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Extraction of *Cuminum cyminum* and *Foeniculum vulgare* Essential Oils and Their Antibacterial and Antibiofilm Activity against Clinically Isolated *Porphyromonas gingivalis* and *Prevotella intermedia*: An In Vitro Study

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Abstract: This study aimed to investigate the antibacterial and antibiofilm activities of the essential oils of *Cuminum cyminum* (*CEO*) and *Foeniculum vulgare* (*FEO*) on the clinically isolated *Porphyromonas gingivalis* (*P. gingivalis*) and *Prevotella intermedia* (*P. intermedia*). Subgingival plaque samples were collected from periodontal pockets (≥6 mm) of patients with generalized severe periodontitis. Microbiological and molecular tests were performed to isolate and confirm *P. intermedia*. A previously isolated *P. gingivalis* was used. The hydrodistillation method was used to extract the *CEO* and *FEO*. The antibacterial activity of the examined oils against the confirmed clinical strains was determined using the disc diffusion and broth dilution methods to determine the minimum inhibitory (MIC) and bactericidal concentrations (MBC). The antibiofilm properties of *CEO* and *FEO* were determined using the tube method. The *t*-test was used for comparisons of inhibition zones. Both *CEO* and *FEO* presented inhibition zones against both clinically isolated bacteria. The *CEO* and *FEO* revealed antibacterial activity with MIC values of 3.125 µL/mL and MBC of 6.25 µL/mL against *P. gingivalis*, respectively. In addition, their MIC and MBC against *P. intermedia* were 0.195 µL/mL and 3.125 µL/mL, respectively. Finally, the essential oils showed moderate to strong antibiofilm activities against the clinically isolated *P. gingivalis* and *P. intermedia*, respectively. This study supports the antibacterial and antibiofilm properties of *CEO* and *FEO* against clinically isolated *P. gingivalis* and *P. intermedia*. Further studies should focus on using these essential oils as an adjunct to periodontal therapy.

Keywords: *Cuminum cyminum*; *Foeniculum vulgare*; *Porphyromonas gingivalis*; *Prevotella intermedia*; antibacterial activity; periodontal disease; dentistry

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1. Introduction

Periodontal diseases are polymicrobial diseases of the supporting structures of the teeth. The advanced stage of the disease, namely periodontitis, might lead to the loss of the supporting structure of the teeth, including the alveolar bone, and, consequently, tooth loss [1]. Although a dental biofilm is necessary for disease initiation, damage to the periodontal tissues is mainly a result of the host's inflammatory-immune reaction to this microbial challenge. In addition, the susceptibility of individuals to periodontitis appears to be impacted by several local and systemic risk factors, such as diabetes, smoking, and systemic diseases [2].

The human oral cavity provides a suitable environment for more than 700 bacterial species. However, only a low percentage might be involved directly in the initiation and progression of periodontal diseases [3]. In almost every patient with periodontitis,

Porphyromonas gingivalis (*P. gingivalis*), known as a keystone pathogen and one of the red complex species, can be found in subgingival biofilm samples [4]. It is a Gram-negative, rod-shaped, anaerobic, and non-motile bacterium colonizing the subgingivally located pockets. Despite its low abundance in the gingival crevice, it can negatively impact the composition of the normal oral flora and encourage the growth of inflammation-inducing microbiota [5]. On the other hand, the orange-complex species include *P. intermedia*, a Gram-negative, anaerobic bacterium with black pigmentation that has been linked to periodontal diseases such as periodontitis, puberty-associated gingivitis, and acute necrotizing ulcerative gingivitis [6,7].

Scaling and root planing (SRP) form the gold standard in periodontal therapy [8]. SRP prepares the periodontal environment for tissue healing by mechanically removing supragingival and subgingival plaques. However, SRP alone cannot remove calculus and other harmful factors from deep periodontal pockets that require additional antimicrobial agents to boost the mechanical debridement of the tooth surfaces [9]. Due to their relatively fewer side effects than antibiotics, numerous herbal extracts have been used as an adjunct to periodontal treatment [10].

Herbal products have recently gained popularity for treating oral and dental diseases, and these products are available as mouth rinses, toothpaste, gels, and chips [11]. The use of natural products such as essential oils (EOs) as antimicrobial agents has increased in medicine and dentistry over the last few decades because of their safety and widespread acceptance by the population worldwide. Herbal products are effective against infections, carcinomas, and cardiovascular and neurological disorders [12].

Cuminum cyminum and *Foeniculum vulgare* are aromatic spices, herbs, and natural food preservatives belonging to the *Apiaceae* family. The *Cuminum cyminum* essential oils (*CEO*) and *Foeniculum vulgare* (*FEO*) are safe natural compounds that can be substituted for synthetic preservatives and additives in the food industry. Additionally, these EOs have been reported to possess a broad range of biological activities [13].

Clinically isolated periodontal pathogens are more virulent than laboratory reference strains that have been sub-cultured for decades since their initial isolation and may have lost critical pathophysiological characteristics. Additionally, significant genetic variations have been identified in laboratory reference strains and their clinical counterparts. This high genetic adaptability raises issues about the usefulness of laboratory bacterial strains for studying "real-world" pathogenesis. Consequently, any investigation based on laboratory strains may underestimate critical pathophysiological aspects exclusive to clinical strains [14]. Furthermore, most laboratory reference strains have reduced a biofilm-forming ability relative to clinical counterparts of the same species [13], suggesting critical genetic shifts affecting bacterial pathogenicity.

Recently, concern has been raised about the emergence of multidrug resistance among some common pathogens; therefore, it is of paramount importance to search for alternative sources of antimicrobial agents for treating many diseases, including periodontal diseases. Many herbal products have been examined for their antibacterial properties against oral pathogens. However, to the best of our knowledge, the antibacterial and antibiofilm properties of *CEO* and *FEO* have not been explored yet. Thus, this present in vitro study aimed to evaluate the antibacterial and antibiofilm properties of *CEO* and *FEO* against clinically isolated *P. gingivalis* and *P. intermedia*.

2. Materials and Methods

2.1. Collection of the Plant's Seeds

The seeds of *Cuminum cyminum* were collected from Shahrazoor district, Sulaimani Governorate, Kurdistan Region, Iraq, in August 2021. The collection was carried out in the field from wild species of the plant under study by trained personnel supervised by the researcher to avoid adulterants or contaminants. *Foeniculum vulgare* seeds were purchased from local markets in Sulaimani City, Iraq. A plant taxonomist in the College of the Agriculture/University of Sulaimani confirmed the identity of the seeds. The study

proposal was approved by the ethical committee of the College of Dentistry at the University of Sulaimani (Approval number: 500 on 21 September 2021).

2.2. Essential Oil Extraction

The EOs were extracted via the hydrodistillation method using the Clevenger apparatus under optimal operating conditions according to the standard protocol [15]. Freshly ground samples were added to sterilized distilled water in a 500 mL round-bottom flask and boiled (balloon heating mantle, Fibroman HT-W, Zhengzhou, China) continuously for 3 h. The EO was collected, dried under anhydrous sodium sulfate, and stored in a sealed amber vial at 4 °C until used.

2.3. Gas Chromatography–Mass Spectrometry (GC-MS)

The ingredients of the EO samples were identified using a GC-MS system composed of an Agilent (7820A/5977B) gas chromatography–mass spectrometry system (Santa Clara, CA, USA) [13].

2.4. Subgingival Plaque Sample Collection and Bacterial Growth Conditions

Samples from subgingival plaques for isolation of *P. intermedia* were collected from patients with generalized severe periodontitis who visited the Periodontics Department, College of Dentistry, University of Sulaimani. Participants with periodontal pockets $\geq$ 6 mm were included. Written and informed consent from patients was obtained before the clinical examination. The samples were collected by isolating periodontal pockets with sterile cotton rolls. A sterilized periodontal curette (Gracy No. 1–2, Hu Friedy Mfg. Co., Chicago, IL, USA) was used to eliminate supra-gingival plaque and/or calculus. A sterile paper point (F2 Dia-ProT™, Diadent Europe. BV, Almere, Netherland) was gently placed into the periodontal pocket until tissue resistance was felt and kept there for one minute. After carefully removing the paper points, they were spread over supplemented Columbia agar media. The agar media contents per 500 mL were 19.5 g of Columbia agar base, 2.5 µg of hemin (Sigma Aldrich, Shanghai, China), 500 µL vitamin K, and 25 mL of human blood. The plates were then incubated for 7–10 days at 37 °C in an anaerobic environment produced by anaerobic gas packs (Thermo Scientific™ Oxoid™ AnaeroGen™, Leicestershire, UK) within an anaerobic jar (BBL®GasPak system, Mississauga, Canada).

2.5. Reviving P. gingivalis and Isolation of P. intermedia

The confirmed *P. gingivalis* strain was obtained from the Microbiology Department of the College of Dentistry, University of Sulaimani, having previously been isolated from deep pockets of patients with generalized periodontitis [16]. Preliminary identification of *P. intermedia* was based on colony morphology, black-pigment production, Gram staining, and anaerobic environment, and finally confirmed using PCR and 16S rRNA gene sequencing techniques. The proven isolated bacterium was stored in a 25% glycerol (Biochem, Cosne-Cours-sur-Loire, France) at −80 °C for future use.

DNAs were extracted from clinical isolate samples via Add Prep bacterial genomic DNA extraction kit (Add Bio Inc., Daejeon, Republic of Korea). A bacterial colony from an overnight bacterial culture was sampled and mixed with one mL of sterilized ultrapure deionized distilled water and vortexed for 30 s (Thermo Scientific LP Vortex Mixer, Seoul, Republic of Korea), followed by the addition of 200 µL of lysis solution and 20 µL proteinase-k solution. After incubation for 10 min at 95 °C, 200 µL of binding solution and 200 µL of absolute ethanol were added and centrifuged. Finally, the genomic DNA was eluted via centrifugation at 13,000 rpm for one minute, and the supernatant was used as a template.

The amplification was performed using specific primer pairs targeted at the 16s rRNA gene to confirm the presence of *P. intermedia* [17]. The specific primers were as follows: 5′-TTT GTT GGG GAG TAA AGC GGG-3′ and 5′-TCA ACA TCT CTG TAT CCT GCG T-3′.

The real-time PCR process was carried out in a total volume of 20 µL that contained 2 µL of reverse primer, 2 µL of forward primer, 10 µL of 2X concentrated Taq polymerase (Add Start Taq Master, Seoul, Republic of Korea), 1 µL of nuclease-free water, and 5 µL of DNA template. The real-time PCR reaction consists of three steps in a digital automated DNA thermal cycler apparatus (VeritiTM 96 well/Thermo Fisher Scientific, Marsiling, Singapore). The DNA sample was denatured initially for 10 min at 95 °C, followed by 35 cycles of amplification (denaturation of the DNA template for 30 s at 95 °C, annealing the specific primers for 30 s at 65 °C, and then an extension of primers at 72 °C for 30 s). The final extension was performed for 5 min at 72 °C for one cycle [18]. The PCR product was electrophoresed at 80 V on a 2% agarose gel for 35 min. Then, 3 µL of ethidium bromide was added to stain the gel. A 100 bp plus DNA ladder was used as a molecular weight marker. Gel purification was performed using the gene JET™ Gel extraction kit (Fermentas, London, UK). Additionally, the standard sequencing for the PCR product was performed via ©Macrogen, Inc., Seoul, Republic of Korea.

2.6. Antibacterial Assays

The disc diffusion assay was used to evaluate the sensitivity of the clinically isolated periodontal pathogens to the tested EOs [19]. The investigator (A. H. R.) was blinded to EO testing, and the results were analyzed with the other investigators later. A swab of the bacterial suspension containing 1.5×10^8 CFU/mL equivalent to 0.5 McFarland turbidity standard was streaked over Petri plates containing Mueller Hinton agar (Oxoid, Thermo Fisher Scientific Inc., Loughborough, UK), enriched with hemin and vitamin K1 under an aseptic environment. Then, sterile 6 mm in diameter empty paper discs (CHMLAB, Barcelona, Spain) were impregnated with 30 µL of different concentrations of each EO and placed on the agar plates. The concentrations were 2.5%, 5%, and 10% of *CEO*, while *FEO* was used at concentrations of 5%, 10%, and 20%. CHX mouthwash at 0.12% was used as a positive control. The Petri plates were left for 15 min at room temperature to allow the diffusion of the oils and then incubated anaerobically at 37 °C for 24 h.

2.7. Minimum Inhibitory and Bactericidal Concentrations

The minimum inhibitory concentration (MIC) of the tested EOs was determined via the broth dilution method [20]. The stock solution was prepared by dissolving *CEO* and *FEO* in an aqueous solution containing 10% dimethyl sulfoxide (DMSO) and 0.5% polysorbate (Tween) 80 (Biochem, Cosne-Cours-sur-Loire, France). CHX mouth rinse 0.12% was used as a positive control, and Muller Hinton broth (MHB) (from Oxoid, Basingstoke, UK) as a negative control to allow bacterial growth. Furthermore, the antibacterial activity of DMSO was examined and showed no effects on antibacterial activity. Two-fold serial dilutions of each EO from 100 to 0.19 µL/mL were made in 10 sterile test tubes containing 900 µL of MHB and 100 µL of bacterial inoculum containing 1.5×10^8 CFU/mL (McFarland turbidity standard 0.5) to yield a final volume of 1 mL per each tube. The tubes were then secured with cotton plugs, incubated at 37 °C under anaerobic conditions in an anaerobic jar for about 24 h, and observed for bacterial growth. The MIC of the EO was determined to be the lowest dose that inhibited the visible growth of the bacteria. After incubation, the tubes were observed for bacterial growth, which is usually indicated by turbidity or a pellet of micro-organisms in the bottom of the tubes [21].

The minimum bactericidal concentration (MBC) was determined by spreading a loopful sample from each test tube containing different concentrations of the EOs on the supplemented MHA Petri plate. The MBC was described as the lowest concentration resulting in no bacterial growth [22].

2.8. Antibiofilm Assays

A qualitative tube method was used to show the effect of the examined EOs in preventing biofilm formation by the clinical strains [23]. In this process, the content of the incubated test tubes used to determine the MIC of the *CEO* and *FEO* that contained

different oil concentrations was carefully discarded. The tubes were washed with sterile phosphate-buffered saline (pH = 7.3) (from Biochem, Cosne-Cours-sur-Loire, France) to remove the planktonic bacteria and left in an inverted position for 45 min to dry completely. Later on, each tube was stained with one mL of 1% crystal violet and incubated at room temperature for 15 min. Then, the excess dye was removed by washing the tubes with sterile distilled water. The biofilm formation was determined by observing a visible film adhering to the bottom and the walls of the tubes and estimated according to the intensity of the violet color as non-adherent (0), weakly adherent (+), which means a robust antibiofilm activity, moderately adherent (++), which means a moderate antibiofilm effect, or strongly adherent (+++), meaning the weak antibiofilm effect of the examined material [23]. All assays were performed in duplicate.

2.9. Statistical Analysis

The obtained data were depicted as mean ± standard deviation and analyzed using SPSS software version 25 (SPSS Inc., Chicago, IL, USA). The *t*-test was used for comparisons, and $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Bacterial Strains

The previously isolated *P. gingivalis* strain used in this study (Figure 1A,E) was obtained from the Microbiology Department, College of Dentistry, University of Sulaimani [16]. *P. intermedia* strain was isolated and confirmed from subgingival plaque samples obtained from patients with periodontitis (Figure 1B). The bacterial identification was initially performed by observing colony morphology, color, growth environment, and Gram staining. The colonies on the plates after 48 h were small, round, opaque, and convex, with black pigmentation developed after one week. The bacterial identification was finally confirmed using real-time PCR technique and DNA sequencing, indicating the presence of *P. intermedia*, as shown in Figure 1C,D.

3.2. GC-MS of the Examined EOs

Based on three successive hydro distillation extractions, the mean yields of *CEO* and *FEO* were 1.9% and 1.75%, respectively. The GC-MS analysis of the *CEO* identified 28 compounds. According to the results, cumin aldehyde (20.74%) was the main compound, followed by γ-Terpinene (20.08%), β-pinene (19.75%), 2-Caren-10-al (18.54%), 1,4-Diethylbenzene (6.73%), and O-Cymene (5.24%). The remaining constituents accounted for about 9%, as shown in Table 1.

On the other hand, The *FEO* contained 17 compounds, of which estragole (67.16%) was the main compound, followed by D-Limonene (13.92%), Fenchone (10.32%), and Anethole (2%). Other constituents were found in trace amounts (Table 2).

3.3. Antibacterial Assays

The findings of the antibacterial activity of the *CEO* against the clinically isolated *P. gingivalis* and *P. intermedia* by the agar disc diffusion assay are presented in Table 3. The clinical strains were sensitive to *CEO* at 2.5%, 5%, and 10% concentrations used in this present study and produced inhibition zones ranging from 6.5 ± 0.27 mm to 16.8 ± 0.4 mm for *P. gingivalis* and from 10.5 ± 0.6 mm to 18.55 ± 0.35 mm for *P. intermedia*. Chlorhexidine (CHX) produced mean inhibition zones of 14.12 ± 0.26 mm and 15.05 ± 0.38 mm against clinically isolated *P. gingivalis* and *P. intermedia*, respectively, as presented in Figure 2A,B.

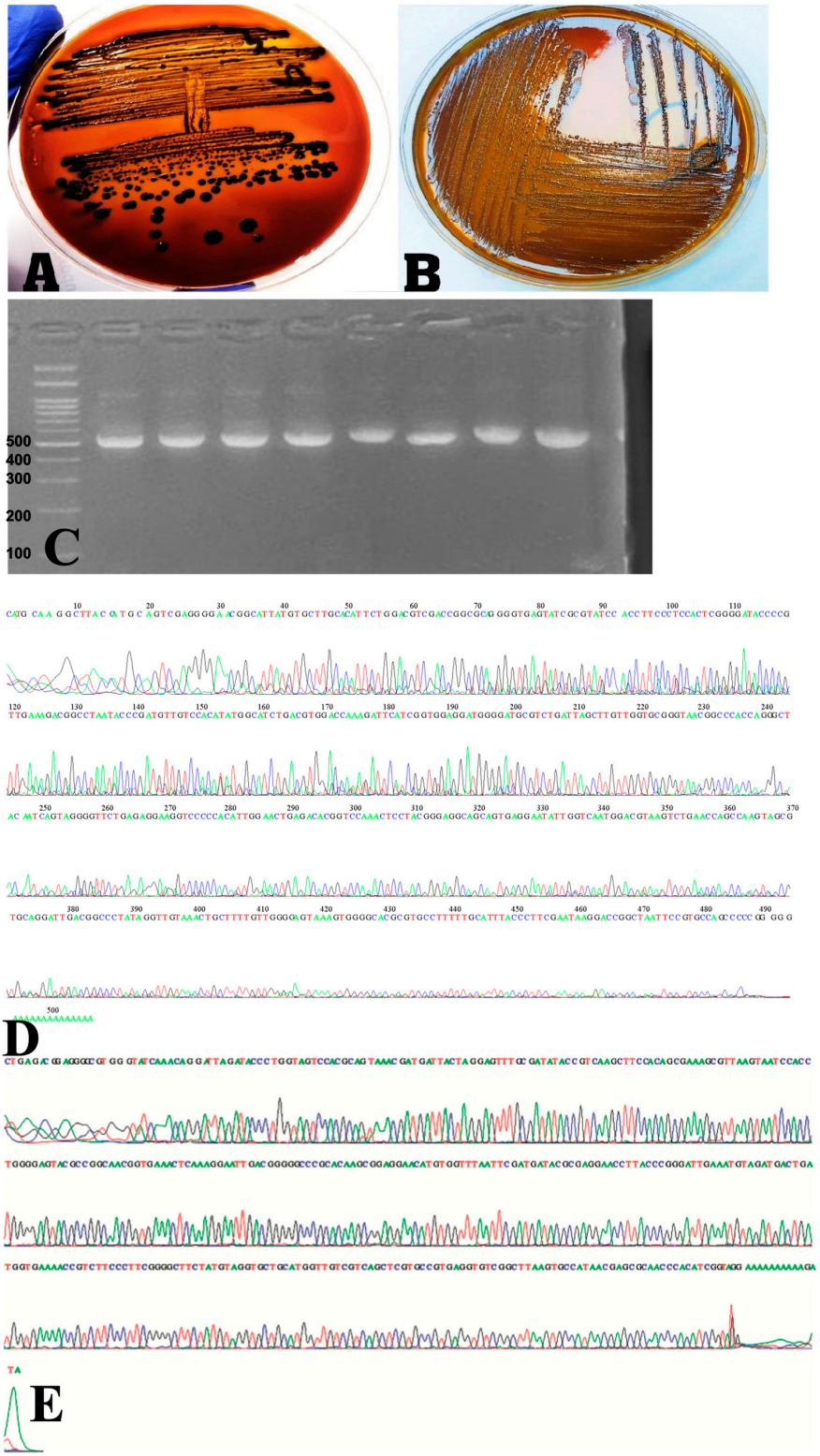

Figure 1. Black pigmented colonies of (**A**) *P. gingivalis* and (**B**) *P. intermedia* on blood agar after ten days of anaerobic incubation. (**C**) PCR products in agarose gel electrophoresis showed multiple bands localized at 575 base pairs, similar to the template size of *P. intermedia* (100 BP ladder). (**D**) 16S rRNA gene sequence graph of the purified colonies of *P. intermedia* performed in Macrogen-Republic of Korea. (**E**) 16S rDNA gene sequence of the purified colonies of *P. gingivalis* conducted in Macrogen, Republic of Korea.

Table 1. Results of GC-MS analysis of the *CEO*.

Peak	Area%	Compound Name	CAS#	MW	MF
1	1.17	α-Pinene	80-56-8	136.23	C10H16
2	19.75	β-Pinene	127-91-3	136.23	C10H16
3	0.53	α-Phellandrene	99-83-2	136.23	C10H16
4	6.73	1,4-Diethylbenzene	105-05-5	134.22	C10H14
5	5.24	O-Cymene	934-80-5	134.22	C10H14
6	20.08	γ-Terpinene	99-85-4	136.23	C10H16
7	0.20	cis-Limonene hydrate	7299-41-4	186.33	C12H26O
8	0.31	2-Octen-4-ol	4798-61-2	128.21	C8H16O
9	0.25	Terpinen-4-ol	562-74-3	154.25	C10H18O
10	2.01	10-Camphorsulfonyl chloride	1000194-76-1	250.74	C10H15ClO3S
11	20.74	Cumin aldehyde	122-03-2	148.20	C10H12O
12	18.54	2-Caren-10-al	151-85-9	150.22	C10H14O
13	0.20	p-Mentha-1,4-dien-7-ol	22539-72-6	152.23	C10H16O
14	0.22	3-Methyl-6-propyl-phenol	31143-55-2	150.22	C10H14O
15	0.43	β-Gurjunene	17334-55-3	204.35	C15H24
16	0.23	Caryophyllene	87-44-5	204.35	C15H24
17	0.16	alpha-Bergamotene	17699-05-7	204.35	C15H24
18	0.45	cis-. beta. -Farnesene	28973-97-9	204.35	C15H24
19	0.44	γ-Muurolene	30021-74-0	204.35	C15H24
20	0.24	β-Bisabolene	495-61-4	204.35	C15H24
21	0.21	Carotol	465-28-1	222.37	C15H26O
22	0.29	Methyl Palmitate	112-39-0	270.45	C17H34O2
23	019	Palmitic acid	57-10-3	256.42	C16H32O2
24	0.15	Methyl eladate	1937-62-8	296.5	C19H36O2
25	0.16	Methyl stearate	112-61-8	298.5	C19H38O2
26	0.15	Asclepic acid	506-17-2	282.5	C18H34O2
27	0.15	Oleic acid	112-80-1	282.5	C18H34O2
28	0.76	Clionasterol	83-47-6	414.71	C29H50O

Area%: Compound percentage; CAS#: Registry number; MW: Molecular weight (g/mol); MF: Molecular formula.

Table 2. The FEO components according to GC-MS analysis.

Peak	Area%	Compound Name	CAS#	MW	MF
1	1.23	α-Pinene	80-56-8	136.23	C10H16
2	0.25	β-Phellandrene	555-10-2	136.23	C10H16
3	0.45	β-Myrcene	123-35-3	136.23	C10H16
4	13.92	D-Limonene	5989-27-5	136.23	C10H16
5	0.39	gamma-Terpinene	99-85-4	136.23	C10H16
6	10.32	Fenchone	1195-79-5	152.23	C10H16O
7	0.26	Camphor	76-22-2	152.23	C10H16O
8	0.18	cis-Carveol	1197-6-4	152.23	C10H16O
9	0.39	Fenchyl acetate	13851-11-1	196.29	C12H20O2
10	0.71	Cumin aldehyde	122-03-2	148.20	C10H12O
11	1.22	Anisaldehyde	123-11-5	136.15	C8H8O2
12	2.00	Anethole	104-46-1	148.20	C10H12O
13	67.16	Estragole	140-67-0	148.20	C10H12O
14	0.22	Apiol	523-80-8	222.24	C12H14O4
15	0.64	Palmitic acid, methyl ester	112-39-0	270.45	C17H34O2
16	0.27	Elaidic acid, methyl ester	1937-62-8	296.5	C19H36O2
17	0.30	Methyl stearate	112-61-8	298.5	C19H38O2

Area%: Compound percentage; CAS#: Registry number; MW: Molecular weight (g/mol); MF: Molecular formula.

Table 3. Inhibition zones of CEO and FEO at different concentrations on clinical strains used in the study.

EO %	Inhibition Zones (mm) (*P. gingivalis*)			Inhibition Zones (mm) (*P. intermedia*)		
	EO	**CHX 0.12%**	***p*-Value**	**EO**	**CHX 0.12%**	***p*-Value**
CEO 2.5%	6.5 ± 0.27	14.2 ± 0.3	0.0001	10.5 ± 0.6	15.05 ± 0.39	0.001
CEO 5%	11.5 ± 0.27	14.1 ± 0.31	0.0001	12.9 ± 0.2	15.1 ± 0.4	0.023
CEO 10%	16.8 ± 0.4	14.05 ± 0.18	0.014	18.55 ± 0.35	15 ± 0.36	0.0001
FEO 5%	7.6 ± 0.39	14.4 ± 0.2	0.001	8.5 ± 0.55	15.3 ± 0.2	0.004
FEO 10%	8.9 ± 0.4	14.3 ± 0.5	0.001	9.4 ± 0.35	15.25 ± 0.3	0.004
FEO 20%	10.7 ± 0.4	14.45 ± 0.25	0.01	10.85 ± 0.4	15.3 ± 0.2	0.001

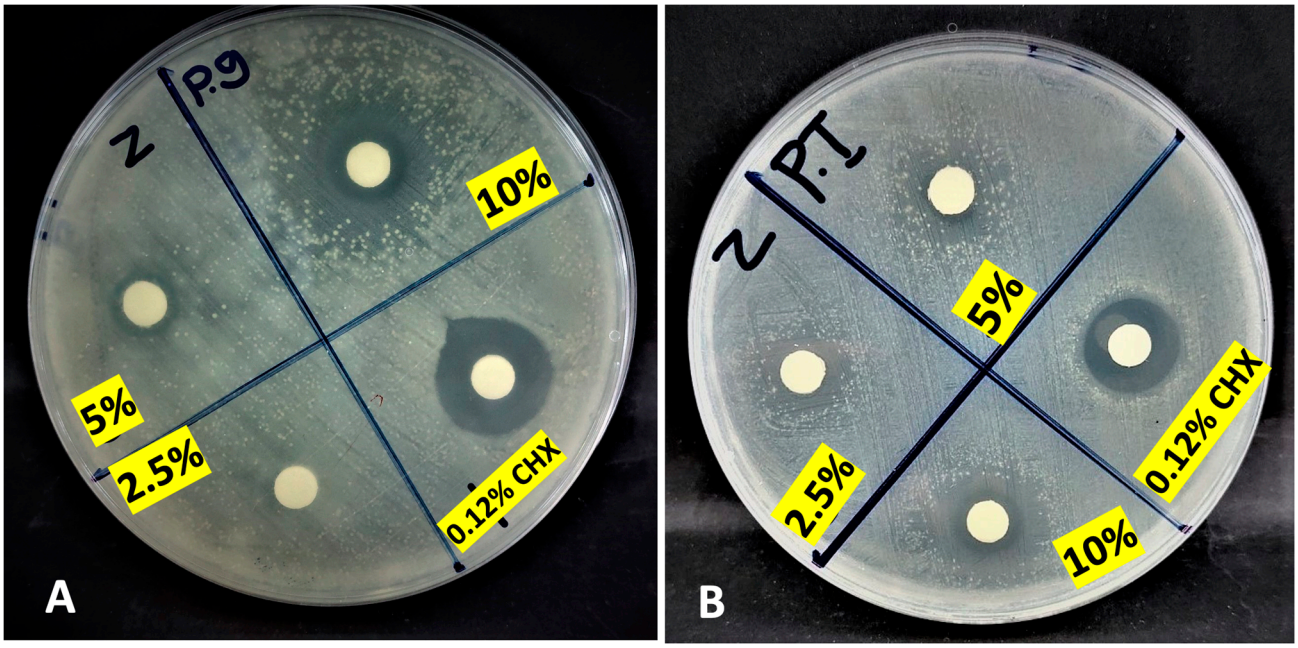

Figure 2. Inhibition zones of different concentrations of CEO (10%, 5%, and 2.5%) on clinical strains (**A**) *P. gingivalis* and (**B**) *P. intermedia*. 0.12% CHX: Chlorohexidine 0.12% as a positive control.

The inhibition zones of *CEO* were increased as the oil concentration was increased. Thus, the difference between various concentrations was statistically significant ($p < 0.001$). The comparison between the mean inhibition zones of *CEO* at 2.5% and 10% concentrations and the mean inhibition zones of CHX showed significant antibacterial activity of CHX with wider inhibition zones against *P. gingivalis* and *P. intermedia* ($p < 0.05$). However, *CEO* at 20% was more potent than CHX and produced more expansive inhibition zones on both clinical strains ($p < 0.05$). Generally, *CEO* at all concentrations and CHX were more potent on *P. intermedia*, producing wider inhibition zones than on *P. gingivalis*.

Regarding the antibacterial activity of *FEO*, the clinical strains were sensitive to the EO at 5% (50 µL/mL), 10% (100 µL/mL), and 20% (200 µL/mL) concentrations and produced mean inhibition zones ranging from 7.6 ± 0.39 mm to 10.7 ± 0.4 mm for *P. gingivalis* and from 8.5 ± 0.55 mm to 10.85 ± 0.4 mm for *P. intermedia*. Comparing the mean inhibition zones produced by CHX with *FEO*, the test revealed statistically significant antibacterial activity of CHX against both clinical strains ($p < 0.05$). Furthermore, CHX was more effective than *FEO* at all concentrations against *P. gingivalis* and *P. intermedia*, as presented in Figure 3A,B.

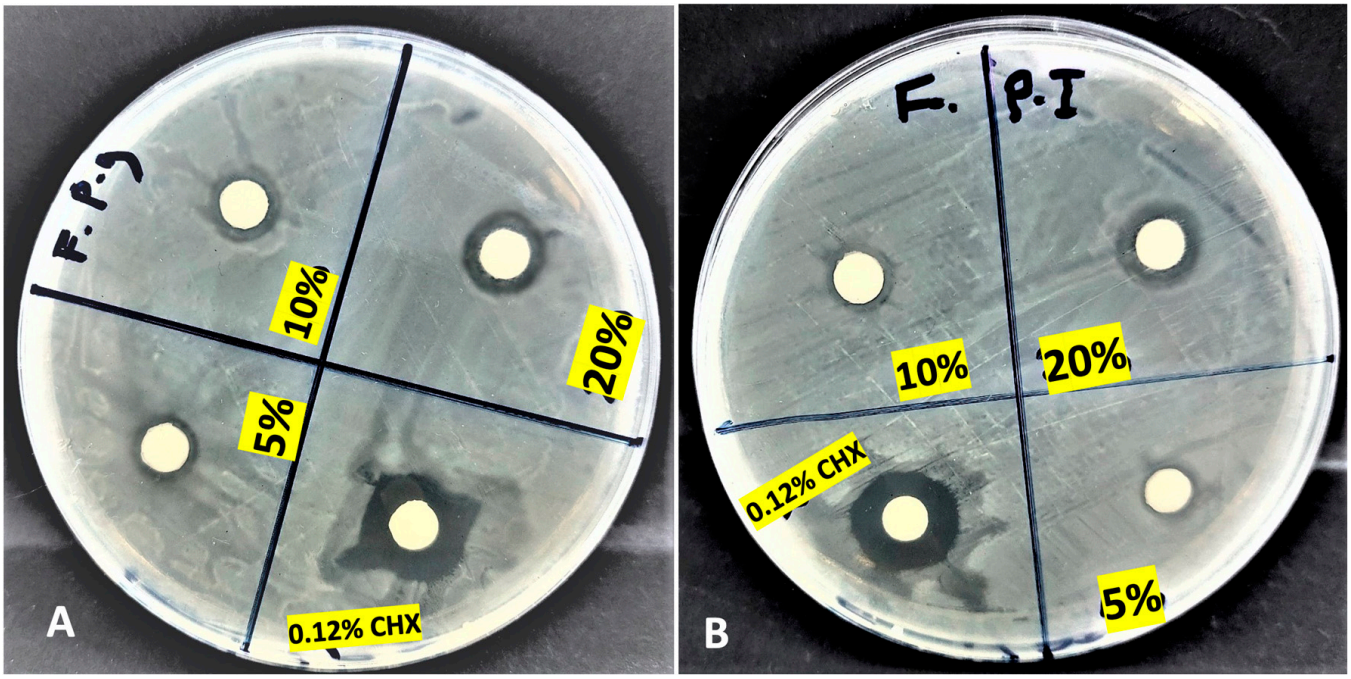

Figure 3. Inhibition zones produced by different concentrations (5%, 10%, and 20%) of FEO and 0.12% of CHX (+) on clinically isolated periodontopathic bacteria (**A**) *P. gingivalis* and (**B**) *P. intermedia*. CHX 0.12% Chlorohexidine 0.12% as positive control.

3.4. Minimum Inhibitory and Bactericidal Concentrations

The MIC values of *CEO* that prevented visible growth of clinically isolated *P. gingivalis* and *P. intermedia* were 3.125 µL/mL and 0.195 µL/mL, respectively, whereas the MBC values were 6.25 µL/mL and 0.195 µL/mL for *P. gingivalis* and *P. intermedia*, respectively, as presented in Table 4 and Figure 4. Regarding the *FEO*, the oil prevented visible growth of the clinically isolated *P. gingivalis* at 3.125 µL/mL as MIC, while the MBC concentration was 6.25 µL/mL. Similarly, the MIC and MBC values of the *FEO* against *P. intermedia* were 3.125 µL/mL, as clarified in Table 4 and Figure 5.

Table 4. MIC and MBC values of CEO and FEO on clinically isolated *P. gingivalis* and *P. intermedia* determined using the broth macro-dilution assay.

Bacteria	*CEO*		*FEO*	
	MIC	**MBC**	**MIC**	**MBC**
P. gingivalis	3.125 µL/mL	6.25 µL/mL	3.125 µL/mL	6.25 µL/mL
P. intermedia	0.195 µL/mL	0.195 µL/mL	3.125 µL/mL	3.125 µL/mL

3.5. Antibiofilm Assays

The results obtained via the qualitative tube method used to measure the effectiveness of the *CEO* and *FEO* on the adherence and biofilm-forming potential of the tested clinical strains are presented in Table 5. The violet color intensity on the tube's walls compared to the negative control tubes was used to assess the antibiofilm-forming ability of the oil against the clinical strains. The results showed that the EOs did not reveal a clear pattern, varying from a nearly complete absence of biofilm at the highest concentration to strong biofilm formation at the lowest concentration used in the present experiment.

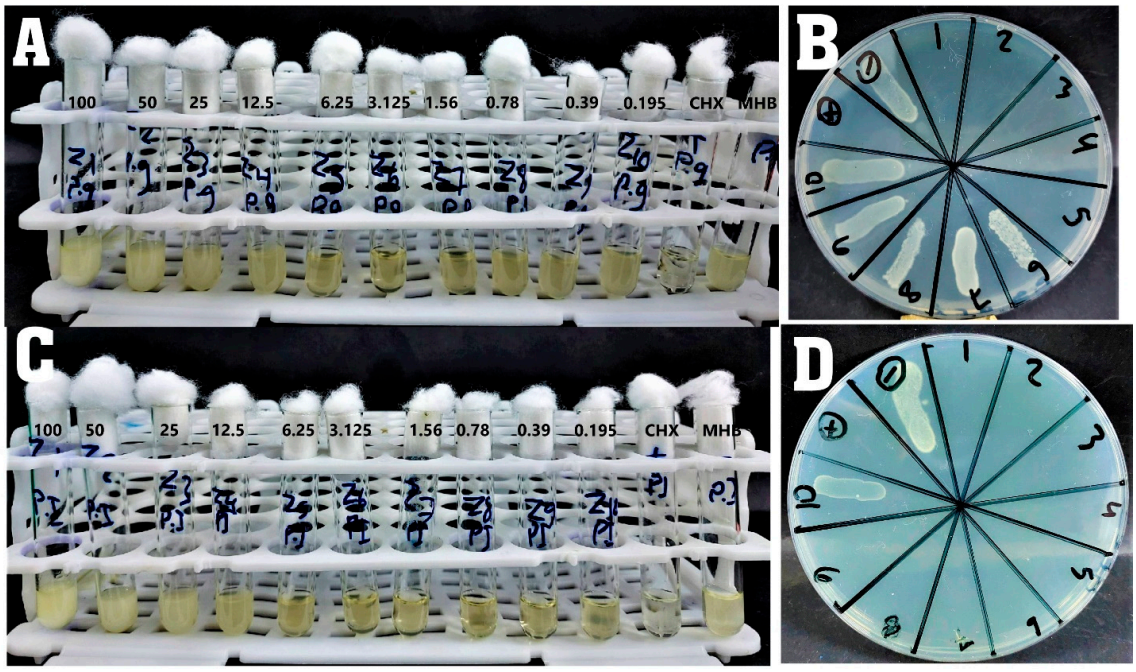

Figure 4. The broth macro dilution method to determine MIC values of CEO on clinically isolated (**A**) *P. gingivalis* and (**C**) *P. intermedia*, respectively. The agar culture method to confirm MBCs of CEO against (**B**) *P. gingivalis* and (**D**) *P. intermedia*, respectively. Concentrations are in µL/mL, while the test tubes labeled (+) and (−) are CHX and MHB, respectively.

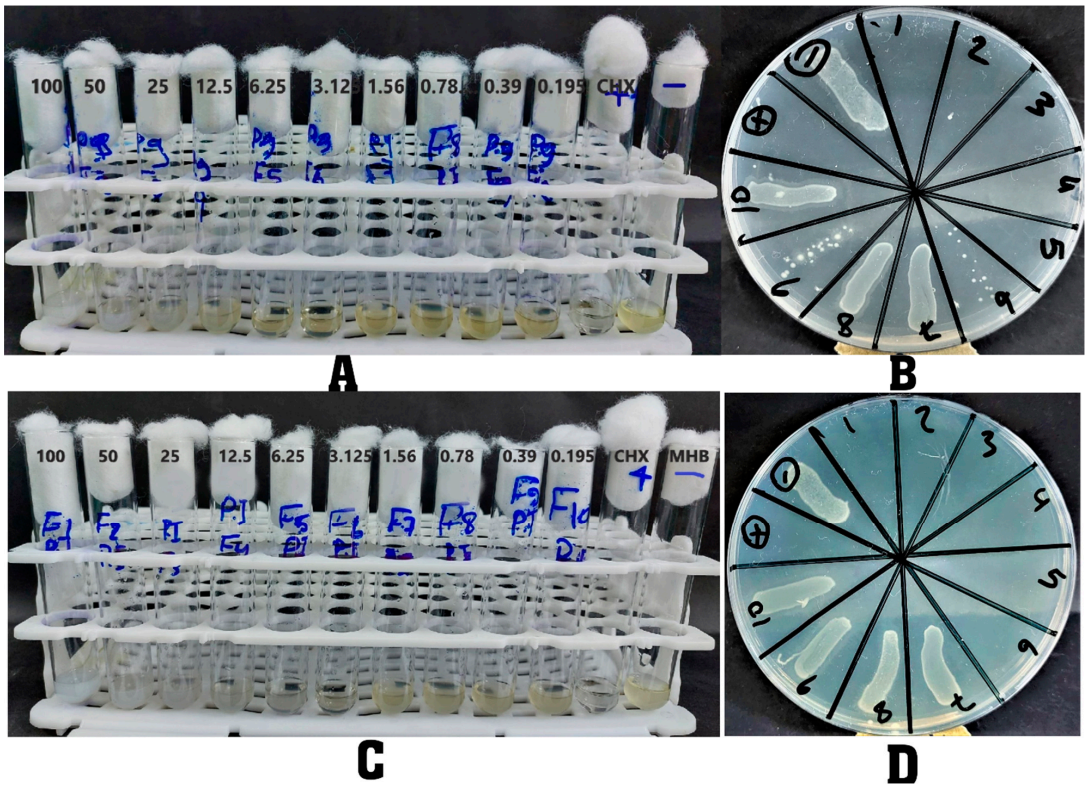

Figure 5. (**A**,**B**) show the minimum inhibitory and bactericidal concentrations of the *FEO* on *P. gingivalis*, while (**C**,**D**) represent the oil's MIC and MBC on *P. intermedia*. Concentrations are in µL/mL, while the test tubes labeled (+) and (−) are CHX and MHB, respectively.

Table 5. Qualitative biofilm formation was judged by observing a visible film lining the walls of the tubes.

Bacteria	*CEO*		*FEO*		CHX (0.12%)	MHB
	1st Tube	MIC Tube	1st Tube	MIC Tube		
P. gingivalis	0	++	+	++	0	+++
P. intermedia	0	+	+	+	0	+++

1st tube: the highest concentration of the dilution series (100 µL/mL), MICtub: test tube containing MIC concentrations of the EOs presented in (Table 4), 0: No biofilm formation, +: Weak biofilm formation (Strong antibiofilm effect), ++: Moderate biofilm formation (Moderate antibiofilm effect), +++: Strong biofilm formation (weak antibiofilm effect), CHX: positive control tube, MHB: negative control tube.

Both the *CEO* (Figure 6) and *FEO* (Figure 7) showed a moderate anti-biofilm effect against *P. gingivalis* and a strong antibiofilm effect against *P. intermedia* at MIC concentrations.

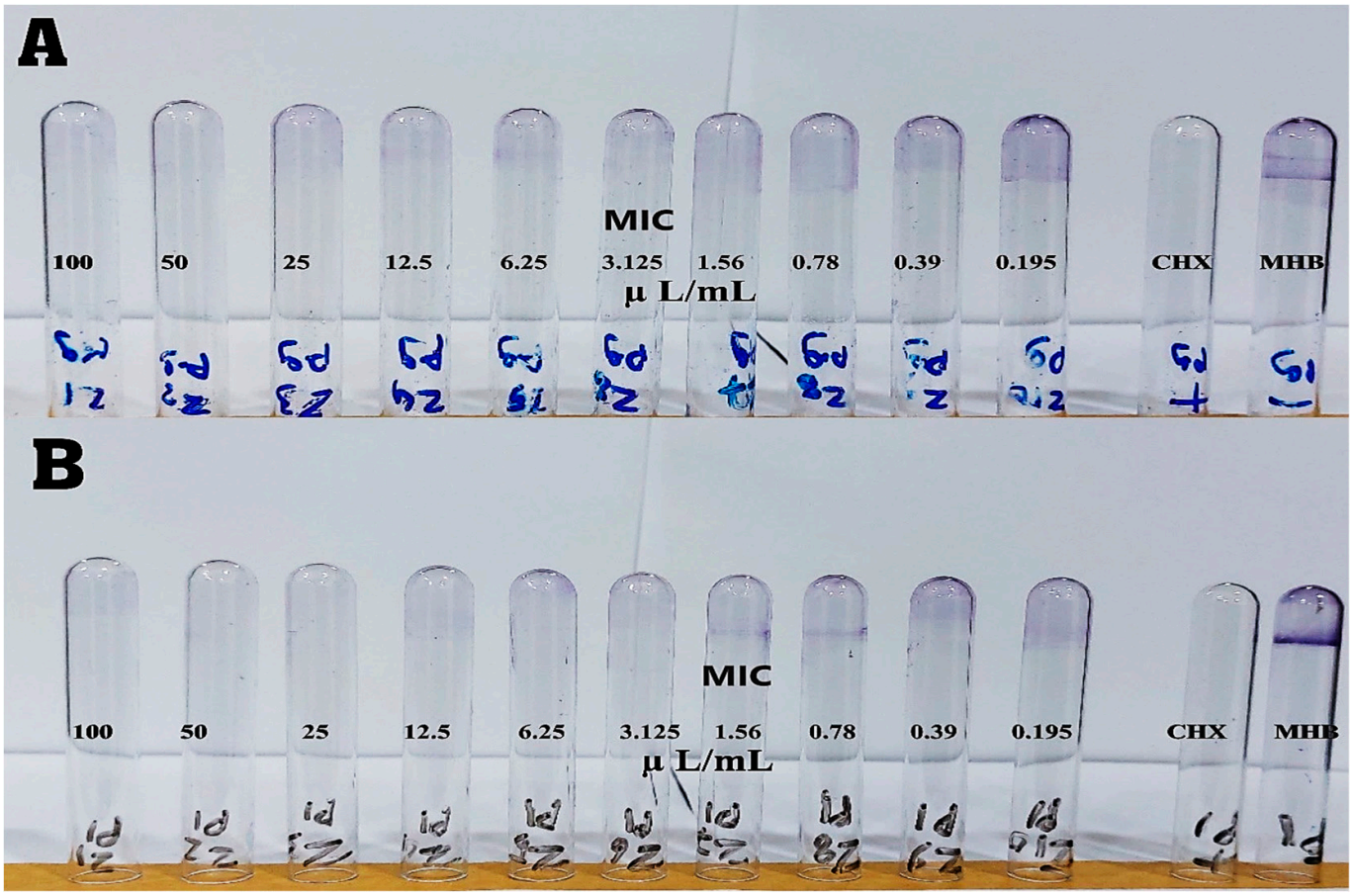

Figure 6. Shows the antibiofilm activity of CEO against (**A**) *P. gingivalis* and (**B**) *P. intermedia*. The intensity of the violet color on the test tubes from left to right (tube 1 to tube 10) represents the amount of biofilm formation by the clinical isolates. Concentrations are in µL/mL, while the test tubes labeled CHX and MHB are (+) and (−) controls, respectively.

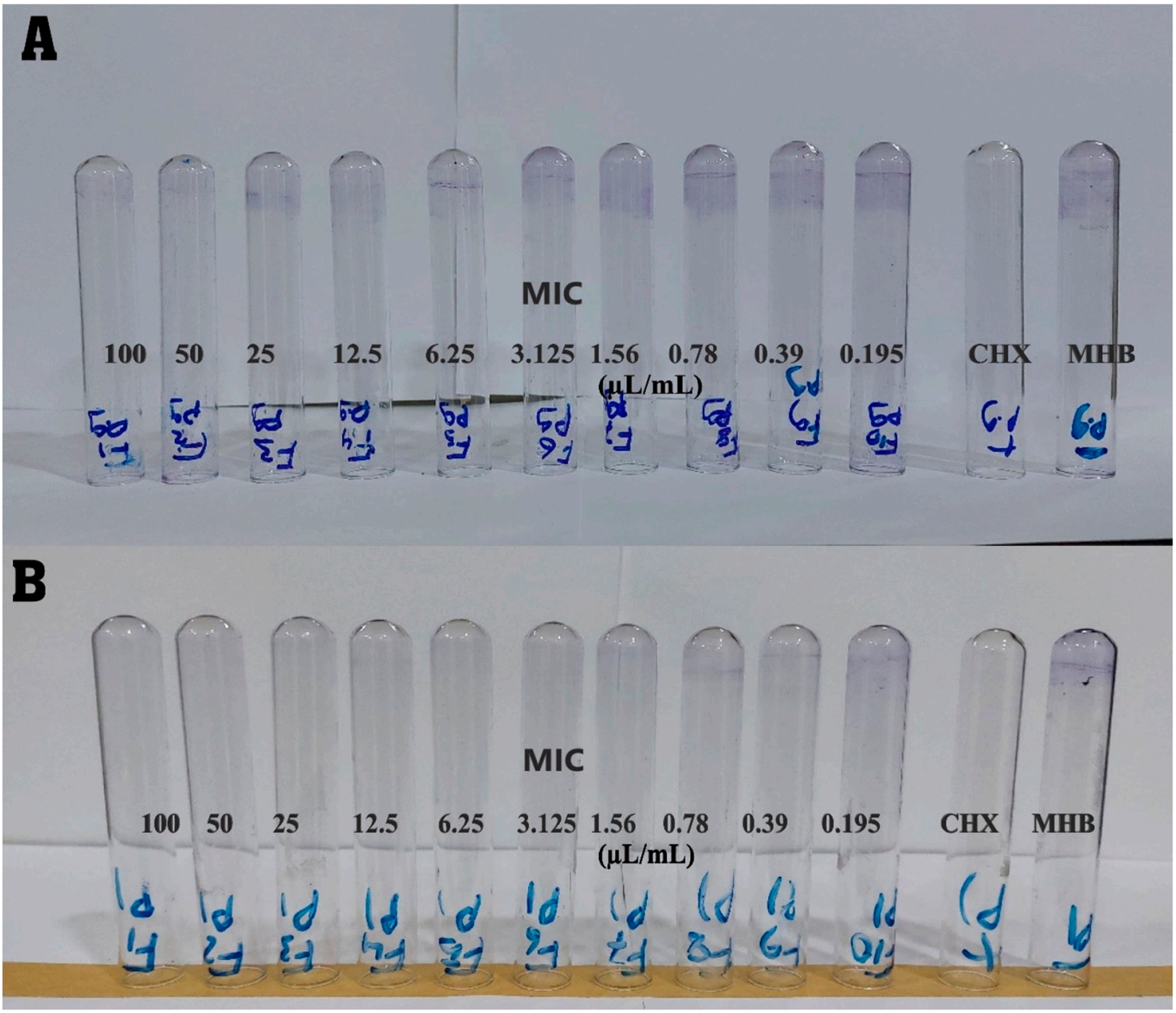

Figure 7. Represents the antibiofilm activity of the FEO against (**A**) *P. gingivalis* and (**B**) *P. intermedia*. The intensity of the violet color on the test tubes from left to right (tube 1 to tube 10) represents the amount of biofilm formation by the clinical isolates. Concentrations are in µL/mL, while the test tubes labeled CHX and MHB are (+) and (−) controls, respectively.

4. Discussion

Over the years, the primary treatment of periodontal diseases has remained constant, comprising the mechanical removal of dental biofilm and calculus by scaling and root planing [8]. However, not all patients might respond to mechanical debridement only [9]. Therefore, antibiotics have been prescribed as an adjunct, albeit prolonged use of these antimicrobials mainly CHX as an adjunct to mechanical plaque reduction can result in a wide range of systemic and local adverse effects, such as tooth staining, taste alterations, burning sensation, and the growth of bacterial resistance [24]. Notably, in this study, CHX has been used as a positive control, and it is apparent that CHX with lower concentration has great antimicrobial and antibiofilm effects against clinically isolated periodontal pathogens. Further, this confirms the importance of using CHX as an adjunct to periodontal therapy. However, chronic use of CHX is associated with the above adverse effects. Thus, finding an alternative to CHX warrants deeper investigation.

It is important to acknowledge that, to the best of our knowledge, this is the first in vitro study to evaluate the antibacterial and antibiofilm activities of *CEO* and *FEO* against the clinically isolated *P. gingivalis* and *P. intermedia*. However, many studies have examined the antimicrobial activity of *CEO* and *FEO* against other pathogenic micro-organisms [13].

The results of this present study revealed that *CEO* has comparable antibacterial and antibiofilm activity against the clinical strains compared to CHX, especially at higher concentrations. Furthermore, *FEO* produced smaller inhibition zones against clinical strains than CHX 0.12% at all concentrations used in this study, while wider bacterial growth inhibition zones were measured at the highest oil concentration when compared to lower concentrations. Generally, natural products like eOs are safer with fewer adverse effects than synthetic antimicrobial agents [25]. Therefore, higher concentrations might be used in future experiments to identify these natural compounds' effective doses and use them as an alternative to systemic and local antimicrobial agents in periodontal therapy.

In agreement with our results, another in vitro study also revealed similar antibacterial and antibiofilm activities of *CEO*, with fewer adverse effects than CHX against *Streptococcus mutans* (*S. mutans*) and *Streptococcus pyogenes* [26]. Other studies tested the antibacterial effect of fennel seed methanolic extract against the cariogenic bacteria *S. mutans*, compared it with CHX 0.2%, and found that CHX was more effective and produced a more significant inhibition zone [27,28]. Furthermore, other researchers found that chewing the seeds for five minutes showed a rise in salivary pH that can prevent demineralization and have an anti-cariogenic property [29].

In this current study, the clinically isolated *P. gingivalis* and *P. intermedia* were sensitive to the *CEO* with mean MICs of 3.125 µL/mL and 0.195 µL/mL, respectively. Further, the MBCs of the oil against clinical strains were 6.25 µL/mL and 0.195 µL/mL, respectively. Furthermore, according to the results, the *CEO* was more potent than *FEO*s against *P. intermedia*, with wider inhibition zones and lower MIC and MBC values. Moreover, the antibacterial efficacy of *CEO* on caries-producing S. mutans with MIC and MBC concentrations of 62.5µg/mL was reported. However, another study reported 0.62 mg/mL and 1.25 mg/mL of cumin seed alcoholic extract as MIC and MBC, respectively, against *S. mutans* [30]. In addition, Vignesh et al. documented the inhibitory effect of cumin with silver nanoparticles against other oral pathogens [31].

Meanwhile, *FEO* showed the ability to inhibit both strains' growth at 3.125 µL/mL, while it eradicated *P. gingivalis* and *P. intermedia* at 6.25 µL/mL and 3.125 µL/mL, respectively. The MIC and MBC concentrations of the *FEO* were similar against *P. intermedia*, indicating the bactericidal profile of the oil. The antimicrobial activity of the *FEO* with MIC of 250 µg/mL was documented against *Micrococcus* spp. by some researchers [32]. The antimicrobial property of the *FEO* was also examined against *S. aureus*, *Bacillus subtilis*, and the MIC was determined to be 1 mg/mL [33].

The discrepancies in MIC values reported by different studies from various regions are mainly attributed to the fact that the chemical composition and the concentrations of active ingredients of EOs are greatly determined by the plant genotype and the influence of environmental factors, including geographical conditions, nature of the soil, temperature, season of collection and harvesting plant, and more importantly, the oil extraction method [34,35].

Based on the results obtained from our study's GC-MS analysis, the *CEO*'s principal components were Cumin-aldehyde, γ-Terpinene, 2-Caren-10-al, and β-Pinene. Cumin aldehyde was the main bioactive compound of *CEO*, rendering most of its pharmacological and clinical significance [36]. Studies on the antimicrobial properties of Cumin-aldehyde have shown that this compound can inhibit growth, cause the death of microbial pathogens, and reduce the negative effects caused by them [36]. Another detected substance, α-pinene, is an organic compound of the polyphenolic (terpene) group, and previous studies have shown its antibacterial properties. In addition, α-pinene also shows significant activity in modulating antibiotic resistance via multiple mechanisms, including inhibition of microbial efflux, decreased membrane integrity, and metabolic disruption [37].

The *FEO* used in this study belongs to the estragole chemotype. The GC-MS analysis detected 17 compounds. Estragole (67.16%) was the main compound. Other compounds, such as D-Limonene at 13.92%, fenchone at 10.32%, anethole at 2%, anisaldehyde, and α-pinene at approximately 1.23%, were also present. The remaining compounds accounted for less than 1%. Estragole, the principal constituent of the *FEO* showed the ability to disrupt the cell membrane, increase nonspecific permeability in the bacterial cell membrane, and enable it to easily pass through the lipopolysaccharide membrane of the Gram-negative bacteria. In addition, fenchone, limonene, and other compounds from *FEO* were found to help accelerate the healing of acute and chronic wounds by protecting the injury site from infection, inhibiting inflammatory cells, and increasing connective tissue formation in the repaired tissue [38]. This finding represents scientific evidence supporting the use of *FEO*, which contains fenchone and limonene, in treating oral diseases, including periodontal diseases.

The discovery of new strategies to eradicate dental biofilms has recently become a popular trend in microbiological dental research. Cuminum cyminum has a remarkable antibiofilm and quorum-sensing inhibitory potential against Gram-negative bacterial pathogens [39]. In this present study, both *CEO* and *FEO* showed moderate and strong antibiofilm activity against clinically isolated *P. gingivalis* and *P. intermedia* at MIC levels, respectively. In agreement with our results, the anti-biofilm effect of *FEO* was examined against selected Gram-positive and Gram-negative bacterial strains and confirmed the oils' capability of preventing biofilm formation at very low concentrations [40]. In addition, the synergistic effect of *FEO* and hydrogen peroxide against *S. aureus* was examined in another study, which found that they have great potential to prevent biofilm formation, promote wound healing, and reduce metabolic activities of attached bacterial cells [41].

Additionally, some recently introduced compounds have been demonstrated to have a significant influence on the oral environment [42]. The use of lysates [43] and postbiotics [44] can modify the clinical and microbiological parameters in periodontal patients. Therefore, these products should be considered in future clinical trials, as adjuvants, in combination with *CEO* and *FEO*.

This study has some limitations, including a limited number of clinically isolated periodontal pathogens examined, and the present in vitro study is conducted by using the isolated bacteria from their usual biological environment that may not fully or accurately predict the same effects on the same bacteria within a whole bacterial community or complex biofilms, which are more virulent than in a planktonic state. Nonetheless, this is the first study to examine the antibacterial and antibiofilm properties of *CEO* and *FEO* against clinically isolated *P. gingivalis* and *P. intermedia*.

5. Conclusions

This current in vitro study provided clear evidence of *CEO* and *FEO*'s antibacterial and antibiofilm properties against clinically isolated *P. gingivalis* and *P. intermedia*. Further studies are suggested to identify and isolate the biologically active ingredients of these oil extracts. Additionally, it is highly recommended to conduct in vivo studies to confirm their effectiveness and use them as an adjunct to mechanical periodontal therapy.

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Informed Consent Statement: Written informed consent has been obtained from the patients to publish this paper.

Data Availability Statement: The data will be available by the corresponding author upon reasonable request.

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