manipulation of the host response by P orphyromonas gingivalis. Eur J Immunol. 2014;44(2):328-38.
2. Hajishengallis G, Lamont RJ. Dancing with the stars: how choreographed bacterial interactions dictate nososymbiocity and give rise to keystone pathogens, accessory pathogens, and pathobionts. Trends Microbiol. 2016;24(6):477-89.
3. Farnsworth NR, Akerele O, Bingel AS, Soejarto DD, Guo Z. Medicinal plants in therapy. Bull World Health Organ. 1985;63(6):965.
4. Abdull Razis AF, Ibrahim MD, Kntayya SB. Health benefits of Moringa oleifera. Asian Pac J Cancer Prev 2014;15(20):8571-6.
5. Jwa SK. Efficacy of Moringa oleifera Leaf Extracts against Cariogenic Biofilm. Prev Nutr Food Sci. 2019;24(3):308-12.
6. Fajriani I. The effectiveness of gargle contain extract of Moringa oleifera leaves to inhibit plaque formation on early childhood caries (ECC). Int J Appl Pharm. 2019;11(4):46–9.
7. Heber D, Schulman RN, Seeram NP. Pomegranates: ancient roots to modern medicine: CRC press; 2006.
8. Dabholkar CS, Shah M, Kathariya R, Bajaj M, Doshi Y. Comparative evaluation of antimicrobial activity of pomegranate-containing mouthwash against Oral-biofilm forming organisms: an Invitro microbial study. J Clin Diagn Res. 2016;10(3):ZC65.
9. Newman MG, Takei H, Klokkevold PR, Carranza FA. Newman and Carranza's Clinical Periodontology E-Book: Elsevier Health Sciences; 2018.
10. Seidel V. Initial and bulk extraction of natural products isolation. Natural products isolation: Springer; 2012. p. 27-41.
11. Ali Redha A, Hasan A, Mandeel Q. Phytochemical determinations of Pomegranate (Punica granatum) Rind and Aril extracts and their antioxidant, antidiabetic and antibacterial activity. Nat Prod Chem Res. 2018;6(4):1-9.

12. Hedayati A, Khosropanah H, Bazargani A, Abed M, Emami A. Assessing the antimicrobial effect of the essential oil of Myrtus communis on the clinical isolates of Porphyromonas gingivalis: An in vitro study. Jundishapur J Nat Pharm Prod. 2013;8(4):165.
13. Gebhardt N. Fluorescence in Situ Hybridization (fish): Application Guide: Springer; 2010.
14. Sakamoto M, Takeuchi Y, Umeda M, Ishikawa I, Benno Y. Rapid detection and quantification of five periodontopathic bacteria by real-time PCR. Microbiol Immunol. 2001;45(1):39-44.
15. Lee PY, Costumbrado J, Hsu C-Y, Kim YH. Agarose gel electrophoresis for the separation of DNA fragments. J Vis Exp. 2012;(62):3923.
16. Wunsch CM, Lewis JP. Porphyromonas gingivalis as a model organism for assessing interaction of anaerobic Bacteria with host cells. J Vis Exp. 2015;(106):53408.
17. Elleuch L, Shaaban M, Smaoui S, Mellouli L, Karray-Rebai I, Fguira LF-B, et al. Bioactive secondary metabolites from a new terrestrial Streptomyces sp. TN262. Appl Biochem Biotechnol. 2010;162(2):579-93.
18. Halkai KR, Mudda JA, Shivanna V, Rathod V, Halkai RS. Biosynthesis, characterization and antibacterial efficacy of silver nanoparticles derived from endophytic fungi against P. Gingivalis. J Clin Diagn Res. 2017;11(9):92.
19. Saquib SA, AlQahtani NA, Ahmad I, Kader MA, Al Shahrani SS, Asiri EA. Evaluation and comparison of antibacterial efficacy of herbal extracts in combination with antibiotics on periodontal pathobionts: An in vitro microbiological study. Antibiotics. 2019;8(3):89.
20. Cavalieri S, Rankin I, Harbeck R, Sautter R, McCarter Y, Sharp S, et al. Manual of antimicrobial susceptibility testing. American Society for Microbiology. Pan American Health Organization. 2005.
21. Lorian V. Antibiotics in laboratory medicine: Lippincott Williams & Wilkins; 2005.
22. Mathur T, Singhal S, Khan S, Upadhyay D, Fatma T, Rattan A. Detection of biofilm formation among the clinical isolates of staphylococci: an evaluation of three different screening methods. Indian J Med Microbiol. 2006;24(1):25-9.
23. Anand BJJRPS. Herbal therapy in periodontics: a review. 2017;3(5):1-7.
24. Hajishengallis G, Lamont RJ. Polymicrobial communities in periodontal disease: Their quasi-organismal nature and dialogue with the host. Periodontol. 2021;86(1):210-30.
25. Hajishengallis G, Darveau RP, Curtis MAJNRM. The keystone-pathogen hypothesis. 2012;10(10):717-25.
26. Kang MS, Oh JS, Kang IC, Hong SJ, Choi CH. Inhibitory effect of methyl gallate and gallic acid on oral bacteria. J Microbiol. 2008;46(6):744-50.
27. Aparecida Procopio Gomes L, Alves Figueiredo LM, Luiza do Rosario Palma A, Correa Geraldo BM, Isler Castro KC, Ruano de Oliveira Fugisaki L, et al. Punica granatum L. (Pomegranate) Extract: In Vivo Study of Antimicrobial Activity against Porphyromonas gingivalis in Galleria mellonella Model. Sci World J. 2016;2016:8626987.
28. Li K, Guan G, Zhu J, Wu H, Sun Q. Antibacterial activity and mechanism of a laccase-catalyzed chitosan–gallic acid derivative against Escherichia coli and Staphylococcus aureus. Food Control. 2019;96:234-43.
29. Sulijaya B, Yamada-Hara M, Yokoji-Takeuchi M, Matsuda-Matsukawa Y, Yamazaki K, Matsugishi A, et al. Antimicrobial function of the polyunsaturated fatty acid KetoC in an experimental model of periodontitis. J Periodontol. 2019;90(12):1470-80.
30. Attallah NGM, Negm WA, Elekhnawy E, Altwaijry N, Elmongy EI, El-Masry TA, et al. Antibacterial Activity of Boswellia sacra Flueck. Oleoresin Extract against Porphyromonas gingivalis Periodontal Pathogen. Antibiotics. 2021;10(7).
31. Fischer CL, Walters KS, Drake DR, Dawson DV, Blanchette DR, Brogden KA, et al. Oral mucosal lipids are antibacterial against Porphyromonas gingivalis, induce ultrastructural damage, and alter bacterial lipid and protein compositions. Int J Oral Sci. 2013;5(3):130-40.
32. Eltigani SA, Eltayeb MM, Ishihara A, Arima J. Isolates from Monechma ciliatum seeds' extract hampered Porphyromonas gingivalis hemagglutinins. J Food Biochem. 2019;43(11):13029.
33. Rao H, Li P, Wu H, Liu C, Peng W, Su W. Simultaneous determination of six compounds in destructive distillation extracts of hawthorn seed by GC-MS and evaluation of their antimicrobial activity. Molecules. 2019;24(23):4328.
34. Howard KC, Gonzalez OA, Garneau-Tsodikova S. Porphyromonas gingivalis: where do we stand in our battle against this oral pathogen? RSC Med Chem. 2021;12(5):666-704.
35. Lynch AS, Robertson GT. Bacterial and fungal

biofilm infections. Annu Rev Med. 2008;59:415-28.

36. Das K, Tiwari R, Shrivastava D. Techniques for evaluation of medicinal plant products as antimicrobial agents: current methods and future trends. J Med Plant Res. 2010;4(2):104-11.

37. Banu KS, Cathrine L. General techniques involved in phytochemical analysis. Int J Adv Res Chem Sci. 2015;2(4):25-32.